Nitrogen use efficiency and nitrogen balance in Australian farmlands

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Abstract

Farms producing crops and animal products occupy 14% of the Australian land mass. Within this agricultural land, 7% consists of intensive industries (dairy, horticulture and viticulture, sugar cane, cotton, irrigated cereals and feedlots) for which the input of fertiliser nitrogen (N) is typical of such industries worldwide. The sugar and dairy industries are adjacent to populated and environmentally fragile water bodies where nitrate (and phosphate) runoff and leaching contributes to water pollution. The nitrogen use efficiency (NUE) of these industries is low but NUE for the inland irrigated rice and cotton industries are relatively high. The remaining 93% of agricultural land grows dryland crops and animal products (wheat, coarse grains, canola, grain legumes, cattle meat, sheep meat, and wool) partly from continuous crops, partly permanent pasture and partly from phased crop-pasture systems. Until the mid-1990s the source of most of the N in dryland crops was from mining the soil organic matter and increasingly, since the 1950s, from N built up from biological N-fixation by legume-based pastures grown in phased rotation. Export of N in products from dryland farms exceeded the input from N fertiliser. Since the mid 1990s N fertiliser input increased to an average of about 45 kg N ha^{-1}, only about half of which is taken up by crops. Of the rest, most is retained in the soil after harvest and about one quarter is lost from denitrification, ammonia volatilisation and leaching. Overuse of N fertiliser in dryland farming is rare because neither products nor fertiliser are subsidised. Arid and semi-arid land occupies 86% of the continent, half of which is not used for production and the other half produces cattle meat, sheep meat and wool with no fertiliser input. The source of N is rain, biological N fixation and redistribution from dust, the amounts of which are greater than the controlled N inputs in the agricultural regions. The feature of N cycling in Australia that distinguishes it from other developed countries is the importance of natural N sources, reflecting the extensive and relatively young agricultural system.

Keywords: nitrogen budget, nitrogen use efficiency, ^{15}N, denitrification, leaching, ammonia volatilisation

Introduction

The emphasis of international research on nitrogen (N) in agricultural systems has changed from the goal of increasing nitrogen use efficiency (NUE) to profitably use N to produce food and fibre, to concerns about the environmental damage from surplus reactive N, particularly from fertiliser, in the natural environment. The purpose of this paper is to summarise both strands of research in Australia. We distinguish between the national and agricultural N balances because the N balance of the vast non-agricultural zones may disguise or even swamp the agricultural N balance. In the agricultural zones, the goals of high NUE and low leakage of reactive N are compatible. We discuss prospects for improving fertiliser NUE.

Soil N levels

The conventional wisdom is that Australian soils are ancient and infertile (PMSEIC 2010). Deep weathering has influenced soil patterns in parts of Australia, particularly in regions that are climatically unsuited for farming (McKenzie et al. 2004). However there are extensive areas of agricultural soils in south-eastern Australia that were enriched by Quaternary aeolian deposits (McKenzie et al. 2004) as well as productive alluvial soils in eastern Australia with minor weathering and even small areas of soil formed on basalt flows that post-date human occupation. Elsewhere in southern Australia soil N levels increased from the pre-farming levels due to biological nitrogen (N) fixation from the extensive use of pasture legumes (Grace and Oades 1994; Ladd and Russell 1983). In many undisturbed Australian agricultural soils the total N content was, by international standards, consistent with their water balance, temperature and texture; for example the average N content of one of the most widespread agricultural soil types, the red-brown earths (Chromosols, Dermosols, Kandosols and Sodosols) was 1.5 g kg^{-1} before intensive agriculture (Stace et al. 1968), comparable with undisturbed soils

in parts of the United States (Arkansas and Mississippi) with a similar mean annual temperature of about 15 °C (Jenny 1941). Levels of natural total N may be low in some Australian soils because they are sandy and located in dry and warm environments. The original nutrient content of soil in Australia before agriculture, as in agricultural soils everywhere, becomes decreasingly relevant to production and off-site effects as fertiliser supplies more of the nutrients removed in crops and livestock.

Change in soil N levels

Long-term experiments in Australia show that the total N (and organic C) content of soils decreases with continuous cropping and crop-fallow systems. Clarke and Russell (1977) reviewed many experiment that quantified N removed by crops during the first half of the twentieth century. The experimental crops received no N fertiliser and the low yield levels and rates of soil-N depletion were unrepresentative of current cropping systems. Table 1 reports more recent observations and experiments where yields were representative of current crops. In some of these cases the rate of decrease with continuous cropping appears to be linear when measured over periods of several decades but is non-linear over a longer period, falling to a new equilibrium. This pattern of decrease is expressed as a half-life of total N in the soil. Averaged over the data in Table 1, the half-life of total N in the soil is about 30 years. The next question is the extent of N mining in Australian cropping lands. To answer this we need to know the number of crops harvested from an average arable field. Based on the annual increase in area of dryland crops (3.2 % from 1850 to 2014), the estimate is 30, assuming that a field is continuously cropped after the first harvest. This assumption is almost certainly an overestimate and the actual number of crops per field is probably less than 30. This estimate, combined with the estimated half-life of soil N, suggests that about half the total N has been mined from cropping land.

Table 1. Decreases in total N in the top 10 cm of soils in dryland cropping systems in Australia.

Cropping system	Location	Years of observations	Half-life of soil total N (years)	Reference
Continuous sorghum	Narayan, Qld	10	18	Russell (1981)
Fence-line comparisons, cereals	119 farms, 6 soil types, S. Qld	1-70	27-67	Dalal and Mayer (1986)
Continuous wheat, no N fertiliser	Hermitage, Qld	14	36	Dalal (1992)
Fallow-wheat, no N fertiliser	Waite Institute, SA	68	40	Grace and Oades (1994)
Continuous wheat, no N fertiliser	Waite Institute, SA	68	48	Grace and Oades (1994)
Continuous wheat, no N fertiliser	Wagga Wagga, NSW	18	27	Helyar et al. (1997)
Continuous wheat, no N fertiliser	Wagga Wagga, NSW	25	18	Heenan et al. (2004)
Continuous wheat, +50 kg N ha^{-1}	Wagga Wagga, NSW	25	22	Heenan et al. (2004)
Wheat-broadleaf with tactical N	Harden, NSW	19	14	Angus et al. (2006)
Continuous cereal, no N fertiliser	Theodore, Qld	23	34	Dalal et al. (2013)

There are few comparable estimates from long-established farming regions internationally, where early farmers undoubtedly mined soil N. In the warm environment of Tanzania the half-life of topsoil N under maize receiving no N inputs over 15 years (Solomon et al. 2000). In the cooler North Dakota environment, topsoil N under long-term wheat-fallow receiving no N inputs for 45 years (Schimel 1986) had an estimated half-life of 65 years.

The modern equivalent of mining soil fertility is to pump natural gas as a feedstock for ammonia synthesis, and this is equally unsustainable in the long term. There is little data about the 'equilibrium' level of soil N after long-term cropping. A notable result among the long-term experiments is that applying N fertiliser to continuous crops had little effect on the depletion rate of soil total N (Russell 1981; Heenan et al. 2004).

Many Australia studies showed that pastures replenish soil total N and C (e.g. Ladd and Russell 1983; Grace and Oades 1994; Helyar et al. 1997), and phased crop-pasture systems dominated the dryland farming systems in southern Australia from the 1950s to the early 1990s. These systems maintained soil N and C with little N fertiliser provided about half the farm grew pastures (Angus and Peoples 2012). It is possible, but expensive, to replenish soil N (and C) in a continuous cropping system when stubble retention is combined with applied fertiliser N, P and S to maintain the ratios of these nutrients in soil (Kirkby et al. 2016).

Factors influencing the ability of soil to supply N to crops include the amount and quality of soil organic matter and residues, disturbance, moisture and temperature regimes (Campbell et al. 1981). An indicator of soil-N supply is the mineral N content in agricultural soils (typically to a depth of 60 cm) before sowing winter crops. Fillery (2001) reported a mean value of 98 kg N ha^{-1} from a survey of experiments after pasture in Western Australia. Results from the laboratory of Incitec-Pivot Ltd representing hundreds of farm samples in Eastern Australia indicate a mean value of 80 kg N ha^{-1} in the top 60 cm. These values are higher than comparable measurements in Western Europe and North America, probably because of the relatively high levels of total N and because the generally high temperatures in Australia promote mineralisation. Potentially mineralisable N stores in south-eastern Australia range from 8% of the total N in burnt systems to 22% after 15 years of residue retention (Gupta et al. 1994).

Fertiliser N use
Before the mid 1990s most of the N fertiliser used in Australian agriculture was for high-value crops such as horticulture and sugar cane. The average application to dryland crops at the time, mostly wheat and barley, was less than 5 kg N ha^{-1}. Crops in south-eastern Australia received less than average and those on less fertile Western Australian soils received more, as did those in Queensland where soil N had not been replenished with pastures. The reason for the generally low rate was not lack of research and extension, but because wheat yield did not reliably respond to applied N at the time (Colwell and Morton 1984).
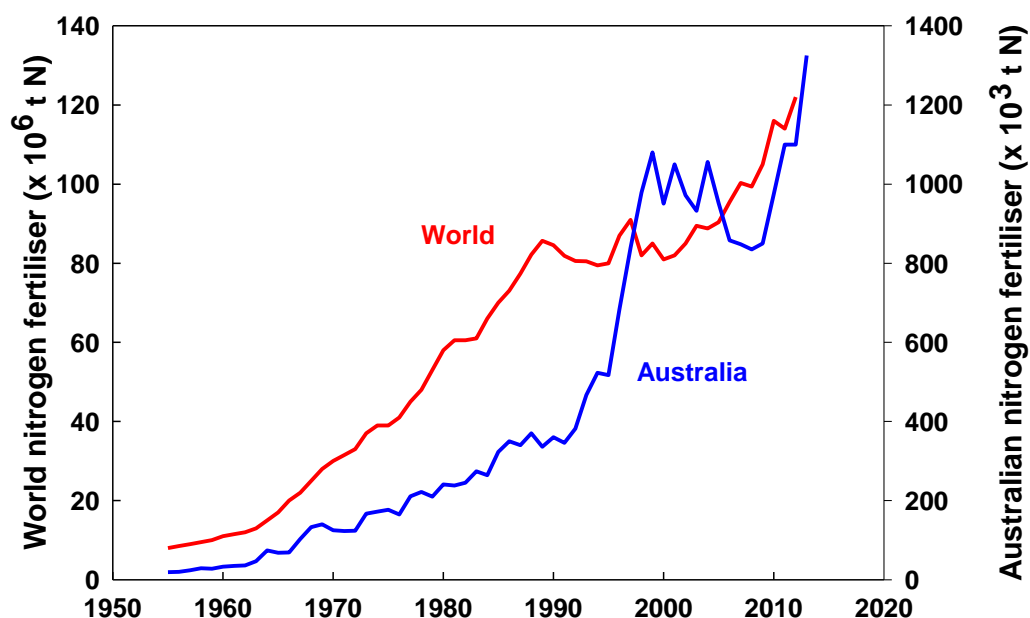

Fig. 1. Changes in N fertiliser use in Australia and the world. The sources are Angus 2001, Fertiliser Australia (www.fertilizer.org.au) and FAOSTAT (www.fao.org).

The growth in N-fertiliser usage in Australia was slow compared to the rest of the world before the mid 1990s (Fig. 1) but for the rest of that decade there was a boom in N-fertiliser use, mostly as inputs to wheat and other dryland crops. This boom closely accompanied increased canola area and lime application. Canola provided the first widely grown break crop in Australia and wheat grown after canola responded more reliably to N fertiliser than wheat after wheat (Angus 2001).

The lime application was needed because canola is acid sensitive and it also enabled other acid-sensitive crops to be grown. Other factors that encouraged farmers to apply N at this time were the availability of efficient fertiliser spreaders and increased premiums for high grain protein. The use of N fertiliser stabilised during the millennium drought of 2002-2009, after which usage resumed its upward course (Fig. 1). Most of the N

fertiliser is now applied to dryland crops at the relatively low rate of 45 kg N ha^{-1} (Table 2). Intensive crops and pastures occupy a relatively small area of land but receive larger application rates.

Table 2. Estimated N-fertiliser use for Australian agriculture, based on estimated areas for 2010-2014 ABARES (2015), national fertiliser use in 2014 is from Fertilizer Australia (www.fertilizer.org.au) and industry fertiliser use is from fertiliser-industry estimates.

		Area (M ha)	Average fertiliser use (kg N ha^{-1})	Total fertiliser use (Mt N)
Dryland crops[*]		24	45	1.08
Intensive farming				
	Cotton	0.44	300	0.09
	Dairy pastures	2.00	100	0.20
	Irrigated cereals[**]	0.31	100	0.03
	Sugar cane	0.36	150	0.06
	Viticulture and horticulture	0.50	100	0.05
Other				
	Sports-fields, parks and gardens	0.1	200	0.02
	Licks and stockfeed			0.03
Total				1.59

[*]Wheat, barley, canola, sorghum, oats, triticale
[**] Rice, maize, wheat

Myers (1984) proposed a simple budget of N inputs and output to estimate N-fertiliser requirement for a single field, in this case a wheat crop which represents the largest crop and consumer of N (Table 3). When this topic was visited previously (Angus 2001), the average N fertiliser application to wheat was 30 kg N ha^{-1}, which represented about one third of the N total supply, the remainder coming from depletion of soil total N and the recent N fixed by pasture legumes and a small contribution from crop legumes. At that time, the supply of N fertiliser worldwide provided about half of the supply to world agriculture (Jenkinson 2001). Applying the same approach to update estimates for all Australian dryland crops in 2014, we estimate that fertiliser provides about 45% of the total N input. In Table 3, mineralisation is partitioned into the contributions from mining the soil and N-fixation by previous pastures. The procedure was to first estimate N-fixation, assuming 50% pasture on the farm from 1950 until 2014 and mineralisation of legume residues according to the rates estimated by Angus and Peoples (2012). The contribution from soil mining was then estimated from the difference between total N-mineralisation and the contribution from N-fixation. Other estimates in Table 3 are the amounts of soil-N retention and losses. Both are based on the fate of ^{15}N fertiliser reviewed in Fig. 2, increased to account for the flow of non-fertiliser N to these pathways. This allocation is based on measurements showing that NUE of native soil N was similar to NUE of fertiliser for dryland wheat (Angus et al. 1998).

Table 3. Nitrogen budget for an average Australian wheat crop, updated from Angus (2001)

		kg N ha^{-1}	Totals (kg N ha^{-1})
Crop N demand	Yield 2.0 t ha^{-1}, 10.5% grain protein	37	
	Straw N (one-third of grain N)	12	
	Rhizodeposited N (34% of total plant N)*	25	74
N supply	Fertiliser	45	
	Rain and dust	5	
	Mineralisation		
	Mining soil N	31	
	N-fixed from previous pastures	31	112
Soil-N retention		24	

			14	38
Losses[**]				

*Wichern et al. (2008)
**leaching, ammonia volatilisation and denitrification of fertiliser and other N

Australian N budget

The land area of Australia is the sixth largest of the ~200 countries but crops and improved pastures make up a relatively small part of the total area and intensive animal industries are relatively small compared with other developed countries (Table 4).

Table 4. Nitrogen balance (M t y^{-1}) of inputs and outputs in Australian regions in 2014, based on the methods of Denmead (1990), McLaughlin et al. 1992) and Galbally et al. (1992) with amounts updated by ABARES (2015) and land areas by ABARES (2010). The transfers represent (1) spatial N movement in dust storms and (2) conversion of organic to mineral N, representing a loss from the soil due to mining and input to the crop.

Zone	Non-agricultural (309 M ha)	Pastoral (355 M ha)	Dryland farming (97 M ha)	Intensive (4 M ha)
Input				
N in rain	0.6	1.2	0.3	
Biological N fixation	0.8	1.1	3.2	0.02
Fertiliser N			1.08	0.36
N offtake in products				
Crop products			-0.9	-0.26
Animal products[*]		-0.02	-0.1	-0.1
Losses				
Ammonia[**]	-1.7	-2.1	-0.6	-0.2
Denitrification			-0.3	-0.6
Nitrate leaching and runoff			-0.1	
Biomass burning (net NO_x)	-0.3	-0.2	-0.1	
Transfers[***]				
N in dust storms	±0.3	±0.3		
Soil organic to mineral N			±0.7	±0.1
Balance	-0.6	-0.1	2.5	-0.6

[*]Empty liveweight of slaughter animals, milk and clean wool
[**] Loss from plant communities, soil, urine, and urea fertiliser
[***] Not included in balance

The estimates of the N balance of the Australian continent follow the work of Denmead (1990), McLaughlin et al. (1992) and Galbally et al. (1992), updated by the area and yield of crops and numbers of livestock, and separated into 4 regions to provide context for the agricultural regions. The regions are non-agricultural land in the arid zone and mountainous conserved forests, pastoral land in semi-arid regions, dryland farmland consisting of the "wheat-sheep" and "high rainfall" zones and intensive industries in irrigated and high-rainfall regions. Small values and well documented data justify reporting with a precision of 10,000 t N, but for other data the precision is 100,000 t N at best.

Table 4 suggests that in the non-agricultural, pastoral and intensively farmed land the N inputs and outputs are similar. In the dryland farming zone there is a positive N balance, mainly due to N-fixation of pastures. This input is probably not associated with the phased crop-pasture system in which pasture area has been decreasing, but due to N-fixation by permanent pastures. Inputs and outputs of N in the zones are discussed in the following sections

Arid rangelands

The inland arid and semi-arid zones make up most of the national estate. Part of these zones have no agricultural production and the rest consist of pastoral properties that produce cattle and sheep with the only N input from atmospheric deposition and biological N fixation. The amounts per hectare are small but the amount over the whole area far exceeds the N offtake in meat and wool. There is no evidence of N accumulation or

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depletion within historical time in these regions but there is a significant amount of N lost and redistributed in dust-storms. These events occur most frequently when strong winds coincide with a period of drought and most of the particles are lifted from the arid inland and some are lifted from farmland. McTainsh et al. (2005) reported an average of 62 dust storms each year from 1960 to 2002 in Australia, most of which remain in the arid zone and only the largest reach the coast. They estimated that a large dust storm in 2002 contained 3.35-4.85 Mt of particulates. A later large dust storm reported by Aryal et al. (2012) contained 10.6% organic matter. Assuming an 'average' dust storm half the size estimated by McTainsh et al. (2005) and an organic matter content of 10.6%, of which an estimated 4% was N, we come up with an annual estimate 0.5 Mt of N redistributed within the Australian continent or blown into the sea. This represents redistribution of 0.7 kg N ha^{-1} across the arid zone.

Other inputs of N in the arid rangelands are from rainfall and biological N fixation. The contribution from rain is calculated from the land area, mean annual rainfall and the N concentration in rainwater, 0.5 mg L^{-1}, based on measurements of Wetselaar and Hutton (1963) and Crockford and Khanna (1997). The main source of N deposited in rain is not thunderstorm activity (Wetselaar and Hutton 1963) but the products of biomass burning and ammonia released from urine voided by grazing animals (Denmead 1990; Galbally et al. 1992). There is likely to be biological N fixation from various symbiotic (e.g. *Acacia* and other leguminous shrubs and forbs, *Casuarina*, *Macrozamia* and lichens) and non-symbiotic associations (Gupta et al. 2006), cyanobacterial soil crusts, free-living microbes and the gut bacteria of cellulose-digesting termites (Evans et al. 2011). Non-symbiotic N-fixation is likely to be greater in northern than in southern Australia because of generally lower soil-N status and high temperatures during the wet season.

Dryland farming
Farming in this zone consists of permanent pasture grazed by sheep and cattle, continuous cropping, and phased crop-pasture sequences. For the period from 1850 to 2000 crop area grew at an average annual rate of 3.2 % (Angus and Good 2004) as crops replaced, and continue to replace, pastures. The expansion has been partly within established farming regions and partly as expansion into new regions in Western Australia, Queensland and along the low-rainfall and high-rainfall boundaries of the cropping region (Kirkegaard et al. 2011). The only source of N for the first century of crop production was from mining the soil, initially through continuous cropping and then through fallow-crop sequences (Donald 1965). Soil N was replenished by biological N-fixation by legumes in improved pastures after the 1950s, triggered by a high wool price and encouraged by a federal bounty on the application of phosphate fertiliser (Henzell 2007). Soil total N is maintained when improved pastures (i.e. with a high proportion of legumes) make up about half the farm area (Angus and Peoples 2012). The area of pastures that significantly contribute to the N balance is difficult to estimate; the total area defined by ABARE (2010) as Grazing Modified Pastures is 72 M ha, but the north eastern part of this zone has relatively few legumes and a major source of N for livestock in the region is urea blocks, which represent much of the 30,000 t of N used as licks and stockfeed (Table 1). A more realistic estimate of the area of pastures that contribute to the N balance is 50 M ha at an annual rate of 60 kg N ha^{-1} (Peoples et al. 2012). This is consistent with the estimate of McDonald (1989) who found annual increments in soil N from legume based pastures ranging from 19-117 kg N ha^{-1} (average 63 kg N ha^{-1}) from 15 studies across southern Australia. Crop legumes also contribute to the N balance of dryland farms but make up only about 5% of the crop area.

Rainfall is variable in most of the dryland farming zone, particularly in the north east. Yield of dryland crops can be partly buffered from the rainfall with fallows and other moisture-retaining methods, but yield is still strongly tied to rainfall during the growing season. The variable yield potential presents a challenge for N management. Applying an average amount of N fertiliser can result in underfertilisation in some seasons and overfertilisation in others. Many graingrowers adopt a tactical approach to N management, aiming to delay application of fertiliser until the yield potential is more predicable during the stem-elongation phase. They then topdress an amount based on a simplified version of the N budget in Table 3 and aim to synchronise the times of N supply and demand. Crops and livestock are increasingly integrated on mixed farms with grazing of vegetative crops by sheep and cattle, the effect of which is greater reliance on fertiliser N rather than biologically fixed N (Virgona et al. 2006). Livestock also graze crop stubble and increase the accumulation of

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soil mineral N, because the consumption of high-C residues reduces N immobilisation (Hunt et al. 2016). Crops growing after canola also benefit from increased N mineralisation (Ryan et al. 2006).

Irrigation and high-rainfall farming
Most of the irrigated land lies on semi-arid plains along the inland rivers of eastern and south-eastern Australia. The yields and N input to summer-growing crops: cotton, rice, maize, wheat, dairy pastures and horticultural and viticulture crops, are high by international standards. The area of irrigation is limited by the amount of irrigation water which, during drought, is conserved for perennial crops and milking cattle. The area of annual crops and hence N-use is therefore highly variable. NUE is generally greater than for dryland crops because N-fertiliser can be washed into the root zone soon after application. Poor irrigation management leads to denitrification losses due to prolonged periods of soil saturation (Mathers et al. 2007). There are no reports of leaching below the root zone but Weaver et al. (2013) measured large accumulation of nitrate at the bottom of the cotton root zone.

High-rainfall farming land is along the south-west, south-east and east coasts where it is concentrated between the Great Dividing Range and the ocean. The largest users of N fertiliser in this zone are dairying, sugar cane and horticulture. Gourley et al. (2012) surveyed Australian dairy farms and found an average NUE of 26%, based not only on fertiliser but also on fodder imported to the farm. Bell et al. (2016) report NUE for sugar cane but no comparable data are available for horticulture. Raised beds are being increasingly employed to manage waterlogged cropping soils across southern Australia (MacEwan et al. 2010).

Nitrogen use efficiency and losses
Nitrogen use efficiency (NUE) is expressed in many ways but in this case we refer mainly to apparent above-ground recovery of fertiliser N (AARFN) because this enables comparisons to be made between species and takes some account of grain protein. There are more complete and complex assessments of NUE that consider both yield and grain protein responses to fertiliser N, and their relative profitability (Fischer et al. 1993; Angus 1995).
For irrigated crops in the semi-arid zone AARFN is over 70% for well managed cotton (Rochester and Bange 2016) and well managed medium-grain rice (Angus et al. 2016), both at yield levels on farms that are high by world standards. AARFN in dryland crops can be much lower. In a survey of 60 commercial dryland wheat crops in south-eastern Australia those that were most likely to give large N responses (mid-season topdressing, early sowing, low N status and following a break crop) the average AARFN was 36% (Angus and van Herwaarden, unpublished). However the average agronomic efficiency (the additional grain per unit of additional fertiliser N) was 13 while the N:grain price ratio was 6.0. At these prices it was profitable for farmers to apply N-fertiliser to grain crops, even with low NUE. The low NUE is consistent with the results of experiments that traced ^{15}N fertiliser applied to 57 wheat crops in Australia (Fig. 2). At maturity 44% of the fertiliser was in crops, 34% in soil and 22% was not recovered, presumably lost by one or more of the processes of denitrification, leaching and ammonia volatilisation. Losses exceeding 20% of applied N are also consistent with dairy (Stott and Gourley 2016; Rowlings at al. 2016) and sugar cane production systems (Bell 2016). Pilbeam (1996) showed that amount of fertiliser N in the soil at maturity was relatively greater in the generally dry environments in Australia than in wetter environments where more of the labelled N was present in the crop. The relatively large amount of fertiliser-N retained in the soil may include some immobilisation which represents a financial loss; the longer the delay before re-mineralisation, the greater the financial loss. Finding ways to reduce immobilisation and losses could increase NUE. For example it may be possible to minimise contact between fertiliser and immobilising and denitrifying microbes by deep banding, concentrating urea or ammonia in mid-row bands or deep bands, or by in-crop side-banding. Concentrating ammonium in bands delays nitrification and hence reduces losses by denitrification and leaching (Wetselaar et al. 1973; Angus et al. 2014). Banding promotes slow release of fertiliser N to the crop.
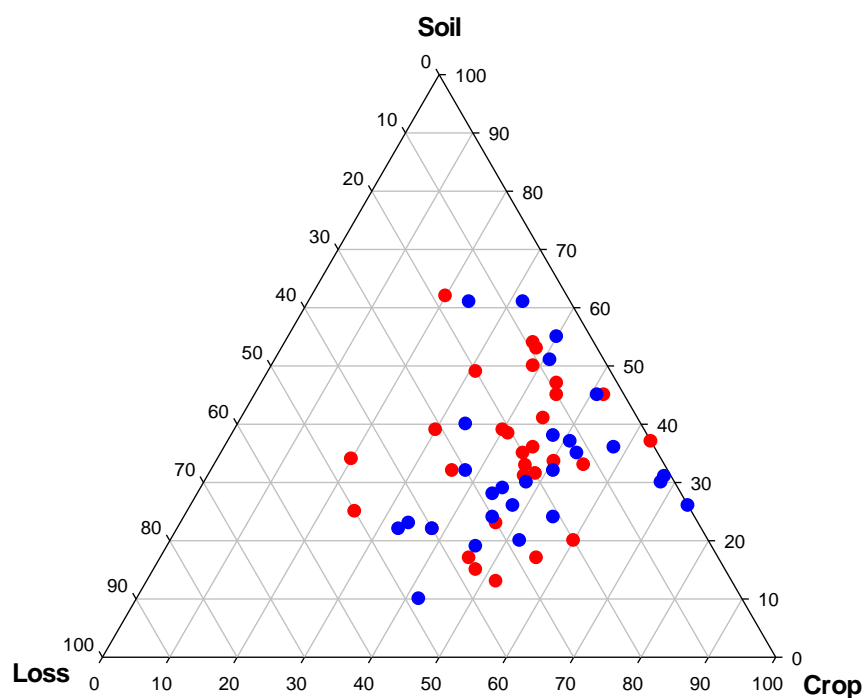
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Fig. 2. Fate of ^{15}N fertiliser applied to Australian grain crops (red for duplex soils and blue for uniform clays). The ^{15}N was measured in above-ground plant parts and soil, both sampled at crop maturity. Unaccounted ^{15}N is reported as loss. The mean for all studies was 44% in crop, 34% in soil and 22% loss and there was little difference between duplex and clay soils. Data sources are Australian experiments reported by Pilbeam (1996) and Freney et al. (1992) and more recent studies by Lam et al. (2012), De Antoni et al. (2014), Bell et al. (2015), Harris et al. (2015), Schwenke and Haigh (2016) and Wallace et al. (2016).

Previous improvements in NUE of Australian dryland crops have come about mostly by increasing crop-N demand through early sowing, controlling root disease, correcting acidity and micronutrient deficiency and by increasing the yield potential by plant breeding. Increasing N demand by the crop leads to more rapid N uptake and so less exposure of the fertiliser to immobilisation and the loss pathways.

Denitrification
Studies reviewed by Chen et al. (2008) and Grace (2015) showed that loss of fertiliser N by denitrification is common in Australian crops. Since most of the fertiliser N is applied to crops and pastures during winter and spring the rate of denitrification is probably limited by temperature. Assuming that most of the N loss estimated from Fig. 2 represents denitrification, the annual N loss from the 1.59 M t of fertiliser N (Table 2) would be <0.35 M t. Less research has been conducted on losses from non-fertiliser sources of N, but the results of Pi et al. (1999) show large losses of N mineralised from organic matter in eastern Australia when the soil is warm and wet. The most widespread occurrence of warm and wet soil is during floods in summer and autumn. One extensive flood on the plains of eastern Australia in January 2011 covered 1.7×10^8 ha, mostly in cropping and grazing land. This area is equivalent to the combined areas of France, Germany, the Netherlands, Belgium, Denmark and Norway, and much of this land remained inundated for over a month in mid-summer. No measurements of denitrification are reported for this event, but it would be reasonable to assume that all the soil NO_3^- would be denitrified. A conservative estimate of the soil NO_3^- is 20 kg N ha^{-1} in the top 0.6 m, based on the lowest values of soil samples that pass through the commercial laboratories and from surveys in research laboratories. Assuming that this amount, the total denitrification from this one flooding event would be 2.6 M t of N. Such flooding events are infrequent but still represent a loss of N comparable with, or greater than, the loss from fertiliser. Denitrification is also significant in water bodies and Harris (2001) concluded that >75% of dissolved N could be lost through this pathway.

Ammonia and nitrogen oxide exchange with the atmosphere
Ammonia escapes to the atmosphere from soil, plants, burning biomass and animal excreta (Denmead 1990). Soils and plants can also capture NH_3 from the atmosphere. The extent of NH_3 loss from native vegetation is uncertain, but Denmead (1990) considered this pathway to be the main net source of ammonia to the atmosphere. The second largest agricultural source of atmospheric ammonia is from the urine of grazing cattle, sheep and kangaroos. The amounts are estimated following the methods of Denmead (1990) with contemporary livestock numbers, which in 2014 amounted to 300 million sheep equivalents. Ammonia loss from the extensive arid zone dominates these losses and the amounts almost balance N deposition in rainfall and may in fact represent the same material. The levels of atmospheric ammonia are relatively low over most of Australia but downwind of large cattle feedlots are comparable with those in western Europe and north eastern North America (Denmead et al. 2014), representing free fertiliser to farms but environmental damage to water bodies and native vegetation growing on poorly buffered soil.

Burning biomass releases nitrogen oxides that, like ammonia, are mostly returned to the land in rain (Galbally et al. 1992). Fires in the arid zone were traditionally started by the indigenous practice of firestick farming (Gammage 2011). This traditional practice of relatively frequent 'cool' burns protected the landscape from less frequent 'hot' wildfires, mostly started by lightning, which are now the main form of biomass burning (Burrows et al. 2006). In the dryland farming zone burning cereal stubbles is usually by 'cool' fires and certainly results in loss of N to the atmosphere. However the long-term reduction in total soil N is not significantly greater than with retained stubble, which decomposes within months or years (Angus et al. 2006). Stubble retention appears to be increasing with the adoption of trash-clearing seed drills which reduce the need for the time-consuming process of burning stubbles.

Ammonia loss to the atmosphere from dryland crops is mostly from hydrolysis of urea applied to the surface of moist alkaline soil which contains urease, typically in plant residues. In Australia the situations in which this is most likely to occur are dairy pastures (Eckard et al. 2003) or crops growing on alkaline soils or with heavy residues where >20% of fertiliser can be lost as ammonia (Turner et al. 2012). The model of Fillery and Khimashia (2016) shows that drilling urea into the soil or topdressing before rain can reduce these losses to zero. In our experience most farmers use rainfall forecasts in planning to topdress urea.

Nitrate leaching and runoff
Nitrate leaches from the topsoil into the subsoil or groundwater when there is nitrate in the soil profile and the water supply exceeds the water-holding capacity. The largest losses are on coarse-textured soil in regions and seasons when the water balance is positive for part of the year, such as in winter-rainfall regions of southern Australia and in the wet season in northern Australia. There are relatively few observations of nitrate leaching in Australia and most of those reported in Table 6 are on the wet fringe of the dryland farming region or in an intensive farming region. Anderson et al. (1998) measured up to 106 kg N ha^{-1} leached from a coarse-textured soil in a relatively high rainfall part of the dryland farming region of Western Australia. Extrapolations to other parts of the region using a simulation model suggested that the long-term mean quantity of leaching varied from zero on a loamy sand in a dry environment to 50 kg N ha^{-1} y^{-1} on a sand in a wet environment (Milroy et al. 2008).

Table 5. Australian examples of nitrate leaching below the root zone and groundwater contamination.

Location	Source of nitrate	Quantity	Reference
Nitrate leaching			
Southeast Queensland	Fallow-wheat with summer rainfall	19 kg N ha^{-1} y^{-1}	Turpin et al. (1998)
Mallee fallow-wheat sequence	Mineralised N during fallows	Accumulation >500 kg N ha^{-1}	D Roget (unpublished)
Sugar deltas and coast	Fertiliser from sugar land	30-50 kg N ha^{-1} y^{-1}	Quoted by Rasiah et al. (2003)
Western Australian crop land	Fertiliser and mineralised N	17-59 kg N ha^{-1} y^{-1}	Anderson et al. (1998)
Southern NSW	Fertiliser and mineralised N	4 kg N ha^{-1} y^{-1}	Poss et al. (1995)
Southern NSW	Annual pasture	9-15 kg N ha^{-1} y^{-1}	Ridley et al. (2001)
Southern Australia,	Pastures where annual rainfall >450 mm	15-35 kg N ha^{-1} y^{-1}	Ridley et al. (2004)
Groundwater contamination			
Southeast South Australia	Annual pasture legumes	7 mg NO_3-N L^{-1}	Dillon (1988)
Central Australia groundwater	Holocene leaching from termite mounds	<80 mg NO_3-N L^{-1}	Barnes et al. (1992)

Along the wetter fringe of the dryland farming regions of eastern Australia winter rainfall is less intense and nitrate leaching is about half the values in Western Australia. In the examples for eastern Australia listed in Table 5 nitrate leaching could be reduced with more intensive management, for example growing perennial rather than annual pastures (Ridley et al. 2001), by earlier sowing of crops and splitting N fertiliser (Anderson et al. 1998) or by double cropping (Turpin et al. 1998). Improved management had less effect on leaching in Western Australia. Nitrate leaching is an infrequent occurrence on most dryland cropping farms in eastern Australia because the soil water-holding capacity is generally sufficient to contain the surplus of rainfall over potential evapotranspiration.

Leached nitrate and particulate N contribute to N pollution of surface water bodies. The concentration of N in most streams that drain areas of permanent pasture in Victoria exceed official guidelines (Ridley et al. 2004), and there is evidence of leaching into shallow groundwater. There are few measurements of nitrate leaching to the water table, which is normally tens of metres below the surface in the dryland farming region. High concentration of nitrate in groundwater is not of much public concern in Australia because relatively small amounts of groundwater are used for human consumption. In fact the largest areas of groundwater affected by high nitrate levels are not due to fertiliser. One is in arid central Australia due to leaching of N that had been biologically fixed by termite gut bacteria in geological time (Barnes et al. 1992). Another large area of high-nitrate groundwater, in a winter-rainfall region of South Australia, is of agricultural origin but from mineralisation of organic N derived from biological N-fixation by clover (Dillon 1988).

Offsite damage from leaching and runoff have been extensively studied in Australia. Damage to the Great Barrier Reef is the most grievous consequences of N (and P) leaching and runoff from near-coastal sugar cane fields and particulate erosion from inland grazed grassy woodlands, discussed in this conference by Bell et al. (2016). There is also N (and P) runoff into the estuaries and coastal lagoon along the southwestern, southern and eastern coastline (Harris 2001). These water bodies periodically become eutrophied by nutrients sourced from diffuse and point sources in cleared catchments. Eutrophication is not exclusively a problem of farming and there is evidence of algal blooms in rivers and lagoons before white settlement, presumably because of concentration of nutrients during drought. Harris (2001) concluded that the N and P discharge to Australian coastal waters was small compared to those in the Northern Hemisphere because of less atmospheric N deposition, lower population densities and less fertiliser use. Eutrophication in Australian coastal lagoons is normally N-limited and there are frequent N-fixing cyanobacterial blooms. It thus becomes important to minimise movement of P into the water courses. For the Gippsland Lakes Roberts et al. (2012) concluded that the most cost-effective methods to reduce contamination were by enforcing existing regulations on the large sources of P from the dairy industry.

Another serious effect of nitrate leaching is acidification of poorly buffered soil in southern Australia (Helyar and Porter 1989). In this case NO$_3^-$ in the topsoil, mostly originating from biological N fixation by pastures, and more recently from N fertiliser, leaches into the subsoil along with alkali and alkali-earth metals, which are then replaced by protons adsorbed onto clay minerals in the topsoil. The lime needed to neutralise this acidity is an additional cost of fertiliser or fixed N that has only partly been met in dryland cropping systems and hardly at all in permanent pastures.

Conclusions
Based on budgetary approximations, the N balance of the Australian continent appears to be slightly positive, mainly because of N-fixation by legume-based pastures in the dry farming zones. In the other zones the N balance is about neutral and the stability of N in the vast arid zone buffers changes in the agricultural zones. The episodic contributions from natural N processes of redistribution in windstorms and denitrification during large floods are comparable with the N amounts in fertiliser.

In the intensive farming zone there are areas of N surplus in the sugar and dairy industries from which reactive N leaks into some coastal and near-coastal water bodies. In southern Australia, at least, the proposed solution is to enforce current regulations, particularly on manure management in the dairy industry.

The net accumulation of N in the dryland farming zone has not caused obvious environmental damage, although soil acidification, partly due to leaching of nitrate to the subsoil, is a less visible but still important problem for both the intensive and dryland farming zones. The obvious solution of applying lime is being implemented for cropping systems. More profitable animal industries will be needed to pay for liming permanent pastures. The N surplus in permanent pastures shows a need for Sustainable Intensification (Godfray al. 2014), for example by introducing more cropping.

The decreased amount of soil N in cropping areas is a consequence of mining existing soil N stocks, which has been partly offset by N-fixation by legume-based pastures grown in phased rotation with crops, and by fertiliser. The future contributions of N-fixation and fertiliser will depend on the relative profitability of cropping and animal industries based on pasture. If the current trend towards continuous cropping continues, meeting crop-N demand with fertiliser rather than mined soil N will be a challenge unless the currently low NUE is improved. New approaches to fertiliser management, combined with mid-season tactical application, are needed to improve NUE and our suggestion is to concentrate on reducing the loss from denitrification and the economic loss from immobilisation. To minimise the potential for N immobilisation and denitrification, a suggested approach is to increase the spatial separation of fertiliser from most of the microbes responsible for these processes, or temporarily inactivate them by placing N fertiliser beneath the microbe-rich topsoil or concentrating it in thin bands.

Acknowledgements

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Fine sediment and particulate organic matter: A review and case study on ridge-to-reef transport, transformations, fates, and impacts on marine ecosystems

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ARTICLE INFO

ABSTRACT

Studies documenting the effects of land-derived suspended particulate matter (SPM, i.e., particulate organic matter and mineral sediment) on marine ecosystems are typically disconnected from terrestrial studies that determine their origin, transport and fate. This study reviews sources, transport, transformations, fate and effects of SPM along the 'ridge-to-reef' continuum. We show that some of the SPM can be transported over long distances and transformed into large and easily resuspendible organic-rich sediment flocs. These flocs may lead to prolonged reductions in water clarity, impacting upon coral reef, seagrass and fish communities. Using the Great Barrier Reef (NE Australia) as a case study, we identify the latest research tools to determine thresholds of SPM exposure, allowing for an improved appreciation of marine risk. These tools are used to determine ecologically-relevant end-of-basin load targets and reliable marine water quality guidelines, thereby enabling enhanced prioritisation and management of SPM export from ridge-to-reef.

1. Introduction

Tropical marine ecosystems such as coral reefs and seagrass meadows are threatened by a combination of both global (i.e. climate change) and local and regional stressors (i.e. increased terrestrial runoff, over- and/or destructive fishing, coastal development and marine based pollution) (Orth et al., 2006; Burke et al., 2011; Grech et al., 2012). These stressors can result in significant loss of ecosystem services impacting on social, cultural, biological and economic values. Management of local and regional scale stressors can be voluntary or involuntary and include: the establishment of marine parks or no/limited-take zones; improved industrial marine activities such as port operations and disposal of dredged material; upgraded sewage treatment plants; and restrictions on the use of pollutants in adjacent catchments. The reduction of diffuse sources of pollutants (e.g. sediment, nutrients and pesticides in terrestrial runoff) is equally urgent, however its effective management is often compromised. This is partly because for each of the constituents, a robust understanding

of its key sources, transport, transformations, fate and effects are required, before effective management strategies can be developed and implemented (e.g. Kroon et al., 2014; Risk, 2014; Yamano et al., 2015; Oleson et al., 2017).

For marine organisms and ecosystems, suspended particulate matter (SPM), and specifically 'organic-rich sediment' is considered one of the most detrimental forms of sediment (Weber et al., 2006, 2012; Bartley et al., 2014). A growing body of research has highlighted the deleterious effects of SPM (see conceptual diagram; Fig. 1), which easily stick to coral tissue and seagrass leaves and are difficult for these species to remove compared to organic-poor calcareous offshore sediments (Weber et al., 2006, 2012; Brodersen et al., 2017). Organic-rich sediments are more easily resuspended due to their lower density, contribute to reductions in water clarity for prolonged periods, locally reduce pH and oxygen conditions, are darker and have a disproportional influence on light attenuation (de Boer, 2007; Storlazzi et al., 2015; Fabricius et al., 2016).
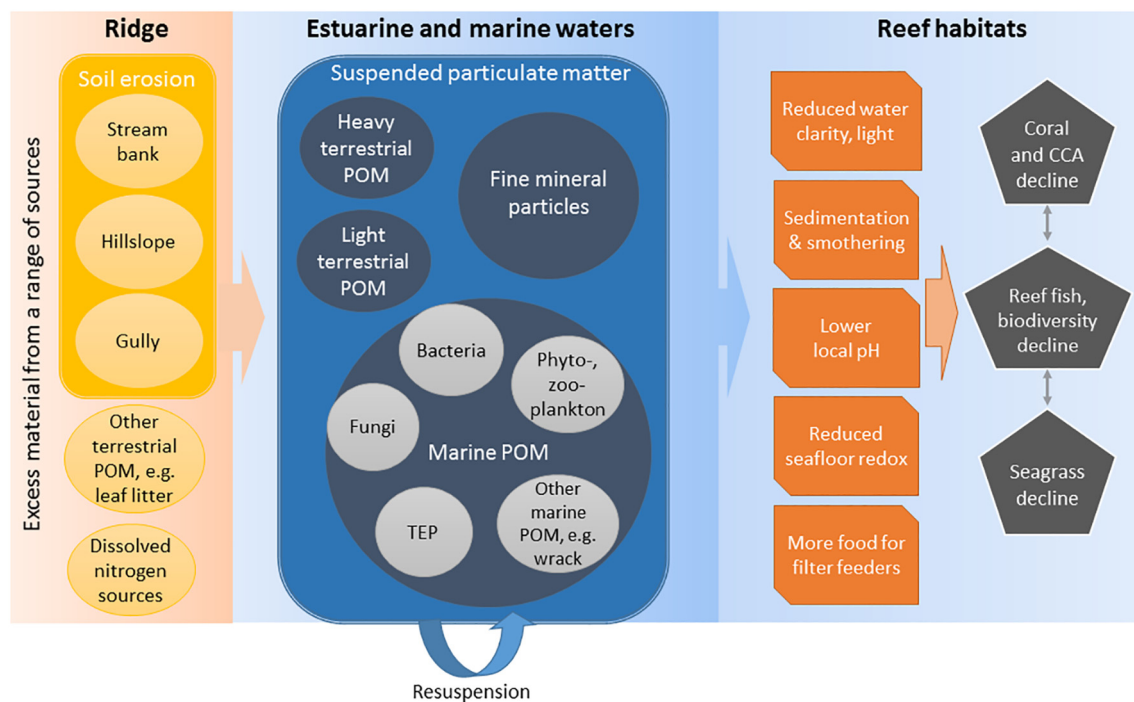

Fig. 1. Conceptual diagram of suspended particulate matter sources, transport processes and tropical marine ecosystem impacts across the ridge-to-reef continuum. The impacts to the marine ecosystem will occur when there is excess soil erosion (above natural and background levels). POM, particulate organic matter; mPOM, marine-derived particulate organic matter; TEP, transparent exopolymer particles; CCA, crustose coralline algae.

A number of recent sediment studies have examined the 'long chain of evidence' linking the 'ridge to the reef'. These identified the role that terrestrial fine-grained (< 63 μm) sediment plays in affecting rates of sedimentation and water clarity in the marine environment (Bartley et al., 2014; Risk, 2014; Gibbs, 2016; Hairsine, 2017; Roberts et al., 2017; Delevaux et al., 2018). However, the distinction between land-derived sediment and nutrient inputs versus marine-derived SPM, the sources of the organic fraction, and their interactions with mineral sediment remain challenging to unravel.

Here we define SPM as a combination of terrigenous (or mineral) sediment and terrestrially-derived particulate organic matter (tPOM) as well as marine-derived POM (mPOM- potentially fueled by terrestrially-derived nutrient inputs; refer to Fig. 1). We also refer to terrigenous sediment and tPOM, combined, as terrestrial sediment. These constituents may be transported as individual particles or aggregated as larger particles (i.e. organic-rich sediment flocs; see Droppo, 2001). Establishing ecological thresholds (e.g. for sedimentation rate, water clarity/Secchi depth, turbidity etc.) that reflect impacts on tropical marine ecosystems is complicated due to the complexity in environmental conditions and ecological processes (e.g. species sensitivities). This limited understanding creates challenges for effectively managing terrestrial sediment export to marine environs and setting meaningful ecologically relevant end-of-basin load targets.

The Great Barrier Reef (GBR), north-east Australia (Fig. 2), provides a unique case study to assess the ridge-to-reef continuum for SPM and demonstrate how integrated research and models can be used together to support systems understanding, establishment of ecological thresholds and management response. Reference will be made to a series of studies addressing the processes linking the effects of terrestrial sediment delivered from the basin to SPM in the marine environment; as well as experiments on coral, seagrass and fish species to establish ecological thresholds for SPM concentration and exposure duration,

sedimentation rates and light (De'ath and Fabricius, 2008, 2010; Collier et al., 2016a; Fabricius et al., 2016; Wenger et al., 2018). Catchment-based research has identified the key terrestrial sediment sources, the dominant contributing erosion process, and the areas where erosion rates have increased due to human development. New techniques are being developed to better isolate and characterise the most ecologically relevant terrestrial sediment fraction that may travel further in the marine environment, contribute to the development of mPOM, and impact marine ecosystems. In addition, biogeochemical models have been developed incorporating our current understanding for broader spatial and temporal coverage, to identify areas of risk within the GBR that are influenced by increased terrestrial sediment export from the adjacent catchment area (Waterhouse et al., 2017). These studies have supported guideline development for the GBR (GBRMPA, 2010), and allowed ecologically relevant end-of-basin sediment load targets to be assigned for the different basins of the GBR (Brodie et al., 2017). However to date, tPOM load contributions have not been separately considered. Our review synthesises this information to provide a more comprehensive conceptual understanding of the importance of POM and provide guidance as to how to best prioritise and manage SPM from ridge-to-reef.

This review follows the key chain-of-evidence pathway established by Bartley et al. (2014; see also Hairsine, 2017). We first describe the general impacts of SPM on reef and seagrass ecosystems, the transport and transformation processes of SPM delivered to the marine environment, and the river basin sources and composition of terrestrial sediment (Fig. 1). We then present a case study from the GBR where this information has been linked from ridge-to-reef, to establish marine thresholds, guidelines, end-of-basin load targets and basin-specific remediation options. This review therefore provides an update on the latest research tools and findings on SPM across the ridge-to-reef continuum.

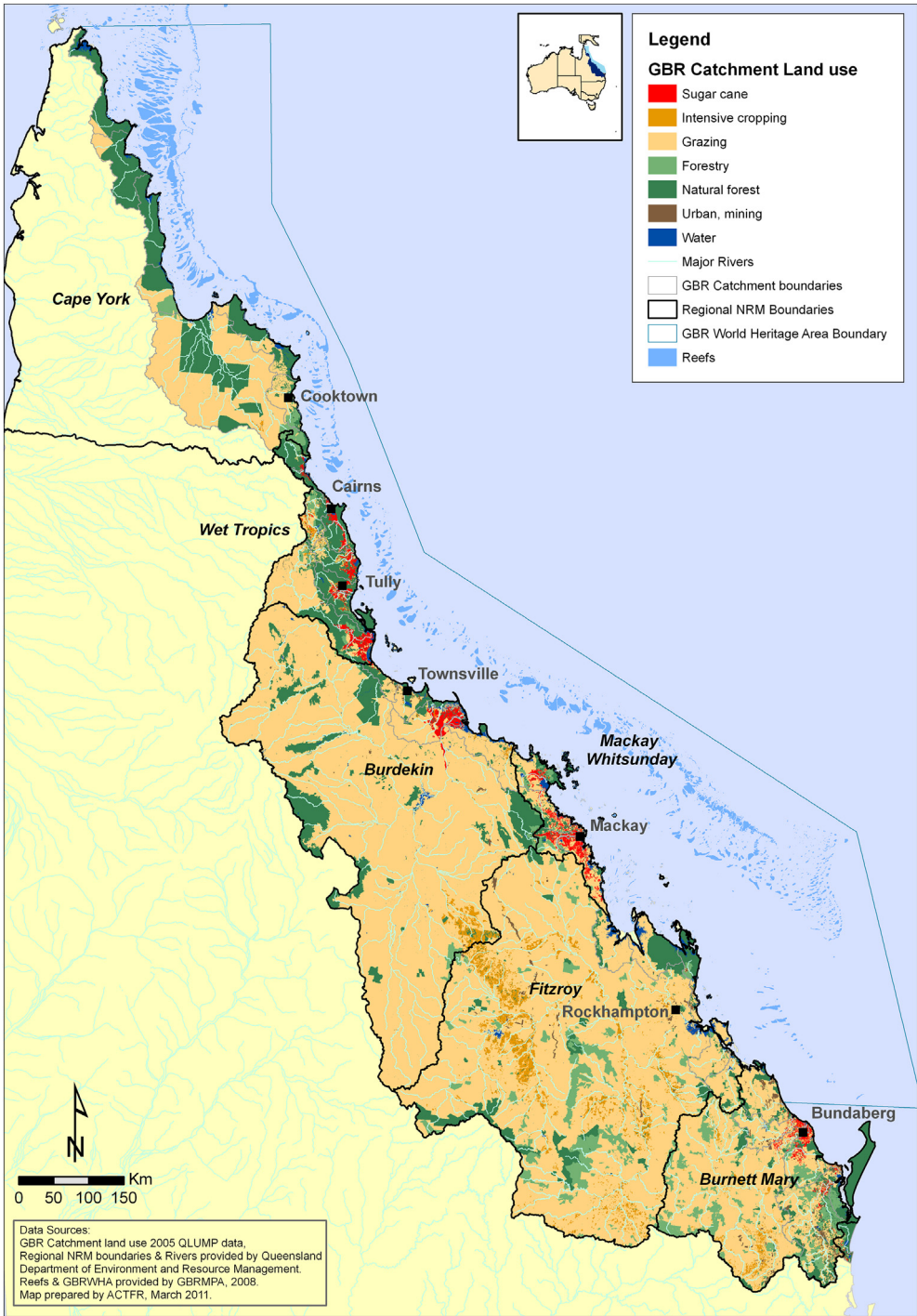Table 1 provides a quick reference to terms and abbreviations used in this review.

Fig. 2. The Great Barrier Reef catchments, primary land use and Regional Natural Resource Management Regions. Source: Brodie et al. (2012).

Table 1

Terms and abbreviations used in this review.

POM	Particulate organic matter
tPOM	Terrestrial POM: POM from catchments and river sources.
mPOM	Marine POM: POM produced by processes in the estuary or marine waters.
HfPOM	Heavy fraction POM
LfPOM	Light fraction POM
SPM	Suspended particulate matter (including POM and mineral particles)
GBR	Great Barrier Reef
CCA	Crustose coralline algae
TEP	Transparent exopolymer particles
Terrestrial sediment	Terrigenous mineral sediment and tPOM

2. Effects of SPM on water clarity and sedimentation on tropical marine ecosystems

2.1. Water clarity

SPM causes at least three separate modes of stress, namely light reduction, disturbance by suspended particles, and sedimentation (Jones et al., 2015; Duckworth et al., 2017). Importantly, SPM increases light attenuation in the water column and alters its spectral composition, therefore reducing the availability of photosynthetically usable light for benthic communities (Strydom et al., 2017). Declining water clarity (turbidity or transparency) is often the first noticeable influence of terrestrial inputs of nutrients and sediments within

coastal and inshore marine environments. Water clarity is one of the strongest water quality indicators, and a strong predictor for ecosystem changes (De'ath and Fabricius, 2010). Ecological impacts depend on the intensity and duration of exposure, the preceding and co-occurring environmental conditions and the communities affected (Collier et al., 2016a; Ferguson et al., 2017; O'Brien et al., 2017; Statton et al., 2018) (Fig. 3). Reduced water clarity can lead to slower growth or even loss of photosynthetic organisms such as coral reefs and seagrasses.

2.1.1. Coral reefs and reef fish

Some reef coral species are highly sensitive to reduced water clarity, predominantly attributable to the loss of light for photosynthesis (Fig. 3a, part 1) (Erftemeijer et al., 2012, Bessell-Browne et al., 2017). Other coral species can feed on the SPM gaining energetic advantage over others as long as light is not limiting (Anthony, 2000; Anthony and Fabricius, 2000). Sensitivity also depends on life stage, with young corals being particularly susceptible to damage from SPM on its own (i.e. without sedimentation or light reduction). SPM can negatively affect fertilisation and subsequent settlement, and the growth and/or survival of coral juveniles (Humanes et al., 2017a, 2017b). However, during times of thermal stress, SPM may be protective against bleaching by shading corals from damaging irradiance (Lesser and Farrell, 2004). Furthermore, feeding on SPM can increase the upper thermal tolerance of some corals (Gregorich et al., 2006; Courtial et al., 2017; Ferrier-Pagès et al., 2018).

The physiological and biological responses of individual corals to reduced water clarity and increased SPM can result in ecosystem-wide shifts. Studies of spatial gradients in coral reef communities have shown that the abundance, biomass and species diversity of corals, invertebrates and fish can be lower on turbid reefs than non-turbid reefs or reefs adjacent to healthier river basins (Fabricius et al., 2005; Mallela et al., 2007; De'ath and Fabricius, 2010; Rodgers et al., 2012; de Bakker et al., 2017), although this is not universally true (Fabricius et al., 2005; Bejarano and Appeldoorn, 2013; Brown et al., 2017). Shifts from corals to macroalgae (De'ath and Fabricius, 2010; Bégin et al., 2016; de Bakker et al., 2017), and at more severe conditions, shifts from macroalgae to heterotrophic filter feeders have been observed (Birkeland, 1988). Responses to reduced water clarity likely differ between species or functional groups such as feeding guilds (Fabricius et al., 2005; Brown et al., 2017).

Reef fishes can be impacted by reduced water clarity and sedimentation indirectly, by changing their coral and seagrass habitats, or directly, as increased sediment loading can have direct behavioural, sub-lethal, and lethal impacts on fish (Wenger et al., 2017). For instance, because increased turbidity can impair visual acuity, activities and processes that require vision can be inhibited, including the ability of recruiting coral reef fish to find suitable habitat (Wenger et al., 2011; O'Connor et al., 2015) and post-settlement movement (Wenger and McCormick, 2013). The ability to find suitable habitat is crucial for development and survival during the very early life history stages (Coker et al., 2009; Feary et al., 2009; Lönnstedt and McCormick, 2011), which may have significant flow-on effects for the adult population (Wilson et al., 2016). Suspended sediment and sedimentation can also inhibit fish foraging (Fig. 3a, part 6). For example, increasing levels of suspended sediment can result in reduced food acquisition (Wenger et al., 2012, 2013; Johansen and Jones, 2013), which can lead to sub-lethal and lethal impacts at high concentrations (i.e. $\geq 60\,\mathrm{mg\,L^{-1}}$) of suspended sediment. Exposure to suspended sediment can also have direct physiological consequences, including damage to gill tissue and structure (Au et al., 2004; Hess et al., 2015), which can alter metabolic rates in some species (Hess et al., 2017). Similarly, suspended sediment embedded in algal turfs suppresses herbivory on coral reefs (Bellwood and Fulton, 2008; Tebbett et al., 2017).

Most studies of the direct effects of suspended sediment on coral reef fish have been laboratory experiments rather than in situ assessments, due to the challenges of disentangling direct from indirect effects in situ (Wenger et al., 2015). Furthermore the composition of sediment used in experiments can be highly variable and do not always consider organic components (i.e. used terrigenous sediment only) and particle size (i.e. coarser fractions > 20 μm) that may have more ecological relevance. A recent in situ study found a direct effect of sediment from logging activity on the abundance of a juvenile coral reef fish (Hamilton et al., 2017), whereas Wenger et al. (2016a) found no relationship between turbidity and reef fish growth rates. Research is considering the direct effects of reduced water quality on coral reef fish. Recent work has endeavoured to develop suspended sediment thresholds for a range of life history stages of fish, which will help ensure that water quality improvement targets are ecologically relevant (Wenger et al., 2018). Consideration of reef fish is particularly important in places where communities are reliant on sensitive species, given social and economic consequences that could occur from declining fish populations (Brown et al., 2017; Hamilton et al., 2017).
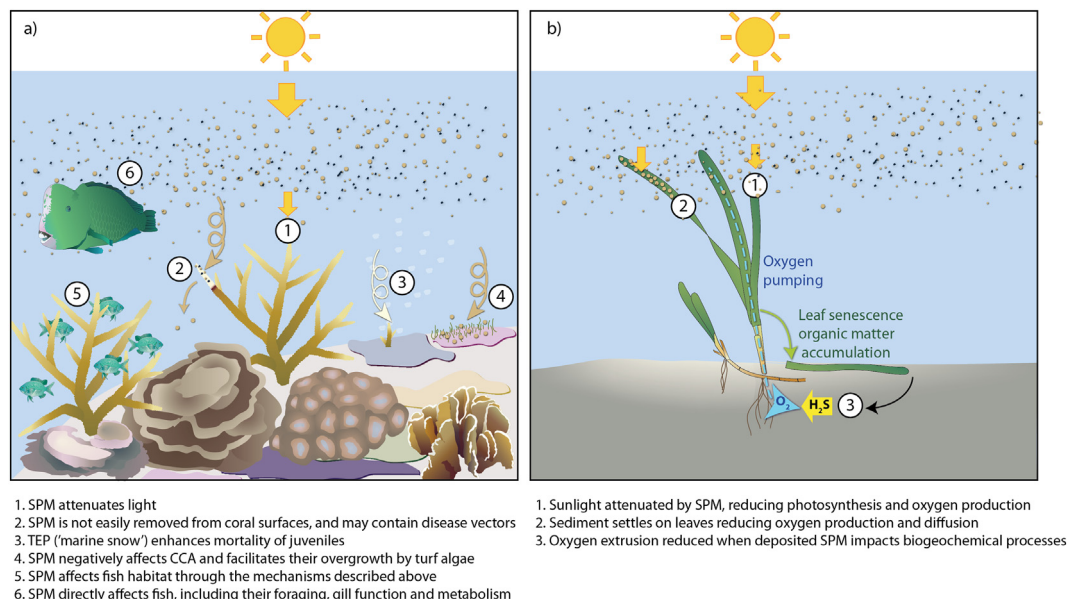

1. SPM attenuates light
2. SPM is not easily removed from coral surfaces, and may contain disease vectors
3. TEP ('marine snow') enhances mortality of juveniles
4. SPM negatively affects CCA and facilitates their overgrowth by turf algae
5. SPM affects fish habitat through the mechanisms described above
6. SPM directly affects fish, including their foraging, gill function and metabolism

1. Sunlight attenuated by SPM, reducing photosynthesis and oxygen production
2. Sediment settles on leaves reducing oxygen production and diffusion
3. Oxygen extrusion reduced when deposited SPM impacts biogeochemical processes

Fig. 3. Summary of the effects of SPM on (a) coral reefs and reef fish, and (b) seagrasses.

2.1.2. Seagrasses

In seagrass meadows, photosynthetic carbon fixation reduces under low light conditions (Fig. 3b, part 1), which necessitates biochemical, physiological and morphological changes to minimise respiratory loss (O'Brien et al., In Press) and to enhance light capture (Zimmerman, 2003; Mackey et al., 2007; Ralph et al., 2007; Collier et al., 2009; McMahon et al., 2013; Hedley et al., 2014; Schliep et al., 2015) (Fig. 3). Mortality occurs if light levels drop below minimum light requirements and reserves are depleted (e.g. Longstaff and Dennison, 1999). When benthic light is negligible (i.e. very high SPM concentrations), the time to mortality ranges from two weeks for small 'colonising' species (sensu Kilminster et al., 2015) to two years for 'persistent' species that can resist mortality by drawing on storage reserves in their large rhizomes (Collier et al., 2009; O'Brien et al., In Press). Feedbacks can enhance or subdue the effects of suspended sediment: if the seagrass patch size and canopy height is appropriate, sediment settle in the meadow, but changes in seagrass induced by high turbidity (meadow patchiness, reduced canopy height) creates a negative feedback whereby sediment cannot settle and resuspension may even be enhanced (Adams et al., 2016), leading to recalcitrant degradation (O'Brien et al., 2017).

2.1.3. Tolerance limits of corals and seagrass to SPM: water clarity

Threshold tolerance limits of SPM can be used to set water clarity targets, which can be based on the proportion of species that must be locally and regionally protected by management of SPM (e.g., 99%, GBRMPA, 2010), the extent and abundance of the habitat to be protected (Steward et al., 2005; Choice et al., 2014; Chartrand et al., 2016; Adams et al., 2015), or growth potential (Larsen et al., 2017). Threshold tolerance limits can also be used to assess ecological risk from SPM (Waterhouse et al., 2017 – Section 5.2). Tolerance limits of corals and seagrass are co-determined not only by the effects of SPM concentration on water clarity, but also the duration (Erftemeijer et al., 2012; Larsen et al., 2017) and periodicity of exposure (Browne et al., 2012). Tolerance limits also vary substantially between species, morphologies and life stages (Erftemeijer et al., 2012), and are likely to vary among populations due to acclimation and/or differences in the population structure (Erftemeijer et al., 2012; Maxwell et al., 2014; Larsen et al., 2017). In corals, they are also influenced by photo- and heterotrophic plasticity (Anthony and Fabricius, 2000). The range in these thresholds among different species has been reviewed elsewhere (e.g. Gattuso et al., 2006; Lee et al., 2007; Erftemeijer et al., 2012). However, SPM incurs at least three separate modes of action including sedimentation, light reduction, and disturbance by suspended particles (Figs. 1 and 3), and these should also be considered when setting water clarity targets. The influence of water clarity also varies depending on the physico-chemical properties at specific locations such as sulphide exposure (Ferguson et al., 2016), water temperature (Collier et al., 2016a) and pH and redox potential (Zimmerman et al., 1997). Specific SPM properties including organic content, particle size, microbial colonisation, elementary composition and flocculation are also likely to affect water clarity tolerance thresholds.

2.2. Sedimentation

2.2.1. Coral reefs

Corals have long been known to be sensitive to sedimentation – the deposition of suspended particles on their surfaces (Fig. 3a) (Rogers, 1990; Fabricius, 2005; Erftemeijer et al., 2012). Tolerance to sedimentation varies between life stages and taxa, and depends on the amount and duration of exposure (Philipp and Fabricius, 2003; Flores et al., 2012). Even the recruitment of some macroalgae such as *Sargassum* is affected by sedimentation (Umar et al., 1998). Coral responses to sedimentation are also strongly dependent on the sediment properties: particle size, contents of organic matter, nutrients, transparent exopolymer particles (TEP, also known as 'marine snow'), and industrial pollutants all co-determine the fate of coral colonies when exposed to sedimentation (Philipp and Fabricius, 2003; Weber et al., 2006, 2012).

Corals can remove coarse calcareous sediment with relative ease, without lasting damage to their surfaces, while they take longer to remove fine and organically-rich sediment; hence the latter often cause colony damage or even mortality (Fig. 3a, part 2) (Weber et al., 2006). Complex microbial successions reflecting sediment biochemistry and nutrient status may develop in the sediments, and the metabolic products of these microbes can contribute to hasten coral damage (Weber et al., 2012). Sediment can also be the carriers of vectors for coral diseases, with disease incidence doubling in areas exposed to elevated levels of sedimentation from dredging events (Pollock et al., 2016). Furthermore, corals that are exposed to suspended sediment enriched with organic matter have a significantly reduced thermal tolerance, with earlier onset of bleaching under heat stress and slower recovery (Fabricius et al., 2013). Conversely, shading of corals via increased turbidity can reduce the UV component of solar radiation and hence lower the severity of bleaching (van Woesik et al., 2012; Morgan et al., 2017). The dredging of seafloor sediment stresses corals through smothering by sedimentation, reduction in light, and suspended sediments, as well as the chemical alteration of conditions (reduced pH, O_2), with the light reduction considered the most fatal impact of dredging on corals (Jones et al., 2015, 2016).

Young corals are particularly susceptible to smothering, especially when sediment contains high concentrations of TEP (Fig. 3a, part 3). SPM on its own (i.e., without sedimentation or light reduction), can negatively affect gamete fertilisation and the growth and survival of coral recruits (Ricardo et al., 2015; Jones et al., 2015; Humanes et al., 2017a). Young *Acropora* were found to sustain high rates of mortality after < 48 h when exposed to sediment enriched with TEP, while no damage was sustained when the same juveniles were exposed to sediment without TEP enrichment (Fabricius et al., 2003). Overall, sedimentation thresholds for young corals appear to be an order of magnitude lower than for adult corals, around 10 mg cm^{-2} d^{-1} (Fabricius et al., 2003).

Crustose coralline algae (CCA) are also known to be highly sensitive to sedimentation, to low pH, and to overgrowth by turf algae which trap sediment (Purcell, 2000; Bessell-Browne et al., 2017) (Fig. 3a, part 4). CCA are filamentous red algae, heavily calcified by calcite embedded in their cell walls. In coral reefs, CCA contribute to limestone formation and the reinforcement of reef surfaces, counteracting wave erosion. Their surface properties also induce the settlement of larvae of corals and many other benthic organisms. CCA cover on the GBR is very low (< 1% cover) on near-shore reefs with high rates of sedimentation, and high (> 20% cover) on offshore clear-water reefs away from coastal influences (Fabricius and De'ath, 2001a). Sediment properties including nutrient content contribute to determining the vulnerability of CCA to sedimentation, as the associated nutrients can facilitate filamentous cyanobacteria, which then outcompete the CCA via overgrowth (Littler et al., 2010). Even mild levels of ocean acidification can detrimentally affect the reproduction and growth of CCA (Bradassi et al., 2013). Organic-rich sediment also can contribute to this effect, whilst calcareous offshore sediment do not alter the pH conditions in their boundary layer by much, and potentially provide a buffer to protect the CCA from dissolution.

2.2.2. Seagrasses

Seagrasses often grow in sediment with low redox potential so they need to pump oxygen through their aerenchyma (i.e. spongy plant tissue) to their below-ground tissues to protect against phytotoxic compounds such as hydrogen sulfide (Brodersen et al., 2015; Enríquez et al., 2001). This process requires oxygen from the leaves, and can be impeded by suspended and settled sediment that either slow photosynthetic oxygen production, or the passive diffusion of oxygen into leaves (Fig. 3b, part 2) (Carlson et al., 1994; Enríquez et al., 2001; Brodersen et al., 2017), which is particularly harmful at night (Olsen

et al., 2018). Fine sediments settled on the surface of leaves can also physically block light penetration to the leaves, enhancing light limitation from suspended sediment (Erftemeijer and Lewis III, 2006).

Sedimentation has another negative feedback on this process as it increases nutrient and organic matter accumulation and enhances the reducing potential of the sediment (Fig. 3b, part 3) (de Boer, 2007). In addition, reduced exudation of oxygen alters the composition of the putative beneficial microbiome around the seagrass roots (Martin et al., 2017) and burial of seagrass from deposited sediment can cause mortality (Campbell and McKenzie, 2004). Deep fine sediment (> 5 cm) deposited on seeds can prevent the emergence of seedlings, hampering recovery from sedimentation events (Jarvis and Moore, 2015) and nutrients associated with fine sediment and organic carbon become biologically available over time, releasing inorganic nitrogen and phosphorus into the water column (Radke et al., 2010), which may drive increased phytoplankton growth, contributing to further shading (Webster et al., 2006).

3. Fine sediment and particulate organic matter in the marine environment – transport, composition and fate

3.1. Estuarine mixing

The transport of terrestrial sediment to the ocean predominately occurs during elevated rainfall-driven river flow events, leading to flood plumes typically of short duration (i.e. < 3 weeks) (Devlin and Brodie, 2005). The extent and movement of the river flood plumes in the marine environment depends on the flow volume, wind speed and direction, ocean currents and Coriolis forcing (Devlin and Brodie, 2005). The transport, deposition and resuspension of SPM depend on several physical and bio-geochemical processes. The reduction in flow velocity and change in ionic strength in the early estuarine mixing zone (salinity 0–5) promotes flocculation and deposition of almost all the coarser material (approx. > 20 μm) and a proportion of the nutrient-enriched finer sediment load within the estuary or near the river mouth (Droppo, 2001; Dagg et al., 2004; Bainbridge et al., 2012; Fettweis and Lee, 2017). Coral reef and seagrass ecosystems do not typically occur in this turbid, low salinity estuarine zone with the exception of some intertidal seagrass species. Coral reef and seagrass species do, however, occur within the zone of influence of river flood plumes, where they are affected by the SPM transported offshore during elevated river flows. During and following flood events, flocculation facilitates the settlement of this terrestrial material and plankton successions (i.e. marine-derived POM) develop from newly delivered and newly released nutrients (Mayer et al., 1998; Dagg et al., 2004; Devlin et al., 2012; Lewis et al., 2014; Franklin et al., 2018).

The composition of SPM carried further offshore by river plumes can consist of individual, fine-grained clay mineral particles or flocculated aggregates (i.e. flocs) bound with both living (microbes, plankton) and non-living (fecal pellets, detritus and its decomposed microbial activity) organic matter, of terrestrially-derived or marine origin (see Droppo, 2001; Fettweis and Lee, 2017). In these biologically-enriched coastal and inshore waters, the bio-mediated production of 'marine snow' (i.e. larger biological aggregates) also occurs where sticky biomass (e.g. TEP) aggregates existing mineral and bio-mineral flocs, further altering rates of sedimentation, resuspension and deposition (Fettweis and Lee, 2017; Lefebvre et al., 2018). Flocs (typically 10–100 μm) and larger marine snow often have different properties (i.e. lower density, organic-rich etc.) compared with the old, consolidated and less-flocculent sediment found on the seafloor, are easily resuspended and strongly alter the light attenuation in the seawater (Storlazzi et al., 2015). Hence, newly delivered terrestrial sediment appears to affect water clarity more readily than the resuspension of historic seafloor sediment (Fabricius et al., 2013, 2014; Lewis et al., 2014; van Maren et al., 2014; Seers and Shears, 2015).

3.2. Marine transport, storage and fate

The geomorphology and bathymetry of the coastline and adjacent submerged continental shelf largely governs the fate of fine terrestrial sediment in the marine environment. Such marine environments can loosely be grouped into open, semi-open and semi-enclosed systems (Delandmeter et al., 2015), with semi-open and semi-enclosed systems trapping a proportion of terrestrial sediment before reaching the open ocean. The distance to a river mouth is another important factor which influences exposure to terrestrial sediment. For example, the nearshore reefs of Papua New Guinea, Philippines, Indonesia, Puerto Rico and Costa Rica reside on narrow submerged continental shelfs close to the coast and can regularly be subjected to direct riverine inputs. Catchment disturbances including mining, logging or agriculture can greatly increase sediment loads with considerable sedimentation damage on nearshore ecosystems including coral reefs (e.g. Cortés and Risk, 1985; Edinger et al., 1998; Acevedo et al., 1989; Munday, 2004; Fabricius, 2005; Ryan et al., 2008; Haywood et al., 2016). Resuspension of deposited sediment has also reduced water clarity on coral reefs from Hawaii and Puerto Rico (Storlazzi et al., 2004; Bothner et al., 2006; Hernández et al., 2009), but much of the terrestrial sediment carried within flood plumes or resuspension events is quickly transported further offshore from these narrow shelfs to much deeper waters (> 150 m) and deposited with little potential for further resuspension.

The Great Barrier Reef can serve as an example to illustrate fates of newly imported sediment on wide continental shelf environs. Here, the fine terrestrial sediment can be transported large distances (> 50 km) in flood plumes, may be deposited in relatively shallow water (< 20 m depth) and become available for wave resuspension (Orpin et al., 1999). While wave resuspension is largely limited to the inner shelf out to the 20 m isobath (Orpin et al., 1999), strong longitudinal currents during tropical cyclones can also resuspend material on the middle shelf (Larcombe and Carter, 2004; Carter et al., 2009).

Sediments are strongly partitioned across the GBR shelf in response to oceanography, geomorphology and storms, resulting in three distinct cross-shelf zones: the inner-shelf dominated by terrestrial sediment (0–22 m water depth), the sediment-starved mid-shelf zone of mixed palimpsest (old, reworked) terrestrial and carbonate sediment (22–40 m), and the carbonate-rich outer-shelf (> 40 m) (Maxwell, 1968; Belperio, 1983; Belperio and Searle, 1988; Larcombe and Carter, 2004). Tropical cyclones influence this partitioning by transporting fine terrestrial sediment to the inner-shelf (from heavy rainfall in the catchment), and by creating the high energy conditions that rework and transport inshore deposited sediments (Larcombe and Carter, 2004; Delandmeter et al., 2015). Even during large cyclonic events, the majority of terrestrial sediment including organic carbon and terrestrial biomarker chemicals are deposited within 15 km from the coastline (Sandstrom, 1988; Ford et al., 2005). A study of a wet tropical GBR river flood event following Tropical Cyclone Winifred (1986) found the majority of terrestrial plant detritus (organic matter) exported from the Johnstone River to be deposited within 2 km of the river mouth (Gagan et al., 1987).

Within the inshore zone (e.g. Keppel Bay) there is continual remineralisation of organic matter long after its delivery and deposition following flood events (Radke et al., 2010). This tidal estuarine zone is an area of high physical and biological activity, and as the terrestrial sediment is continually reworked nutrients are released for many months afterwards (Alongi and McKinnon, 2005; Robson et al., 2006; Radke et al., 2010).

Most fine SPM inputs over the past 8000 years in the GBR are likely stored within the inshore and coastal zone (0 to 20 m depth) including river estuaries, coastal deposits such as mangroves, intertidal and subtidal mud flats, within the framework of inshore coral reef deposits, and bay fill deposits (Belperio, 1983; Carter et al., 1993; Orpin et al., 2004; Brooke et al., 2006; Browne et al., 2012; Lewis et al., 2014; Delandmeter et al., 2015; Ryan et al., 2016). In the GBR, bay-fill

deposits are most extensive within north-facing embayments (i.e. sheltered from prevailing SE winds) or bays which receive inputs from the large river basins (Belperio, 1983; Orpin et al., 2004; Brooke et al., 2006). Much of this older SPM is likely to be stripped of its bioavailable nutrient content and buried at depths that limit resuspension and hence is fairly inert in the marine environment. However, the exact 'residence time' of this SPM in the GBR is uncertain (e.g. Brodie et al., 2012).

4. River basin sources and transport of fine sediment and particulate organic matter

4.1. Fine terrigenous sediment

Soil erosion can occur through physical or biogeochemical processes generally related to local climate, topography, tectonics, geology and land use (Verheijen et al., 2009). Due to the non-linear interaction between these variables, soil erosion rates are extremely variable in time and space (García-Ruiz et al., 2015). Once soil is eroded and enters a waterway, sediment (detached soil) can move in solution, in suspension or as bedload.

Globally, humans have increased the transport of riverine eroded sediment by 2.3 ± 0.6 billion MT per year (Syvitski et al., 2005). The erosion, transport and delivery of sediment from agricultural areas to coastal ecosystems are complex (Bartley et al., 2014) and the opportunity for the deposition and storage of sediment is significant (Fryirs, 2013). This means that high rates of erosion do not necessarily correspond to high rates of sediment delivery (Walling, 1983, 1999). Therefore, understanding and predicting the delivery of sediment from ridge-to-reef is more complicated than simply predicting catchment erosion risk. Understanding of the hydrological regime and connectivity of sediment from the erosion source to the basin outlet is required (Bracken et al., 2015). Basin size, shape, river network pattern and rainfall-runoff regime all influence the ability of sediment to be delivered to the marine system. Changes in climate and tectonic activity can influence erosion and sediment delivery (Bartley et al., 2018), and some areas can generate large volumes of sediment without human disturbance. For example, steep, wet forested areas may have similar sediment yields to flat, dry grazing pastures.

The rates from different land use types should not necessarily be compared to each other, but instead how much these rates have changed over time at a single location. The evaluation of contemporary (< 100 year) erosion rates at a site needs to include the contribution of the natural, long term (> 100 year) or background erosion rates. Increasingly, techniques such as cosmogenic isotopes, OSL dating and similar approaches are being used to benchmark natural and accelerated erosion rates due to land use impact (Hewawasam et al., 2003; Gellis et al., 2004; Bartley et al., 2015; Coates-Marnane et al., 2016). Such approaches could also be used to bench-mark the recovery process or assess reductions in soil erosion as a result of remediation (e.g. Vanacker et al., 2007).

Soil properties (e.g. particle size and density), vegetation cover and topography are also important factors controlling sediment production from erosion processes such as gullying and landslides (Zhao et al., 2016; Loch et al., 1998). Insights into the delivery of sediment from ridge-to-reef have increased with the use of geochemical fingerprinting techniques which link the chemical signature of the marine sediment to soil and sediment within the basin. Studies from Australia and other parts of the world suggest that marine sediment are often dominated by basaltic soils (Douglas et al., 2003, 2006; McCulloch et al., 2003a; Takesue and Storlazzi, 2017), however, soils formed on granitic and sedimentary lithologies can also contribute to marine systems (Bainbridge et al., 2016). In some cases geochemical tracers have also been useful to demonstrate that specific rivers are not contributing sediment to marine areas, often because these rivers are carrying sediment composed of coarse quartz dominated material (Araújo et al., 2002). The larger and more geologically and hydrologically diverse a

basin is, the more challenging it can be to isolate source lithologies (Maher et al., 2009). This is because numerous chemical, biological and physical processes can alter sediment as it moves through a basin disconnecting the source to sink process (Koiter et al., 2013; Laceby et al., 2017).

4.2. Terrestrially-derived particulate organic matter

Terrestrially-derived POM is produced in river basins through different mechanisms including plant senescence (Sollins et al., 1985; Webster et al., 1999), soil formation (Fontaine and Barot, 2005; Kemmitt et al., 2008) and freshwater productivity (Vannote et al., 1980; Webster and Meyer, 1997; Thorp and Delong, 2002). Soil erosion contributes to the export of large quantities of tPOM downstream (Ludwig and Probst, 1996; Lal, 2003; Beusen et al., 2005), redistributing landscape tPOM laterally, vertically and/or longitudinally from ridge-to-reef (Gregorich et al., 1998; Ran et al., 2014; Ma et al., 2016).

There is large variability (spatial and temporal) in the quantity and type of tPOM delivered to streams (Kendall et al., 2001; Cross et al., 2005; Tank et al., 2010), characterised by two distinct fractions: light fraction POM (LfPOM), constituted by a mixture of the remains of plants, animals and microorganisms at various stages of decomposition (Gregorich et al., 2006) and heavy fraction POM (HfPOM), which is attached to fine mineral sediment particles (i.e. silt and clay) through chemical bonds (Horowitz and Elrick, 1987). Climate has been identified as a major control of tPOM, not only affecting basin and riverine erosion dynamics (Valentin et al., 2005; McKenzie-Smith et al., 2006; Jung et al., 2012), but also direct inputs from riparian and catchment vegetation (Tank et al., 2010; Rowland et al., 2017).

Forested basins have significantly higher LfPOM inputs than non-forested basins (Golladay, 1997; Webster and Meyer, 1997). Deforestation and land-use change affect the relative proportions of LfPOM and HfPOM fractions in tPOM exported by rivers (Beusen et al., 2005; Ochiai et al., 2015), with an increased contribution from the HfPOM fraction associated with increased soil erosion and reduced vegetation biomass inputs (Kao and Liu, 2000; Garzon-Garcia et al., 2017). Additionally, land use change modifies the dominant vegetation type in catchments affecting both the LfPOM and HfPOM components of soil, with consequent changes in the characteristics of tPOM exported downstream (Garten and Ashwood, 2002; Marwick et al., 2014) and tPOM in-stream processing (McTammany et al., 2003; Dodds et al., 2004; Kominoski et al., 2007). In river basins severely affected by erosion, the source of sediment (surface versus subsurface erosion) and soil type are important controlling factors on tPOM contributions and dynamics (Garzon-Garcia et al., 2015, 2018).

The riverine transport of tPOM is complex and mediated by fractionation and mixing, deposition or sourcing in riverine habitats and biological transformations which change tPOM characteristics (Blair et al., 2004; Tank et al., 2010; Bouwman et al., 2013), often resulting in downstream tPOM composition different from that at its source (Webster et al., 1999; Gomez et al., 2010; Garzon-Garcia et al., 2017). tPOM biological instream processing is influenced by its chemical and physical properties, the biota and environmental factors (e.g., temperature, nutrients, redox) (see review by Tank et al., 2010).

As the various fractions of tPOM have different bioavailabilities to microbial mineralisation (Mayer et al., 1998; Bianchi and Bauer, 2011), the relative mix of LfPOM and HfPOM has an important effect on tPOM bioavailability during river transport and as it is deposited in stream habitats and floodplains (Tank et al., 2010; Guenet et al., 2014). LfPOM has a higher carbon (C) to nitrogen (N) ratio than HfPOM (Rowland et al., 2017) and N mineralised during LfPOM decomposition tends to be re-immobilised by microflora (Sollins et al., 1984) enhancing N retention in rivers (Dodds et al., 2004). Depending on its decomposition stage it can provide fresh C and other nutrients that can fuel microbial processes in rivers and downstream marine ecosystems (Guenet et al.,

2014). HfPOM has narrower C to N ratios and decomposes more slowly than LfPOM, but has larger net N mineralisation than the LfPOM in parent soils (Sollins et al., 1984). LfPOM decomposition rates vary widely depending on N and P content, lignin, tannins, leaf structure and particle size (Wang et al., 2004; Yoshimura et al., 2008; Tank et al., 2010). HfPOM decomposition rates depend on sediment particle size and intrinsic sediment properties (e.g., organic matter composition, C and N content, clay mineralogy) (Mayer et al., 1998; Bianchi and Bauer, 2011).

Indeed, the composition of the tPOM fractions would likely have considerable influence on the SPM in the estuarine and marine zone and specifically on the development and formation of sediment flocs, bioavailable nutrient generation rates and loads and consequent effects on marine ecosystems.

5. Tools for guiding management: case study in the Great Barrier Reef, Australia

Using the 'ridge-to-reef' approach outlined above, this section presents a case study of how current knowledge of reduced water clarity and sedimentation in the GBR lagoon and the drivers of these conditions can be used in conjunction with ecological risk assessment, and the definition of pollutant load reduction targets to guide management priorities for SPM in the GBR region. This application can be used to inform management of tropical marine ecosystems and the associated pressures from external influences such as land runoff.

5.1. Evidence of reduced water clarity and sedimentation in the GBR lagoon

Increased delivery of terrestrial sediment to the GBR lagoon following mining and agricultural development of the catchment (c. 1850) have been well documented using a variety of approaches including sediment cores (Walker and Brunskill, 1997; Lewis et al., 2014), coral cores (Lewis et al., 2007) and modelling (Kroon et al., 2012). This change also coincides with an increase in the frequency of large and extreme freshwater discharge events to the GBR (Lough et al., 2015), resulting in an increase in loads of terrestrial constituents to be exported and likely an increase in the extent of influence within the GBR lagoon. Distinctive changes in water quality parameters, biological assemblages and coral core geochemistry have been observed across terrestrial gradients with distance from the river mouth of many developed catchments in the GBR (van Woesik et al., 1999; Fabricius et al., 2005; Udy et al., 2005; Cooper et al., 2007; Lewis et al., 2012). While it is challenging to quantify the changes in these gradients as a result of increased terrestrial inputs, long coral core records document enhanced terrestrial influence over the past 150 years (McCulloch et al., 2003b; Lewis et al., 2007, 2012, 2018; Jupiter et al., 2008; Lough et al., 2015). Furthermore, declines in seagrass meadow area and abundance, coral condition and certain reef fish have been measured following large river discharge events (Preen et al., 1995; Collier et al., 2012; Petus et al., 2014; Thompson et al., 2014; Wenger et al., 2016b) which are likely associated with prolonged reductions in water clarity (Fabricius et al., 2016). Daily remote sensing observations over 12 years show that water clarity throughout the inshore and midshelf GBR remains significantly reduced for up to 6–8 months in years with high river sediment discharges but not in years with low river discharges (Fabricius et al., 2016). Reduced water clarity (increased turbidity) is also strongly associated with reduced species diversity of hard corals and octocorals, increased cover of macroalgae and reduced crustose coralline algae on the inshore GBR (Fabricius and De'ath, 2001a, 2001b; De'ath and Fabricius, 2010).

Recent advances in optical models have enabled accurate mapping of the areas impacted by sediment deposition and reduced water clarity associated with river flood plumes (Baird et al., 2016b; Brodie et al., 2017). They also facilitate disentanglement of the influences of discharges from various rivers and sediment resuspension due to winds

and tides, highlighting the substantial role of resuspension in the observed nearshore optical conditions (Baird et al., 2017; Wolff et al., 2018). The eReefs marine models for the GBR (Baird et al., 2016a; Herzfeld et al., 2016; Skerratt et al., 2018) provide large-scale, near real-time modelling of relevant processes in open access (http://ereefs. info) at a level of detail not previously available. The modelled processes include transport, deposition and resuspension of tPOM, mPOM and mineral sediment; adsorption and desorption of nitrate from mineral sediment surfaces; remineralisation of tPOM and mPOM; generation of mPOM through uptake of inorganic nutrients by phytoplankton, planktonic processes; and the effect of each SPM component on light quality and PAR through the water column.

Recent work with these models has demonstrated the possible role of very fine mineral sediment and organic flocs in delivering terrestrially-derived materials to the outer reef (Margvelashvili et al., 2018), though reduced water clarity in the mid- and outer reef following high-flow years could also be explained by transport of nutrients in the form of phytoplankton (Robson et al., 2017).

5.2. Assessing ecological risk for prioritised ridge management

Ecological risk-based assessments are used globally to guide management of coastal and marine ecosystems (e.g. Burke et al., 2011; Doubleday et al., 2017; Pittman et al., 2017; Brodie et al., 2013; Waterhouse et al., 2012, 2017), and are often used to guide where and how management interventions could reduce stressors (SETAC, 1997). The assessment frameworks are heavily dependent on knowledge of the current status of pressures and drivers of an ecosystem state, and the impacts of those pressures on the receiving environment.

A key example in the GBR is the recent work of Waterhouse et al. (2017) which assessed the likelihood of reduced water clarity linked to fine terrestrial sediment inputs, and the associated consequence of this exposure to seagrass. Predicted dispersion of end-of-basin fine sediment loads and frequency of exposure to turbid conditions were included as a proxy for SPM. In addition, the difference between modelled estimates of current annual light attenuation and pre-development light attenuation scenarios were used to assess 'anthropogenic influences' of SPM.

Reduced light was used as an example of the consequence of fine sediment exposure to seagrass habitat (Waterhouse et al., 2017). The consequence of fine sediment exposure was calculated using modelled estimates of when benthic light (eReefs outputs) did not meet light thresholds ($< 6\,\text{mol photons}\,\text{m}^{-2}\,\text{day}^{-1}$ for 42–100% days in the whole year (Collier et al., 2016b)), as seagrass loss is almost certainly expected if fine sediment loads increase in these habitats (see Fig. 4b). The most frequent failure to meet the benthic light threshold (Fig. 4b) was on the inner shelf next to basins with high anthropogenic fine sediment loads and in seagrass habitats influenced by the Burdekin River. The Burdekin River ultimately poses the greatest potential risk to seagrass habitat in the GBR, calculated as the product of the likelihood of SPM exposure and the consequence of reduced light (Fig. 4c). This highlights the Burdekin River basin as a priority area for management. Marine areas receiving discharge from the Burdekin River in the northern GBR are most likely to be exposed to higher concentrations of SPM than in other regions (Fig. 4a) (Waterhouse et al., 2017). This is related to the relatively large total runoff volumes from the Burdekin River, steeper and dissected terrain in areas close to the coast, and a mixture of vulnerable soil types that have had a range of land use disturbances over the last century (Bainbridge et al., 2014; Furuichi et al., 2016; Bartley et al., 2018).

In this step, the relevance of on-ground management priorities and outcomes to SPM is constrained by not accounting for the end-of-basin nutrient contributions (i.e. tPOM) that are likely to contribute to particulate organic matter production in the marine environment.

These results are being used by the Australian and Queensland governments to guide management priorities in the current GBR water
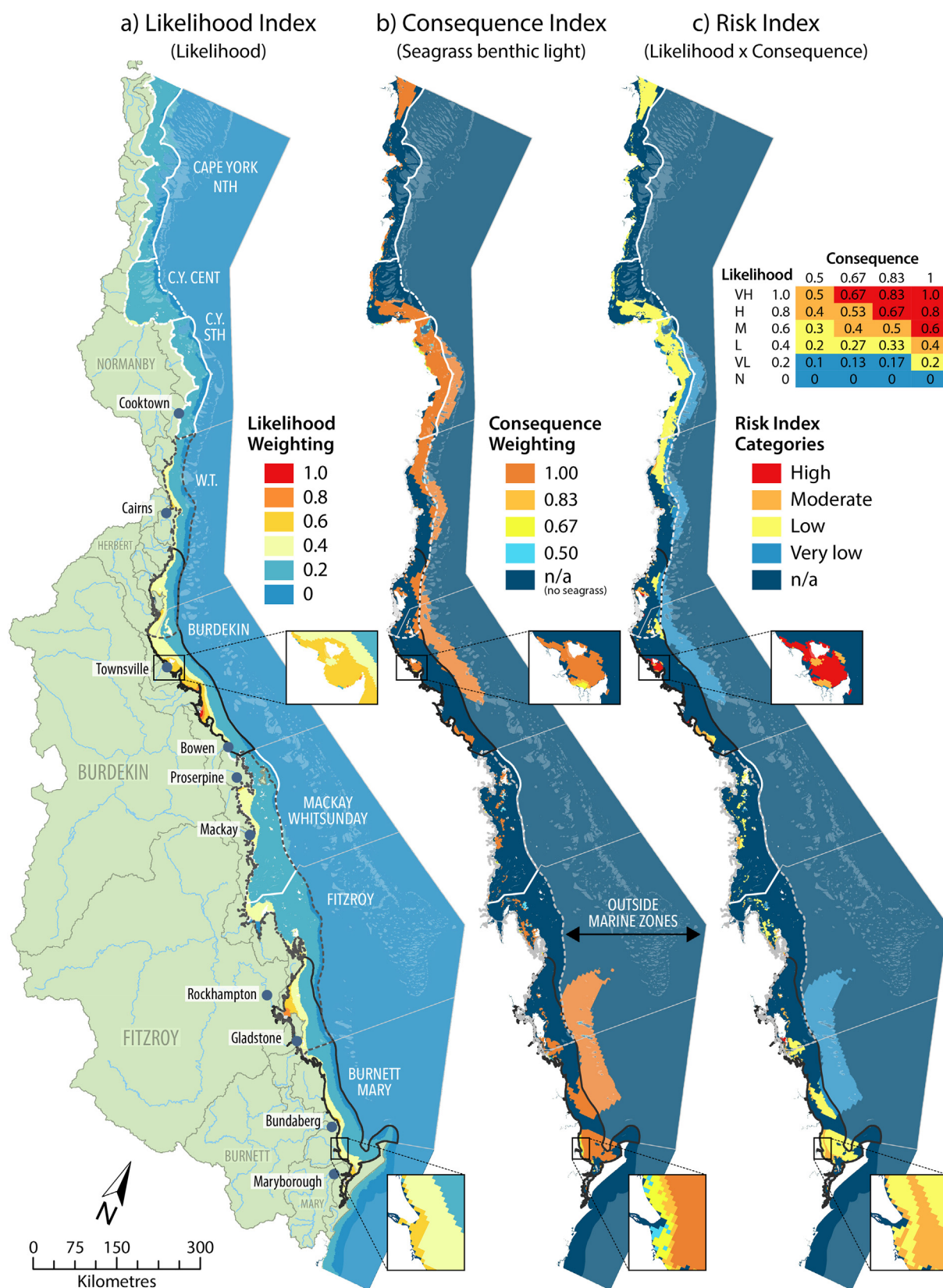
Fig. 4. Inputs representing a) <u>likelihood of exposure</u> of anthropogenic fine sediment to seagrass, b) an example of the <u>consequence of exposure</u> using an analysis of the failure to meet benthic light thresholds for seagrass, and c) the estimated <u>risk</u> from anthropogenic fine sediment to seagrass. Source: Waterhouse et al. (2017).

quality policy, the Reef 2050 Water Quality Improvement Plan (Queensland and Australian governments, 2017). This policy recognises that the ability to reduce ecological risk is largely driven by the reduction of anthropogenic pollutant delivery to the GBR, and hence, the

government has defined end-of-basin load reduction targets (Brodie et al., 2017). The targets are calculated as the reduction in the modelled annual average tonnes of fine sediment at the end-of-basins, and expressed as a percentage reduction from a baseline anthropogenic load

(2013) to be achieved by 2025 (Brodie et al., 2017). The basin-specific reductions range from 40% to 0% (i.e. the need to maintain current levels), and the basins with the highest fine sediment reduction requirements align with the results of the risk assessment outlined above (Waterhouse et al., 2017).

5.3. GBR basin sources and prioritised on-ground management

Identification of basin sources that contribute the greatest risk from fine terrestrial sediment to GBR ecosystems enables catchment managers to focus their efforts for prioritised on-ground management. As described above, the Burdekin basin contributes the highest loads of fine terrestrial sediment delivered to the GBR (McCloskey et al., 2017) and hence presents the highest risk in terms of ecological impact (Waterhouse et al., 2017). However, the Burdekin basin is large (130,000 km²; the same size as England) and identifying which parts are contributing most to the excess sediment was needed to help prioritise areas for targeted remediation.

A number of studies have identified the Bowen-Bogie-Broken (BBB) and Upper Burdekin tributaries as major sources of river runoff and sediment (noting this largely applies only to the mineral sediment as organic matter sources have not been systematically evaluated at the whole-of-basin scale). These studies have used a combination of mineral magnetics (Maher et al., 2009), geochemical tracing (Furuichi et al., 2016) and clay minerology tracing data (Bainbridge et al., 2016) as well as monitoring data (Bainbridge et al., 2014). The Upper Burdekin dominates overall freshwater runoff and has a considerable sediment load (Bainbridge et al., 2014), however, the BBB basin dominates both increased anthropogenic sediment yield (Bartley et al., 2015) and fine sediment yield (Bainbridge et al., 2016). This is largely because a considerable proportion of the sediment, and particularly the coarser size fractions, transported from the Upper Burdekin are trapped in the Burdekin Falls Dam (Lewis et al., 2013). Bartley et al. (2015) estimated that, on average, current sediment yields in the BBB area have increased ~7.47 ± 3.71 times over long term (~100 to > 10,000 years) erosion rates.

To understand which erosion processes are contributing to the excess sediment, fallout radionuclide studies using ¹³⁷Cs and ²¹⁰Pb have been carried out in the Bowen catchment, which suggest that sediment is delivered from a relatively small proportion of the catchment which has vulnerable soils that are well-connected to the stream network. These are primarily where subsoil is exposed in scalds, rills and gullies (Wilkinson et al., 2013, 2015; Hancock et al., 2014). It is possible to identify these features in the landscape, but the critical gap in our understanding is the effectiveness of erosion remediation options for controlling these sources of sediment at property and sub-catchment scales.

Organic matter sources associated with terrestrial sediment export have received less attention in the GBR catchments. A recent isotope tracing study in Moreton Bay, 300 km south of the GBR, identified tree vegetation litter as a dominant source of tPOM (measured as organic carbon), even though grasses dominate the vegetation cover in this catchment, and subsoil erosion is the main sediment source in the Moreton Bay catchments (Garzon-Garcia et al., 2017). This suggests that although sub-surface sediment dominates the mineral sediment contribution to freshwater and marine areas, surface erosion and litter input (i.e. LfPOM) may dominate the source of tPOM. Further understanding of these source contributions in GBR catchments is required for effective on-ground management of both mineral sediment and tPOM (see also Judy et al. 2018).

Given the strong scientific evidence demonstrating that the Burdekin basin, and the BBB catchment within the Burdekin, is a major source of anthropogenic sediment delivering to high risk marine areas, the Queensland Government through the Queensland Reef Water Quality Program allocated $15 million to the BBB catchment known as the 'Landholders Driving Change' Major Integrated Project (https://ldc.

nqdrytropics.com.au/). This funding is being used to promote adoption of improved erosion management practices, and to trial a range of land remediation practices to help reduce the amount of runoff and erosion coming from this area.

Conceptually, erosion can be mitigated by (i) managing the distribution and timing of grazing pressure away from erosion features like scalds, gullies and streambanks, and by setting stocking rates that maintain ground cover and forage to reduce surface runoff that fuel erosion processes (Thorburn and Wilkinson, 2013; Thorburn et al., 2013; Kroon et al., 2016), and/or (ii) direct site remediation of large scale alluvial gullies. A number of projects are currently evaluating the effectiveness of these approaches on land condition, erosion and sediment loss. These approaches will be evaluated against their contribution to the Reef 2050 Water Quality Improvement Plan fine sediment load targets for the Burdekin basin, which is a 30% reduction in the anthropogenic fine sediment load by 2025 (Queensland and Australian governments, 2017). The project also has a strong socio-economic foundation acknowledging that humans, and the industries that sustain them (grazing, farming and mining) are an integral part of the system.

5.4. Current ridge-to-reef management gaps and concluding remarks

Considerable progress has been made over the past decade to understand the sources, transport, transformations, fate and effects of SPM across the ridge-to-reef continuum for the GBR, and to conceptualise these components into a framework for effective management prioritisation. However, there are still avenues for further investigation to better refine and improve our knowledge of the GBR ridge-to-reef continuum including our understanding of SPM sourcing and transport processes, the way we currently monitor and model SPM including terrestrial sediment, tPOM and mPOM contributions, our ability to manage for the most ecologically relevant components, and their thresholds:

GBR sources, **transport**, **fate and associated ecological effects of SPM**

- There is limited characterisation of the SPM component in river flood plumes that impinge on coral reef and seagrass meadows. Better characterisation will (i) refine source identification (to erosion type and LfPOM or HfPOM), (ii) improve our understanding of the bioavailability, transport potential (i.e. including role in marine snow formation) and fate and (iii) clarify the likely impact on the health of marine ecosystems.
- The influence of increased terrestrial sediment runoff on GBR turbidity regimes, and specifically the resuspension potential of newly delivered SPM compared to the existing and 'abundant bay-fill' sediment on the seafloor requires further refinement (see Orpin et al., 2004; Lewis et al., 2014; Fabricius et al., 2014, 2016). Quantifying the influence of 'newly delivered' SPM on GBR turbidity regimes is the subject of current research activity.
- Further, the contribution of dissolved and particulate nutrients on the formation of mPOM in association with terrestrial fine sediment has not been quantified, and hence its influence on water clarity (i.e. turbidity) and sedimentation in the marine environment is constrained. Although high river loads do seem to be associated with reduced water clarity in the GBR throughout the year (Fabricius et al., 2014, 2016) it has not yet been firmly established whether the mechanism is delivery of fresh very fine mineral particle sizes (Margvelashvili et al., 2018) or mid shelf transport of nutrients in the form of marine phytoplankton nourished by catchment nutrients delivered with river flows (Robson et al., 2017), or even increased upwelling associated with the weather conditions that cause high river discharge.
- The impact of SPM on marine eutrophication depends not only on the total load (i.e. quantity), but also on the quality (i.e. nutrient

enrichment status) of the fine sediment and tPOM. Prioritisation of management actions to reduce nutrient loads associated with sediment will differ from prioritisation to reduce sediment alone – here, a multiple objective prioritisation will result in additional water quality benefits. Additionally, further research on the influence of the intrinsic soil properties on sediment generation, transport and delivery is required.

- Organic carbon plays an important role in mediating the effects of terrestrially-derived SPM on phytoplankton growth in the GBR. Since vegetation litter is a significant source of organic carbon, the type of plants used in revegetation will likely influence the quality of the SPM leaving the terrestrial environment. An understanding of the interactions between mineral sediment, LfPOM and HfPOM is required to refine restoration strategies for optimal water quality outcomes.

GBR monitoring and modelling:

- There is a lack of standardised measurement and terminology for SPM across the ridge-to-reef continuum. For example, end-of-river terrestrial sediment concentrations and loads are reported as total suspended solids, and total tPOM loads are rarely reported. Organic carbon is not routinely sampled in GBR monitoring programmes across the catchments, end-of-river or flood plumes even though organic carbon is a strong indicator of the potential effects of terrestrially-derived SPM on phytoplankton growth (Garzon-Garcia et al., 2018). Quantification of total tPOM loads exported by the GBR basins cannot be quantified without the measurements and load calculations of both nitrogen and organic carbon.

- We lack a standard definition of the most ecologically relevant, terrigenous mineral particle size, and therefore lack consistency in terminology and size-fractions to focus upon across ridge-to-reef monitoring and modelling. International studies primarily refer to fine-grained terrestrial sediment as the < 63 μm (i.e. clay and silt fraction), although organic matter is adsorbed more readily/enriched on finer clay-sized particles. Based on our observations in the GBR, the majority of terrigenous mineral particles carried offshore in river flood plumes are < 20 μm i.e. ~ fine-silt and clay-sized fractions (Bainbridge et al., 2012; Lewis and Bainbridge, unpublished data) and is likely the most ecologically relevant size fraction of concern. Sediment particle size should be included in routine end-of-basin and marine sampling efforts to provide further confirmation.

- Parameterisation of the eReefs marine model has highlighted gaps in our current knowledge of fine sediment and POM generation in the GBR river basins, as well as particle size distributions, decay rates and processing at the freshwater-marine interface. So far, these gaps have been filled through a combination of expert opinion and careful use of relevant biogeochemical data from other systems (Robson et al., 2018), however, ongoing collaboration between modellers and observational scientists is essential to identify these gaps and further improve upon the models.

GBR management of the ecologically relevant component:

- Whilst ecological responses to the chronic exposure of suspended sediment and chlorophyll *a* concentrations (De'ath and Fabricius, 2010) have been used to set water quality guidelines (GBRMPA, 2010), further work is required to set guidelines and end-of-basin load reduction targets which fully capture the influence of SPM and its associated complexities (e.g. incorporate properties such as mineralogy, particle size and tPOM contributions). These complexities need also to be considered in determining light attenuation thresholds (see Petus et al., 2018) and in ecological studies investigating SPM effects on marine organisms.

- Determining SPM thresholds for coastal marine ecosystems will

continue to involve many considerations. These include the separate modes of action of SPM on coastal marine ecosystems, the physicochemical properties at specific locations, and population-level acclimation. These complications require a combined research approach of controlled ecotoxicological studies (e.g. Flores et al., 2012) on sentinel species, with less controlled but more holistic and environmentally realistic field studies.

Our review highlights the various components that need to be synthesised to understand how SPM affects vulnerable marine ecosystems and how the composition of SPM may vary from source to sink for effective management. Indeed, the GBR case study has demonstrated how research across the ridge-to-reef continuum has been used to (i) identify the impacts of SPM on key marine habitats and processes at risk and set meaningful guidelines for ecosystem protection (ii) use information from (i) to help set end-of-basin fine sediment load targets and; (iii) identify the high priority catchment sources and processes contributing terrestrial sediment to these marine ecosystems. Reef management could be improved by taking a ridge-to-reef approach integrating research and models to bring together the most ecologically relevant components of SPM to tropical marine ecosystem health, linking this material of most risk through the estuarine and marine transport processes that alter the composition and properties of SPM, and finally, the characterisation and source identification of terrigenous mineral sediment and tPOM that provide the greatest contributions. Elements of this approach including the study of estuarine SPM terrestrial and marine sources towards an improved understanding of its fate and impact have been applied internationally including the Louisiana coastal zone (Mississippi River) and the Gironde Estuary in France (Mayer et al., 2008; Savoye et al., 2012). Finally, end-of-basin load targets and marine ecosystem thresholds need to be explicit for SPM, and ideally the different constituents of SPM, due to their fundamentally different properties in transport and impacts. These challenges allude to the next frontier of integrated research on SPM across the ridge-to-reef continuum and provide 'a ray of light' for the future of tropical marine ecosystems.

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A brief story of nitrogen fixation in sugarcane – Reasons for success in Brazil

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Some of the authors of this publication are also working on these related projects:

Análise do perfil de expressão dos genes gumD, cpaB, eglA e luxI em Gluconacetobacter diazotrophicus PAL5T colonizando plantas de cana-de-açúcar por qRT-PCR. View project

Response of the C4 energy Pennisetum purpureum commercial hybrid and new PCEA genotype to inoculation with diazotrophic bacteria for bioenergy application purposes View project

Review:

A brief story of nitrogen fixation in sugarcane — reasons for success in Brazil

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Abstract.   Sugarcane was first introduced into Brazil in 1532, in São Vicente (São Paulo State) by the Portuguese. Since the first cane selection and breeding programs started in Brazil, both local and introduced material were used. In none of the breeding programs were large amounts of nitrogen fertilizer utilized, and this may be the reason why today the best materials have little demand for nitrogen fertilizer, and an effective association has developed between endophytic nitrogen-fixing bacteria and the plant. In some cases high inputs of associated biological nitrogen fixation have been observed. The oil crisis also played a role in the sugarcane story, since the alcohol-from-cane-juice (PRO-ÁLCOOL) program installed to find a substitute for gasoline in cars, stimulated the selection of highly efficient varieties with low nitrogen fertilizer input. The recent promising results involving the inoculation of micropropagated sugarcane plants with endophytic diazotrophic bacteria, along with the ongoing Brazilian sugarcane plant and bacterial genome programs, suggest that the success of the Brazilian sugarcane business may continue for many years to come, considering the potential to be exploited.

Introduction

Sugarcane (*Saccharum officinarum* L.) was first grown in south-east and western India, and nowadays is cultivated in the belt 35° N and 35° S, from sea level to 1000 m altitude, on a wide range of soil types (Malavolta 1994). The 'commercial' sugarcane planted today is a hybrid of *S. officinarum* and other species with agronomic characteristics of disease and drought resistance. It has been cultivated in over 120 countries, with a total of 19.4 million ha [Food Agricultural Organization (FAO) 1999]. The major sugarcane cultivated areas are in Brazil and India, followed by Cuba and China, whereas the greatest production (tons) is observed in Brazil, India, China and Mexico (Table 1). The Brazilian sugarcane program was intensified in the 1970s due to the petroleum crisis, since at that time around 84% of its oil needs were imported (Urquiaga *et al.* 1999). The government created the alcohol program (called PRO-ÁLCOOL) envisaging the substitution of gasoline with alcohol for running cars, a 'biofuel' that is known to be less polluting and today has evolved to support more than one million jobs. One aspect that may have contributed to the success of the PRO-ÁLCOOL program comes from the positive energy balance of this crop system in Brazil, in contrast to other countries. Silva *et al.* (1978) made the first approximation of the energy balance from ethanol

production from sugarcane under Brazilian conditions. The overall basic energy balance ratio (e/h) was 2.43. However, if it is assumed that all factory power is derived from bagasse (which today is universal practice), this value increases to 4.53, and if no nitrogen is applied to the plant, it increases to 5.79 (Boddey 1995). In USA, the use of high rates of chemical fertilization (200–400 kg N ha^{-1}) and mechanization contribute to the drastic reduction of the energy balance to almost 1. More recently, Macedo (2000) adjusted the observed data of the energy balance for the agro-industry localized in São Paulo State (COPERSUCAR - Cooperativa dos Produtores de Açúcar e Álcool do Estado de São Paulo Ltda), and the output/input reached the value of 9.2 (Table 2). The input corresponds to the energy consumed to produce sugarcane (i.e. agricultural operations, transport, fertilizer, lime, herbicides, seeds, equipment) and ethanol (i.e. chemicals, lubricants, buildings and equipment), whereas the output refers to the energy related to the ethanol produced and that generated from the burn of the bagasse.

In the last ten years, the number of cars being manufactured to run on hydrated ethanol (95%) has decreased drastically to less than 1% of the market, due to the lower petroleum price and higher price of sugar in the international market, although there are still three million cars on the road running on this alternative fuel. Although the

Table 1. Planted area, yield and productivity of sugarcane cultivated in the major sugarcane-producing countries of the world
Source: FAO (1999)

Country	Planted area (ha)	Yield (million ton)	Productivity (ton ha⁻¹)
Australia	415 000	3 922 000	88.97
Brazil	4 860 266	333 314 400	68.58
Cuba	1 100 000	35 000 000	31.82
China	1 048 000	89 388 023	85.30
India	4 150 000	282 249 894	68.01
Mexico	627 200	46 000 000	73.42
Philippines	358 000	26 287 000	73.42
South Africa	311 000	21 248 256	68.32
United States	401 130	32 406 000	80.79
Mauritius	65 000	3 500 000	53.84
World	19 404 768	1 274 697 080	65.69

program has declined, Brazilian gasoline still contains 20–24% of this biofuel. The mixture contributes to a better global environment since less CO (57%), hydrocarbons (64%), NO_x (13%), and zero lead, are emitted to the atmosphere (Boddey 1995). Although production of alcohol derived from sugarcane has been maintained at an almost constant level over the last 15 years (12 billion L year⁻¹), the sugarcane area and yield are still increasing in Brazil (Ministério da Agricultura 2000). The planted area (ha) increased from approximately 2 (year 1975) to 4.8 million, while total production has increased from around 75 to 315 million tons of sugarcane (Fig. 1). Breeding programs have introduced new materials every year and, according to the breeders, it is still possible to increase Brazilian sugarcane yields by about 20% (Fernandes and Irvine 1987). Today, the sugarcane agribusiness is responsible for 2.3% of the gross

national product, which is equivalent to approximately six billion dollars.

In contrast to other Graminaceous crops, sugarcane cultivated in Brazil neither depletes soil nitrogen reserves nor suffers a decline in yield after many decades, or even centuries, of cane cropping, suggesting that this crop may benefit significantly from inputs from biological nitrogen fixation (BNF; Boddey *et al.* 1995). The search for BNF in sugarcane plants began in the 1950s when Johanna Döbereiner and her colleague Alaídes Ruschel isolated the nitrogen-fixing bacteria *Beijerinckia fluminensis* from the rhizosphere of this plant (Döbereiner and Ruschel 1958). Later, an attempt to quantify the BNF in sugarcane plants was carried out by Döbereiner's group using the acetylene reduction technique (ARA; Döbereiner *et al.* 1972). Although the methodology is the subject of criticism, they could detect small BNF contributions that were attributed to nitrogen-fixing bacteria present in the rhizosphere of the
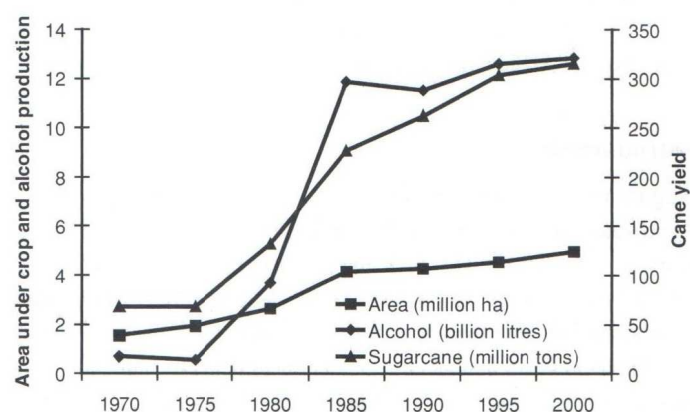

Fig. 1. The evolution of sugarcane planted area, yield and alcohol production in the last 30 years in Brazil.

Table 2. Energy balance in sugarcane and ethanol production under Brazilian conditions
Adapted from Macedo (2000)

Items	Averages		Best values	
Sugarcane production (total)	189.87		175.53	
Agricultural operations and transport	65.02		61.97	
Fertilizers	66.96		56.09	
Lime, herbicides, seeds and equipment	57.89		57.89	
Ethanol Production (total)	46.08		36.39	
Electricity (bought)	0.00		0.00	
Chemical and lubricants	7.34		7.34	
Buildings and equipment	38.74		29.05	
External energy flows (agriculture and industry)	Input	Output	Input	Output
Agriculture	189.87	0.00	175.53	0.00
Industry	46.08	0.00	36.39	0.00
Ethanol produced	0.00	1996.37	0.00	3045.27
Bagasse surplus	0.00	175.14	0.00	328.54
Totals (external flows)	235.95	2171.51	211.92	2373.81
Output/Input	9.2		11.2	

plant. Other attempts to quantify BNF in sugarcane plants were carried out by Ruschel and her collaborators in Piracicaba (São Paulo) using $^{15}N_2$ gas (Ruschel *et al.* 1975). These authors detected reasonable amounts of nitrogen derived from BNF being incorporated in 90-d old plants, but this amount could not account for total nitrogen accumulated in the plant tissues. Later, a long-term experiment carried out with ten sugarcane varieties grown in a concrete tank filled with soil supplemented with ^{15}N-labelled organic matter, demonstrated that most of the nitrogen accumulated by some sugarcane varieties was derived from BNF (Urquiaga *et al.* 1992). Almost at the same time, many endophytic nitrogen-fixing bacteria (i.e. *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *H. rubrisubalbicans*) were found colonizing many sugarcane varieties (Cavalcante and Döbereiner 1988; Baldani *et al.* 1997). The role of these endophytic nitrogen-fixing bacteria in this association is still unclear. However, there is much evidence supporting their contribution to the nitrogen accumulated in the plants.

The importance of the endophytic nitrogen-fixing bacteria/sugarcane association to Brazilian agriculture is such that the genome program called SUCEST (Sugarcane Expressed Sequence Tag), created in 1999 to study functions involved with morpho-physiological and genetic aspects of the plant [Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) 2000], included the study of two cDNA libraries from plants infected with the endophytic nitrogen-fixing bacteria *G. diazotrophicus* and *H. rubrisubalbicans* to determine the role of these organisms in the association (FAPESP 2000). An earlier investigation carried out by Vargas *et al.* (1999) found that a gene called *Misa2* is strongly induced in plants infected with either *Herbaspirillum* spp. or with a mixture of nitrogen-fixing bacteria. More recently, data mining in the SUCEST bank showed new forms of glutamine synthetase present in sugarcane plants (A. Hemerly, pers. comm.). Besides the plant project, two bacterial genome programs have been established in Brazil: one on *G. diazotrophicus* in Rio de Janeiro and another on *H. seropedicae* in Paraná State. The knowledge of the genome of both plant and endophytic nitrogen-fixing bacteria should allow the identification of new genes, and their genetic manipulation, to determine specific functions. For example, the role of the bacteria in the association, how the endophytic interaction occurs (molecular signalling), and how the fixed nitrogen is transferred to the plant tissues, are among the questions still not answered. Many new genes have already been discovered in the sugarcane plant, the functions of which are still unknown. The manipulation of these genes as well as those involved in the nitrogen, carbon, and photosynthetic metabolisms could improve the efficiency of the nitrogen-fixation process, therefore maximizing the potential of the association.

Plant propagation *versus* bacterial dissemination

Sugarcane has been considered a highly efficient in the conversion of light into chemical energy, because it possesses a C_4 pathway. Sugarcane is propagated from cuttings (setts). Roots formed from the sett die off when the roots from the shoots start to develop and take their functions of absorption and anchorage. At this stage, a high metabolic activity seems to be operating, since high nitrogenase activity has been detected in pre-germinated setts (15–30 d; F. Lopes Olivares, pers. comm.), usually followed by an increase of the endophytic diazotrophic population (Perin *et al.* 1999). Therefore, the question that arises is how to explain the presence of these bacteria within the setts, considering that the sugarcane programs are today based on plants originating from seeds collected after inter-specific crossing which have been sown into nurseries prepared with sterilized peat. To better understand the internal plant colonization and dissemination of these endophytic diazotrophic bacteria, it is necessary to have a wider view of the sugarcane story in Brazil.

One could speculate that these endophytic bacteria (mainly *G. diazotrophicus*) arrived with the introduction of sugarcane plants (setts) that originated from the Island of Madeira and were brought by the Portuguese in 1532, or later, when the noble varieties originating from the South Sea Islands were brought to the western hemisphere between 1768 and 1793 (Machado *et al.* 1987). From the West Indies and French West Indies, at least four noble varieties were distributed throughout the Caribbean and the Americas. The best variety, called Caianne, was spread throughout Brazil in 1803, and a sugar production of over $100\,000$ ton ha^{-1} was reported by 1853. New materials were introduced between 1857 and 1881 from Mauritius and Reunion (Machado *et al.* 1987), but unfortunately these came together with a disease that caused a decline in the Caianne productivity and forced breeding programs to be established. These programs are continuously furnishing new varieties with agronomic and industrial characteristics superior to the older varieties. An interesting aspect that might have contributed to the maintenance of endophytic diazotrophic bacteria in sugarcane was the failure of the botanist to detect seed formation in the first collected materials, leading to the conclusion that sugarcane was only propagated by setts (Machado *et al.* 1987). This was true for the varieties Creoula and Bourbon, the most planted sugarcane varieties until 1880. In the meantime, it was observed that sugarcane could propagate by seeds, and this was a very important stage in the sugarcane breeding program, that culminated with the crossing of a *S. spontaneous* and a *S. officinarum* by Soltwedel in 1887 (cited by Machado *et al.* 1987). Since then, new varieties have been introduced in virgin areas, or in fields continuously cultivated with sugarcane.

Local dissemination by insects also seems to play an important role in the ecology of the sugarcane/bacteria

association. This hypothesis was raised by Caballero-Mellado and Martinez-Romero (1994) while studying the diversity of isolates of *G. diazotrophicus* based on the work of Ashbolt and Inkerman (1990), who first published the presence of this bacterium within the mealybug, *Saccharococcus sacchari*. Another possible pathway of dissemination could be the mycorrhizal spores present in the sugarcane soil and known to contain diazotrophic bacteria such *Azospirillum*, *Klebsiella*, *Burkholderia*, *G. diazotrophicus*, and others (Paula *et al.* 1991; Reis *et al.* 1999). Trash left in the field is another source of dissemination, since diazotrophic bacteria have been already detected in these materials (Reis *et al.* 1994), although it is still not clear how the plant infection occurs (Arcanjo *et al.* 2000). Our group is investigating the possibility that these bacteria are present in the soil in a viable but non-cultivable stage and, once stimulated by plant root exudates, are able to infect and colonize the plants, but no clear picture has yet arisen.

More recently, it was demonstrated that graminaceous plants besides sugarcane that are propagated by seeds, such as *Eleusine coracana* (Loganathan *et al.* 1999) and coffee plants (Jimenez-Salgado *et al.* 1998), are also colonized by the endophytic bacteria *G. diazotrophicus*. However, no proof that the seeds were free from this specific bacterium was shown. In addition, attempts to isolate *G. diazotrophicus* from sugarcane seeds and fuzzes, either using enriched semi-solid medium or polymerase chain reaction methodology using species-specific primers, were unsuccessful (S. Arcanjo, pers. comm.), suggesting that the bacteria were somehow disseminated in the field.

However, a study on the genetic diversity of *G. diazotrophicus* carried out by Caballero-Mellado and Martinez-Romero (1994) using strains from Mexico and Brazil, showed that the diversity was very limited. Results from our laboratory also confirmed the narrow genetic diversity of this species through the analysis of 45 *G. diazotrophicus* isolates, originating from a sugarcane germplasm bank maintained for around 30 years at the same site, using the internal transcribed spacer/restriction fragment length polymorphism (ITS/RFLP) method (S. Tavares, pers. comm.). A genetic diversity study with strains originated from different countries, including those from where sugarcane probably originated, could give a better picture of the evolution of this species and how it was disseminated among the sugarcane fields.

Nitrogen fertilizer *versus* biological nitrogen fixation

High BNF contributions were observed in certain Brazilian sugarcane varieties cultivated for 22 months in large pots containing soil (64 kg) labelled with ^{15}N (Lima *et al.* 1987). Values varying from 109 to 175 kg N ha^{-1} were observed in hybrid varieties, while values were much lower on *S. barberi* (Chunee) and *S. officinarum* (Caianne) (56 and 22 kg N ha^{-1}, respectively; Urquiaga *et al.* 1992). The small amounts of

nitrogen applied to the crop during the breeding programs might have contributed to the development of an efficient association between endophytic diazotrophic bacteria and the sugarcane plant. The response of the planted cane crop to nitrogen fertilizer is generally very low or null, although the same does not apply to the first, second, and third ratoon (Carnaúba 1990). The response varies from country to country and depends on the soil type, region and management, and whether the crop is irrigated or rain-fed [International Fertilizer Industry Association (IFA) 1999]. In Brazil, the amount of nitrogen recommended by the COPERSUCAR for non-irrigated modern varieties is 50 kg N ha^{-1} year^{-1} to the plant cane crop, and 100 kg N ha^{-1} year^{-1} to the first ratoon (COPERSUCAR 2000). Much higher amounts of nitrogen fertilizer are used in other countries such as Hawaii, USA, India, Mexico, Philippines, and South Africa (Table 3). Several reports have shown that very high doses of nitrogen fertilizer can have a negative effect on the yield and sucrose content of the stalks (Table 4, and see Humbert 1963 cited by Malavolta 1994; Wiedenfeld 1998). In addition, other papers have shown the detrimental effect of elevated nitrogen fertilizer on the population of *G. diazotrophicus*, one of the endophytic

Table 3.   Nitrogen fertilizer levels applied to sugarcane plants grown in different countries
Source: IFA (1999)

Country	Nitrogen fertilizer (kg ha^{-1})
Argentina	100
Australia	150–250
Brazil	50
India	100–300
Mexico	120–200
Philippines	120–200
South Africa	80–120
USA — Hawaii	300–400

Table 4.   Effect of nitrogen fertilizer on sugarcane yield and amount of sugar
nd, not determined

Nitrogen (kg ha^{-1})	Cane (ton ha^{-1})	Sugar (%)	(ton ha^{-1})
Data adapted from Malavolta (1994):			
177	245	9.5	23.0
277	267	9.0	23.7
377	260	8.5	22.2
Data adapted from Wiedenfeld (1998):			
(kg ha^{-1})	(ton ha^{-1})	(%)	(kg ha^{-1})
0	92	nd	132
50	95	nd	128
100	98	nd	126
150	96	nd	125

diazotrophic bacteria colonizing sugarcane plants (Table 5, and see Fuentes-Ramírez et al. 1999; Muthukumarasamy et al. 1999; Reis Jr et al. 2000a).

Although there is a high level of nitrogen removal from the system via stems taken to the mill and material burned off before cutting (1.5–2.0 kg N ha^{-1} per ton of plant cane), it has been found that nitrogen reserves in the soil are not exhausted, and the level of productivity is maintained (Orlando Filho et al. 1980). This suggests that BNF plays an important role in the nutrition of the sugarcane crop. A BNF contribution was first demonstrated by Ruschel et al. (1975), using $^{15}N_2$ gas. At that time, the diazotrophic population detected in the rhizosphere soil and roots did not seem to be able to account for the BNF observed, although unidentified diazotrophs were detected within stems, and the nitrogenase activity varied among sugarcane genotypes (Ruschel and Vose 1982). The BNF variation among varieties was confirmed later by the work of Lima et al. (1987) and Urquiaga et al. (1992), using the ^{15}N isotope dilution technique and nitrogen balance. These authors showed much higher BNF contribution (60%) than the work done by Ruschel and co-authors (17%). These results were recently confirmed using material collected from different sugarcane regions in Brazil and analysed with the $\delta^{15}N$ technique (Boddey et al. 2001). Recent work carried out by Sevilla et al. (2001) using $^{15}N_2$, confirmed that G. diazotrophicus fixes nitrogen inside the sugarcane plants. However, the amount of nitrogen fixed was much lower than that observed by Lima et al. (1987) and Urquiaga et al. (1992). This was probably due to differences in plant age and growth conditions. The first authors used 30-d old micropropagated plants (8-cm tall) inoculated with the bacteria and grown in Murashige and Skoog (MS) medium. After washing several times with sucrose-free MS medium, the plants were transferred to a new sterile tube and exposed to $^{15}N_2$ for 24 h. The two Brazilian experiments were carried out with 12- to 18-month old plants (2 to 3-m tall) grown in tanks

containing soil labelled with ^{15}N. Therefore, the differences should be expected since, for plants originated from micropropagation and grown in the field, the nitrogen fixation process seems to be higher later in the growth cycle (A. Oliveira, pers. comm.). Growth of sugarcane in the field is also influenced by water supply, soil fertility (N, P, and K), and the molybdenum level in the soil, which is known to interfere with BNF. Such high nitrogen fixation values were corroborated by the discovery of endophytic diazotrophic bacteria such as G. diazotrophicus, H. seropedicae, H. rubrisubalbicans, Azospirillum spp., and Burkholderia spp. (Döbereiner 1992) colonizing these varieties. Recent work using the enzyme-linked immunosorbent assay (ELISA) method showed very high populations of G. diazotrophicus and Herbaspirillum spp. colonizing different parts of the plants (Table 6, and see Li and MacRae 1992; Boddey et al. 2000). These values are much higher than those obtained using the most probable number (MPN) technique, as shown in Table 5. Boddey et al. (2000) discussed both methods applied to quantify the nitrogen-fixing endophytes in sugarcane plant tissues, and outlined several factors that interfere with quantification of the bacteria. It is known that populations of these diazotrophs are influenced by the water status and nutritional state of the plant, including the nitrogen level (Reis Jr et al. 2000b), tissue utilized (roots, leaves, stems; surface-sterilized or not), and plant age (Fuentes-Ramírez et al. 1999). If these materials are from plants previously inoculated and grown either in sterilized or field conditions, this also influences the diazotrophic populations (Sevilla et al. 2001). Despite either under- or over-estimation of the numbers of endophytic diazotrophic bacteria present within the plant tissues, both

Table 5. Log number of *Herbaspirillum* spp. and *Gluconacetobacter diazotrophicus* colonizing roots and stems of two sugarcane varieties (SP 79–2312 and SP 70–1143), grown with and without nitrogen fertilizer

Adapted from Reis Jr et al. (2000b). Values followed by the same letter within a column are not significantly different at $P=0.05$ according to Tukey's test. + N treatments were fertilized with 300 kg N ha^{-1}

Sample type	SP 79–2312		SP 70–1143	
	(+ N)	(− N)	(+ N)	(− N)
Herbaspirillum spp.				
Roots	3.52	3.71	3.65	4.05
Stems	1.94	1.73	2.09	2.19
G. diazotrophicus				
Roots	1.92^b	2.36^a	2.28	2.49
Stems	1.67^b	2.19^a	1.81	1.85

Table 6. Detection of *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *H. rubrisubalbicans* in different tissues of field-grown sugarcane by the ELISA technique using purified polyclonal antibody raised against strain PRJ2 (*G. diazotrophicus*), HRC54 (*H. seropedicae*), and HCC103 (*H. rubrisubalbicans*)

Adapted from Boddey et al. 2000. All values are expressed per g fresh weight of plant tissue. nd, not determined

Sample type	Sterilized	Not sterilized
G. diazotrophicus		
Roots	9.0×10^5	4.0×10^7
Rhizomes	4.9×10^5	5.9×10^6
Leaves	1.8×10^6	2.0×10^7
H. seropedicae		
Roots	1.9×10^6	1.3×10^7
Rhizomes	1.2×10^5	4.2×10^6
Leaves	nd	nd
H. rubrisubalbicans		
Roots	3.6×10^6	2.4×10^6
Rhizome	nd	6.8×10^5
Leaves	7.4×10^5	3.9×10^6

methods can be used to compare the population among different sugarcane varieties. Using these methods, Reis Jr *et al.* (2000*b*) and Boddey *et al.* (2000) could not detect significant quantitative or qualitative differences in the diazotrophic population between sugarcane varieties that could explain the differences in BNF observed by Urquiaga *et al.* (1992).

Although several nitrogen-fixing bacteria have been isolated from different parts of sugarcane plants, there are still doubts about the major bacterium responsible for BNF. Inoculation of micropropagated sugarcane plants with endophytic diazotrophic bacteria has confirmed their potential for use in agriculture (Baldani *et al.* 2000). More recent data have shown that inoculation with a mixture of these endophytes seems to be the best strategy for improvement of the plant/bacteria association (Oliveira *et al.* 2000). Similar results were demonstrated for four varieties of sugarcane grown in India, where inoculation with a mixture of different diazotrophic bacteria plus mycorrhizal fungi produced a response equivalent to that following a half-dose of the recommended nitrogen fertilizer (Muthukumarasamy *et al.* 1999). It is not clear how these different bacteria interact, although a synergistic effect (phytohormones and BNF) seems to play a role at different stages of sugarcane growth. It has already been demonstrated that nitrogen-fixing bacteria, including *G. diazotrophicus*, are able to produce indole-3-acetic acid (IAA; Fuentes-Ramírez *et al.* 1993). These authors observed differences in the amount of IAA produced by strains of *G. diazotrophicus* and suggested that phytohormones could help initial root development and, consequently, plant growth. Large differences in root mass were observed in micropropagated sugarcane plants inoculated with a mixture of nitrogen-fixing bacteria and grown in the field for 12 months (A. Oliveira, pers. comm.). An additional effect of a plant growth-promoting factor, provided by inoculation with the *G. diazotrophicus* Nif minus mutant, under conditions where mineral nitrogen was not limiting, was also demonstrated by Sevilla *et al.* (2001).

Final considerations

During all these years the researchers in this specific field have accumulated considerable evidence on how diazotrophs can individually infect and colonize monoaxenic plants, but have not answered the question of which micro-organism(s) are responsible for the nitrogen-fixing activity detected under natural conditions. In any circumstance, the research teams must work together in a practical way to improve the BNF in these agro-ecosystems. The Brazilian sugarcane cropping system may be an important example for the other countries, but a lot must be done to convert this knowledge to a practical technology for use on-farm.

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Review

Relating sediment impacts on coral reefs to watershed sources, processes and management: A review

CrossMark

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HIGHLIGHTS

- This paper reviews the impact of sediment delivery to coral reefs.
- The sources, processes and management options of excess sediment are discussed.
- The synthesis is based primarily on *measured* data sets.
- The approaches and outcomes are relevant to coral reefs around the world.

ARTICLE INFO

ABSTRACT

Modification of terrestrial sediment fluxes can result in increased sedimentation and turbidity in receiving waters, with detrimental impacts on coral reef ecosystems. Preventing anthropogenic sediment reaching coral reefs requires a better understanding of the specific characteristics, sources and processes generating the anthropogenic sediment, so that effective watershed management strategies can be implemented. Here, we review and synthesise research on measured runoff, sediment erosion and sediment delivery from watersheds to near-shore marine areas, with a strong focus on the Burdekin watershed in the Great Barrier Reef region, Australia. We first investigate the characteristics of sediment that pose the greatest risk to coral reef ecosystems. Next we track this sediment back from the marine system into the watershed to determine the storage zones, source areas and processes responsible for sediment generation and run-off.

The review determined that only a small proportion of the sediment that has been eroded from the watershed makes it to the mid and outer reefs. The sediment transported >1 km offshore is generally the clay to fine silt (<4–16 μm) fraction, yet there is considerable potential for other terrestrially derived sediment fractions (<63 μm) to be stored in the near-shore zone and remobilised during wind and tide driven re-suspension. The specific source of the fine clay sediments is still under investigation; however, the Bowen, Upper Burdekin and Lower Burdekin sub-watersheds appear to be the dominant source of the clay and fine silt fractions. Subsurface erosion is the dominant process responsible for the fine sediment exported from these watersheds in recent times, although further work on the particle size of this material is required. Maintaining average minimum ground cover >75% will likely be required to reduce runoff and prevent sub-soil erosion; however, it is not known whether ground cover management alone will reduce sediment supply to ecologically acceptable levels.

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Contents

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1. Introduction

Suspended sediment plays an important role in freshwater and marine biogeochemical processes and food webs (Krumins et al., 2013; Wood and Armitage, 1997). Yet excessive sedimentation and turbidity have been shown to have deleterious effects on coral reefs in Australia (Fabricius, 2005; Fabricius and Wolanski, 2000; Rogers, 1990; Uthicke et al., 2012), Africa (Vankatwijk et al., 1993), Pacific Islands (Golbuu et al., 2011), Hawaii (Wolanski et al., 2009), Indonesia (Crabbe and Smith, 2005), Madagascar (Maina et al., 2013) and the meso-American reefs (Andrefouet et al., 2002). For large coral reef systems such as the Great Barrier Reef (GBR), there is general agreement that increased sediment from agricultural regions is impacting on coral reefs (De'ath et al., 2012) and other adjacent habitats such as seagrass beds (Waycott et al., 2005). The growth of coral reefs have fluctuated considerably in the past (8500 year record) independently of anthropogenic impact (Browne et al., 2012) and many reefs have coexisted with very high water turbidity for millennia (Larcombe et al., 1995). Therefore, quantifying the impact of anthropogenic sediment delivery from agricultural land use change since European settlement, against the high variability of natural sediment loads in tropical rivers (Syvitski et al., 2005), is a challenging and contested research area. Few studies have been able to trace sediment from its watershed source through to the marine zone, accounting for all erosion and depositional processes, particularly in large (>100,000 km²), geologically diverse watersheds (Douglas et al., 2006; Takesue et al., 2009). There have, however, been multiple independent studies, that have evaluated individual aspects of the source, delivery and fate of sediment to the marine system. It is rare that these papers are evaluated together to provide an understanding of sediment movement across the watershed-to-marine-ecosystem continuum.

This paper presents a review of the literature that examines the link between sediment impacts on coral reef ecosystems and the amount, source and processes contributing sediment from the 130,000 km²
Burdekin River watershed, Australia. First, we evaluate the response of coral reef ecosystems to elevated levels of sediment (Section 3) and assess the evidence for an increase in anthropogenic derived sediment fluxes to the Great Barrier Reef (GBR) (Section 4). We then trace the sediment back to the watershed source (Section 5) and the erosion process generating the sediment (Section 6). The key drivers of this erosion (Section 7) and the potential management response for reducing sediment erosion and delivery are then discussed (Section 8). A synthesis of the findings and areas of further research are presented in Section 9. In each section, we aim to identify specific sediment characteristics that link impacts on reef ecology to the source and transport processes in the watershed. Our review focuses on the Burdekin River watershed and surrounding marine waters as it is the largest contributor of anthropogenic derived fine sediment to the GBR lagoon (Kroon et al., 2012). The results of this review will be relevant to all watersheds upstream of coral reefs, and of particular interest to dry-tropical watersheds that have undergone land degradation due to cattle grazing. Excessive amounts of nutrients (nitrogen, phosphorus, carbon and silica) are also known to affect coral reef ecology (Hallock and Schlager, 1986); however, for brevity, nutrients are not assessed in this review as they have been dealt with in detail elsewhere (e.g. Fabricius, 2005).

2. Setting the scene: the Burdekin River watershed

The Burdekin watershed is ~130,000 km² and drains into the Great Barrier Reef Lagoon south of Townsville on the east coast of Australia (Fig. 1). It has an annual average rainfall of 727 mm and the largest mean annual runoff of any of the GBR watersheds at 10.29×10^6 ML (Furnas, 2003). The rainfall and runoff regime is highly variable in both space and time (Petheram et al., 2008; Rustomji et al., 2009) with rainfall generally higher near the coast (often >2000 mm) than in western areas (<600 mm). The Burdekin has highly pronounced wet and dry seasons on an annual time scale, and long periods of below average rainfall can be punctuated with tropical depressions

Fig. 1. Major sub-watersheds of the Burdekin watershed. The Inner, Middle and Outer shelf delineations correspond to the 20 m and 40 m bathymetric depths based on Orpin et al. (2004a).

that can bring >1000 mm of rainfall in a few weeks. The Burdekin watershed is composed of 6 sub-watershed areas including the Upper Burdekin (~29% of the total area), Cape River (~15%), Belyando (~27%) and Suttor sub-watersheds (~13%) all above the Burdekin Falls Dam (BFD). The Bowen sub-watershed (~8%) and Bogie and lower East Burdekin (~8%) are below the Burdekin Falls Dam (Fig. 1).

The marine zone influenced by periodic runoff from the Burdekin watershed has been estimated at ~47,000 km² (Devlin et al., 2012), which includes ~246 coral reefs and 73 seagrass beds (Devlin et al., 2011). The inner reefs (including some tidally exposed reefs) and islands (e.g. Magnetic Island and Palm Island Group) are located on the inner shelf <20 km offshore in water depths of ~20 m. The middle shelf is between 20 and 50 km offshore with water depths of 20–40 m, and the outer shelf is ~100 km from the mouth of the Burdekin river at water depths of 50–90 m (Belperio, 1983; Larcombe et al., 2001) (see Fig. 1).

The Burdekin watershed is dominated by cattle grazing (~91%) which occurs largely on native pastures within open woodland

communities (DSITIA, 2012). There are also small areas of dryland cropping in the Belyando–Suttor region (~70,000 ha, Dight, 2009), and sugar cane dominates the lower floodplain (occupying <1% of the watershed; although most of the sugar lies out-side of the hydrological watershed boundary). The pastures are located on sedimentary (~59%), igneous (35%) and metamorphic lithologies (~6%) (Furnas, 2003). The sedimentary soils are generally located in the south western areas (Belyando and Suttor) and the igneous derived soils (granodiorites and basalts) dominate the north of the watershed (Dight, 2009). Vegetation clearing has resulted in 25% less vegetation in 2009 compared with European settlement (Great Barrier Reef Marine Park Authority, 2012). This loss includes areas of forest, woodlands, sedgelands and wetlands. Woody tree thickening has also occurred in many parts of this watershed (Scanlan et al., 1996a).

Degradation of rangelands and pastures is well documented for many parts of the world (e.g. Milton et al., 1994), and the Burdekin watershed is no exception (McKeon et al., 2004). Cattle numbers have

increased in the Burdekin from ~0.05 million in 1860 to ~1.4 million in 2010–11 (Australian Bureau of Statistics, 2012). This period of land use change and intensification has been correlated with increased suspended sediment loads that are recorded within coral cores offshore of the Burdekin River mouth (Lewis et al., 2007; McCulloch et al., 2003). These studies suggest that there has been a five- to tenfold increase in the delivery of sediments with the highest fluxes occurring during the drought-breaking floods. Erosion in the Upper Burdekin has been particularly severe in the first half of the 20th Century with 12.5% or 6900 km^2 of this area considered to be impacted by soil erosion resulting in an estimated soil loss of 8.63 million tonnes (Burdekin Project Committee, 1976, p. 648). A period of accelerated degradation occurred in the Burdekin in the mid-1980s that was considered to be due to the adoption of more hardy tropical cattle breeds, use of feed supplements and accelerated market fluctuations, combined with a number of years of well below average rainfall (McKeon et al., 2004). Following the drought of the 1980s, six out of eight land types surveyed in the Dalrymple Shire were found to have >30% of sites with sheet and scald erosion (DeCorte et al., 1994). At present, ~8% of the Burdekin is considered to be in D-condition, defined as areas with <40% vegetative ground cover (Abbott et al., 2008; Karfs et al., 2009). Large areas of the Burdekin watershed are shown to have modelled hillslope erosion rates in excess of 5 t/ha/yr (McKergow et al., 2005), and subsequently this watershed has been identified as a high priority area for controlling hillslope erosion within Australia.

2.1. Particle size terminology

References to sediment particle size made in this paper follow the (rounded) Udden–Wentworth size classification for sand (>63 μm), medium and coarse silt (16–63 μm), fine silt (4–16 μm) and clay (<4 μm) (Leeder, 1982). These size classes, although standard, are often augmented with additional class fractions to represent specific processes in fluvial or marine systems. For example, the 10 μm threshold, or sortable silt fraction, is used in oceanography to distinguish silt sizes >10 μm that behave differently under wave and current processes (McCave et al., 1995). Silt <10 μm generally behaves in the same way as clay, and silt >10 μm responds more readily to hydrodynamic processes offshore. Therefore the sortable silt fraction is often referred to in publications describing sediment transport processes in the near-shore zone (e.g. Orpin et al., 1999).

3. Ecological impacts of anthropogenic sediment on coral reef ecosystems

The influence of sediment on the growth and distribution of corals was recognised more than a 100 years ago (Wood-Jones, 1912). Through field observations and laboratory experiments these early studies demonstrated that corals have species-specific tolerances to sedimentation (Crossland, 1928; Edmondson, 1928; Mayer, 1918; Vaughan, 1919). Importantly, these studies recognised that sediment can have interactive effects, including with other variables such as salinity and temperature, on coral growth and survival.

3.1. Ecological impacts of sediment on coral reefs

Some coral species can tolerate very high sedimentation rates (Sofonia and Anthony, 2008) and turbidity (Browne, 2012), and recovery from short-term or low levels of sedimentation has been observed (Philipp and Fabricius, 2003; Wesseling et al., 1999). However, most coral reef organisms are negatively affected by smothering (sedimentation) and reduced light availability for photosynthesis due to turbidity in the water column (Dubinsky and Stambler, 1996; Fabricius, 2011). High sedimentation rates may reduce larval recruitment, making the settlement substratum unsuitable (Dikou and van Woesik, 2006; Hodgson, 1990), and growth of adult corals is inhibited through reduced

photosynthesis (Philipp and Fabricius, 2003; Riegl and Branch, 1995; Telesnicki and Goldberg, 1995). Extensive or excessive sediment exposure can also result in coral disease (Haapkyla et al., 2011) and mortality (Philipp and Fabricius, 2003; Victor et al., 2006), and cause a shift to macroalgal dominance (De'ath and Fabricius, 2010; Dikou and van Woesik, 2006; Golbuu et al., 2011). Polyps of many coral species exhibit sediment rejection behaviour (Vaughan, 1919) comprising of ciliary currents, tissue expansion, and mucus production (Stafford-Smith and Ormond, 1992). The exact responses to sedimentation depend on the coral species, duration and amount of sedimentation, and sediment composition (Dubinsky and Stambler, 1996; Weber et al., 2006). Increased suspended sediment concentrations also affect juvenile reef fish through interference in recruitment success via suppression of chemical cues for settlement from the planktonic stage and reducing growth via interference in feeding (Wenger et al., 2011, 2012).

3.2. Composition and characteristics of the anthropogenic sediment that is affecting coral reefs

Laboratory based experiments using sediment collected from local rivers discharging into the GBR suggest that grain size and organic and nutrient-related sediment properties are key factors determining sedimentation stress in corals after short-term exposure (Philipp and Fabricius, 2003; Weber et al., 2006, 2012). Stress levels in coral were strongly related to the organic and nutrient-related parameters in the sediment, weakly related to the physical parameters and unrelated to the geochemical parameters measured. Weber et al. (2006) found that silt-sized and nutrient-rich sediments can stress corals after short exposure (<36 h) due to microbial processes leading to reduced oxygen and the formation of toxic hydrogen sulphide, while sandy sediments or nutrient-poor silts affect corals to a lesser extent. The nutrient enhanced sediments are also prone to forming sticky flocs of 'marine snow' which can have detrimental impacts on corals within 1 h of settling (Fabricius and Wolanski, 2000). Although recent research has shown that some corals can develop and thrive in nearshore areas with high turbidity (Browne et al., 2012; Palmer et al., 2010), according to Rogers (1990) 'normal' sedimentation rates for offshore coral reefs appear to be less than 10 mg cm^{-2} d^{-1}, and typical suspended solid concentrations are less than 10 mg L^{-1}. De'ath and Fabricius (2008) refined these estimates for the GBR and suggest that a daily maximum sedimentation rate of 15 mg cm^{-2} d^{-1} or a mean annual rate of 3 mg cm^{-2} d^{-1} should guard against excessive coral mortality which equates to average suspended sediment concentrations of 1.6 mg L^{-1} in winter and 2.4 mg L^{-1} during the summer wet season.

3.3. Summary: ecological impacts of anthropogenic sediment on coral reef ecosystems

In summary, the sediment of most concern to marine ecosystems is the nutrient/organic rich silt and clay sized (<63 μm) fractions (Weber et al., 2006) that have persistent concentrations of >10 mg L^{-1} (Rogers, 1990). In coastal waters, this equates to average sediment concentrations of 1.6 mg L^{-1} in winter and 2.4 mg L^{-1} during the summer wet season (De'ath and Fabricius, 2008). Resuspension of sediment in windy conditions or strong tidal currents in shallow waters (<10 m) leads to conditions where TSS concentrations are above the GBR water quality guidelines (De'ath and Fabricius, 2008; Great Barrier Reef Marine Park Authority, 2009), and this threatens coral reefs through reduced light for photosynthesis (Fabricius, 2005). To improve the characterisation and provenance tracing of sediments present in the ecologically significant areas (e.g. coral reefs and seagrass meadows) further knowledge of the particle size, nutrient content and mineralogy of the material that is being deposited and re-suspended from near-shore sites is required.

4. The fate and characteristics of anthropogenic terrestrial sediment delivered to coral reef ecosystems

Many factors control the delivery of terrestrial sediment from end-of-river to marine systems, including tides, wind and wave direction, land position and distance from terrestrial inputs (Woolfe and Larcombe, 1998). The fate of the sediment also depends on its particle size, mineralogy and attached materials (e.g. organic matter, nutrients, chemicals). In many cases, it is not necessarily the plume itself but rather the continual reworking of the sediment delivered by the plume, that has an impact on the marine ecosystem (Storlazzi et al., 2009). Here, we review the current knowledge on the post-European delivery of sediment from the Burdekin River to the GBR.

4.1. The quantity of anthropogenic sediment being delivered to coral reef systems

Cores of reef sediment and corals have indicated both increases (Fleitmann et al., 2007; Lewis et al., 2007; McCulloch et al., 2003) and decreases (due to reservoir construction; Hungspreugs et al., 2002) in terrestrial sediment fluxes to coral reefs since the 1900s. In the more recent studies, the trace element to calcium ratios in corals have been used to quantify the link between changed land use, runoff and sediment yield. Studies from the Burdekin watershed suggest a five to tenfold increase in the delivery of sediments to the marine zone since European settlement, with the highest fluxes occurring during the drought-breaking floods (Alibert et al., 2003; McCulloch et al., 2003). In general, fine sediment delivery to off-shore coral from the Burdekin watershed increased after ~1860 correlating strongly with the introduction of sheep and cattle and associated land clearing (Lewis et al., 2007; McCulloch et al., 2003). Levels of mercury in sediment cores from Bowling Green Bay at levels 25 times background (or pre-European), is further evidence that sediment from the Upper Burdekin watershed, in this case from gold mining in the period 1880–1914, reached the marine areas offshore (Walker and Brunskill, 1997). Similar (coral core) techniques have been used offshore from the Pioneer and O'Connell Rivers, Queensland (Jupiter et al., 2008; Lewis et al., 2012a), Africa (Fleitmann et al., 2007), Hawaii (Prouty et al., 2010; Takesue et al., 2009) and Madagascar (Grove et al., 2012; Maina et al., 2012) showing similar patterns of sediment increase to coral reefs following the expansion of agriculture.

4.2. The location and characteristics of the anthropogenic sediment

Many coral reefs have developed under the influence of terrigenous sediments (Johnson and Risk, 1987; Lewis et al., 2012b), and reefs have evolved and changed under fluctuating fluvial sediment delivery (Palmer et al., 2010). The transport and re-suspension processes offshore from the Burdekin River have led to a strongly sediment-partitioned shelf, with modern riverine sediment located adjacent to the coastline and carbonate-dominated sediments located on the middle and outer shelf (Belperio, 1983).

Over the past ~5500 years, ~80–90% of the contemporary sediment from the Burdekin River has been captured in the Burdekin delta (Fielding et al., 2006) or Bowling Green Bay to the north of the Burdekin River mouth (Orpin et al., 2004a). The coarser fractions of this sediment have formed chenier ridges and the Cape Bowling Green sand spit. The finer sediments are interspersed between the mudflats and are located in the low energy marine waters within ~10 km of the coast (Belperio, 1983; Orpin et al., 2004a). This deposited material is then strongly influenced by sediment re-suspension by waves and turbulent mixing (Larcombe et al., 1995; Larcombe and Wolfe, 1999; Orpin et al., 1999, 2004b), where it is driven northwards via longshore drift (Lambeck and Woolfe, 2000). Though most terrestrially derived sediment is deposited near the coast, recent research by Fabricius et al. (2013) has shown that when the influence of wave and tidal conditions were

removed from turbidity records, mean turbidity increased significantly with river flow.

Turbid plumes of sediment are visible each wet season extending up to 10 km offshore from the Burdekin River mouth (Brodie et al., 2010; Devlin et al., 2012; Schroeder et al., 2012). Only the very fine silt and clay sized (<16 µm) sediment is present in these plumes (Bainbridge et al., 2012; Devlin et al., 2012). For the Burdekin River flood plume, Bainbridge et al. (2012) determined that suspended sediment concentrations can drop from >500 mg L^{-1} in the river at zero salinity to <10 mg L^{-1} at salinities with concentrations near 5–10 (dimensionless salinity units), which is approximately 10 km offshore. This considerable reduction in suspended sediment concentrations is fostered through flocculation processes (Bainbridge et al., 2012).

Research from other watersheds found that suspended sediments (<100 µm) will undergo flocculation when they encounter salty water which increases their sinking rate (Webster and Ford, 2010), trapping >50% of the modern sediment load in the estuary (Bostock et al., 2007). Ayukai and Wolanski (1997) also determined that highly turbid sediment delivered from the Fly River (New Guinea) settled out when salinity reached ~23. Although most of the sediment settles out at higher salinities, the wave and tidal energy is often sufficient to maintain turbidity at elevated levels (Lambrechts et al., 2010), and fine sediment may remain available for re-suspension many years after a given flood event.

4.3. Summary: the fate and characteristics of anthropogenic terrestrial sediment delivered to coral reef ecosystems

In summary, trace element to calcium ratios in coral cores identified that the amount of fine sediment (silt and clay) leaving the Burdekin River has increased at least 5 times over the last 150 years (McCulloch et al., 2003). This increase is linked to changes in animal numbers and vegetation, with the highest sediment fluxes occurring during the drought-breaking floods (when ground cover is low; see Section 7.1). Interestingly, ~80–90% of the contemporary sediment from the Burdekin River has been captured (or stored) in the Burdekin delta (Fielding et al., 2006). Only sediment <4 µm (clay) is transported more than 5 km offshore, and sediment <16 µm is transported <3 km from the river mouth (Bainbridge et al., 2012). However, all fine sediment fractions (<63 µm) can be transported to the river mouth where they can be re-suspended (Fabricius et al., 2013) and potentially impact on marine ecosystems. Understanding the sources of the <4–16 µm sediment is a priority for understanding the impact of land use change on outer shelf reefs (see Fig. 1).

5. Estimating sediment yields from sub-watersheds

At a global scale, fluxes of terrestrial sediment to coastal areas have been substantially modified by humans (Syvitski et al., 2005; Walling, 2006). Increases in these fluxes are due to soil erosion, associated with changes in surface runoff, urbanization, deforestation, agricultural practices, and mining. On the other hand, reductions in sediment fluxes to coastal areas are primarily due to retention within impoundments (Syvitski et al., 2005; Vorosmarty et al., 2003). This section reviews our knowledge of the amounts and sources of sediment within the Burdekin watershed.

5.1. The quantity of sediment being exported from watersheds to the end-of-river

A range of techniques have been used over the last 30 years in the Burdekin watershed to estimate end of watershed sediment yields. These include simple empirical models (e.g. Belperio, 1979; Moss et al., 1993; Neil et al., 2002), watershed sediment budget modelling (McKergow et al., 2005), measured sediment loads (Furnas, 2003; Joo et al., 2012) and integrated modelling and monitoring data

(Kroon et al., 2012). The most recent statistical analysis suggests that the mean-annual contemporary fine sediment load from the Burdekin watershed is ~3930 kt year^{-1} (with an annual range between 4 and 15,741 kt year^{-1}) based on a 24 year monitoring data set (Kuhnert et al., 2012). The sediment flux for the Burdekin River exhibits high inter-annual variability (Rustomji et al., 2009). The sediment flux per unit area (t/ha) is lower than average by world standards (Walling and Fang, 2003); however, when evaluated according to sediment yield per unit runoff (t/ha/mm) it is relatively high by world standards (Thorburn et al., 2013).

5.2. Identifying the dominant sub-watershed signal for the anthropogenic sediment

Determining the dominant (sub-watershed) source of sediment in a basin requires a combination of techniques including direct sediment flux monitoring, sediment provenance tracing and watershed modelling (Walling et al., 2011). In the Burdekin watershed, monitoring of the suspended sediment export from the five main sub-watersheds (Upper Burdekin, Cape, Belyando, Suttor and Bowen), the Burdekin Falls Dam overflow and end of basin (Clare gauge) suggests that the Upper Burdekin, Bowen and Lower Burdekin/Bogie sub-watersheds dominate the total sediment load, and deliver ~27%, 45% and 26% of the annual fine (<63 μm) sediment load over a five-year study period (Bainbridge et al., in review) (Table 1). The same sub-watersheds are also the dominant source of the clay and fine silt <16 μm sediment fraction (Bainbridge et al., in review).

Similar research in the adjacent Fitzroy watershed found that sediments deposited in Keppel Bay were from a combination of sedimentary, granitic and basaltic soil types, yet the greatest increase of fine sediment (<10 μm) since European settlement was from the Tertiary basalts (Douglas et al., 2008; Smith et al., 2008). Most of the basaltic sediment appears to have come from cultivated cropping rather than grazed areas (Hughes et al., 2009a). Importantly, the basaltic soils have been identified as the major contributor to phosphorus fluxes, due to their higher phosphorus concentrations (Douglas et al., 2006). It is therefore likely that the 70,000 ha of dryland cropping on basaltic soils in the Belyando and Suttor sub-watersheds in the Burdekin also contribute dis-proportionately to these tributaries; however, a detailed geochemical study of the sediment sources is required to confirm this hypothesis.

To put the 5 years of measured sediment yield data set into context, and obtain estimates of sediment delivery from un-sampled sites, watershed modelling has also been used to assess the relative sources of sediment within the Burdekin watershed (Kinsey-Henderson et al., 2007). Despite known uncertainties with the prediction of sediment sources and erosion processes (see Section 6) the watershed models are consistently within 30% of the long term measured TSS loads for large watersheds (>2000 km^2) (Wilkinson, 2008). There also appears to be general agreement between watershed models and measurement-based TSS load estimates for the relative contribution of sub-watersheds to Burdekin River TSS yield. While watershed models also predict the detailed spatial patterns within sub-watersheds, finer-

resolution input data and process understanding are required before these predictions will be reliable (McKergow et al., 2005).

5.3. Summary: estimating sediment yields from sub-watersheds

In summary, 24 years of monitoring data at the end of the Burdekin River estimates that an average of ~3930 kt/yr of fine sediment reaches the estuary (Kuhnert et al., 2012). Sub-watershed monitoring and watershed modelling are consistent in identifying that the Upper Burdekin, Bowen and Lower Burdekin/Bogie sub-watersheds dominate basin fine sediment delivery.

6. Identifying and quantifying the erosion and sediment storage processes

Following the identification of the major geographic sources of sediment, it is important to determine which erosion process is predominantly responsible for the sediment loss so that appropriate restoration strategies can be implemented. Sediment can be eroded from surface (hillslope) erosion or from gully networks or river banks within the channel network (I.P. Prosser et al., 2001). Following erosion, there are numerous opportunities for sediment to be deposited within the watershed before a small proportion of the eroded material is delivered to the marine system (as discussed in Section 4.2).

Traditional erosion studies have primarily been interested in gross erosion for the purpose of evaluating soil loss from agricultural land (e.g. Scanlan et al., 1996b). Specific information about the ecologically relevant particle size of the sediment has not, until recently (Bainbridge et al., 2012), been a major consideration in many hillslope and watershed studies. This has limited our ability to link erosion processes across the watershed-to-marine continuum. This section will describe the various erosion and depositional processes, including identification of the ecologically threatening fraction (where possible), within the Burdekin watershed.

6.1. The contribution of hillslope erosion to sediment delivery

Sheetwash or hillslope erosion generally dominates sediment budgets in cultivated areas (Walling et al., 2011) and is considered to dominate in grazed landscapes where the rainfall erosivity is high and seasonal ground cover is low when the peak rainfall occurs (Lu et al., 2003). In the Burdekin watershed, measured rates of hillslope erosion for fine sediment (<63 μm) vary from <0.02 t/ha on the flatter dry hillslopes (Bonnell and Williams, 1987) to ~2.3 t/ha for moderately grazed areas (Hawdon et al., 2008) and up to ~8 t/ha for areas with low (<10%) ground cover (Bartley et al., 2010a). Due to the difficulty and cost of obtaining erosion measurements across an area the size of the Burdekin watershed, most of the hillslope erosion estimates have been calculated using a modified form of the Universal Soil Loss Equation (USLE) within the SedNet model (Thorburn and Wilkinson, in press; Wilkinson et al., 2009). SedNet modelling across the whole of the Burdekin watershed suggested that hillslope erosion was the

Table 1
Estimates of the measured sub-watershed contributions over a four year period (2005–2009) of the <16 μm and <63 μm fractions (summarised from Bainbridge et al., in review). These values have taken into account the relative trapping efficiencies of the various particle size fractions (Lewis et al., 2013). They are then compared with SedNet modelled TSS (<63 μm) sediment for the Burdekin watershed (Kinsey-Henderson et al., 2007).

Sub-watershed	Watershed area (%)	% contribution of clay and fine silt (<16 μm) (averaged for 2006–2009)	% contribution of <63 μm sediment (averaged for 2006–2009)	Modelled % total suspended sediment (TSS) (<63 μm)
Upper Burdekin	29%	30%	27%	21%
Cape	15%	1.6%	1.3%	6%
Belyando	27%	0.9%	0.7%	8%
Suttor	13%	1.6%	1.1%	14%
Bowen	8%	42%	45%	27%
Bogie and Lower Burdekin River	8%	24%	26%	24%
Burdekin River (End-of-basin)	100%	100%	100%	100%

dominant process delivering fine sediment to stream networks with ~67% of end of watershed loads coming from hillslope erosion, and the remainder, ~27% and ~6%, coming from gully and bank erosion, respectively (I. Prosser et al., 2001). However, parameterisation of the USLE has not yet been appropriately constrained by local field measurements of ground cover and soil erodibility.

More recently, a series of field sediment budget and tracing studies in the Upper Burdekin determined that although hillslope erosion can dominate fine sediment loads during drought years when ground cover is low (Bartley et al., 2007), sub-surface or channel erosion dominates sediment yields in the longer term (Wilkinson et al., in press-b) (see Section 6.2). Sediment source tracing indicates that approximately 60–80% of fine river sediment is derived from sub-surface soil (Wilkinson et al., in press-b). Importantly, recent tracing using ^{7}Be have shown that hillslope soils may be a contributor to this sub-surface soil loss, with rilled, scalded and badland areas on hillslopes being sediment sources of comparable importance to vertical channel banks (Hancock et al., 2013). While understanding of the source contributions will continue to be refined, it is now clear that the bulk of fine sediment delivered from the Burdekin basin to the GBR is derived from a very small proportion of the basin area well-connected to the stream network, where subsoil is exposed in scalds, rills, gullies and streambanks (Wilkinson et al., in press-b).

There are a number of reasons for the discrepancy in the ratio of sediment sources including (i) that most modelling projects have used estimates of hillslope erosion based on a static relationship between land use class (C) and erosion, and direct vegetation (and rock) cover measurements were generally not used (McKergow et al., 2005); (ii) where used, remotely sensed vegetation cover imagery has inadequate resolution in dissected terrain to identify bare ground areas that can dominate hillslope erosion (Bartley et al., 2010b); (iii) the degree of discrepancy between models and source tracing is unclear because modelling has lumped sheetwash and rill erosion, while source tracing has lumped channel, gully and rill erosion (Wilkinson et al., in press-b), and (iv) gully erosion data inputs to the models have had poor spatial representation (Kuhnert et al., 2010).

These recent sediment tracing and field studies have superseded the previous modelled estimates of erosion process contributions to fine sediment yield. Similar over-estimation in the modelled contribution of hillslope sheetwash erosion has been identified in the Fitzroy basin (Hughes and Croke, 2011). Therefore, any further discussion of erosion processes in this paper will refer specifically to studies that have used direct measurements or tracing techniques. Subsequent watershed sediment budget modelling (not yet published) is incorporating the new understanding of erosion sources (Waters et al., pers. comm.).

Research from other semi-arid rangeland areas around the world suggest that end of watershed sediment yields are a poor indicator of soil erosion on hillslopes as considerable amounts of sediment can move within hillslopes, but not necessarily be delivered to streams (Ritchie et al., 2009). There also appears to be a cover threshold of ~40% that distinguishes between surface and channel erosion dominance. At sites with <40% cover, sediment yields can be dominated by surface erosion (Bartley et al., 2007; McIvor et al., 1995; Nichols et al., 2012) whereas once cover increases above this level, surface erosion decreases and sediment yields are dominated by channel sources (Bartley et al., 2010b). It is therefore possible that the source of sediment has 'switched' between hillslope and channel sources over time when ground cover has moved across this 40% threshold. It would also depend on the dominant soil type, location in the watershed, and arrangement of ground cover (Bartley et al., 2006). It is also likely that many gullies were initiated when ground cover was <40%, and then developed and continued to grow even when ground cover improved (see Section 7).

6.2. The role of channel erosion in sediment delivery

There is considerable recent evidence from the tropical rangelands of Northern Australia demonstrating that sub-surface or channel erosion is the dominant source of sediment contributing to watershed sediment yields (Brooks et al., 2009; Caitcheon et al., 2012; Hughes et al., 2009b; Tims et al., 2010; Wasson et al., 2002). Sediment source tracing in the Burdekin basin is consistent with these observations, with subsurface soil dominating river sediment in the Upper Burdekin and Bowen sub-watersheds (Wilkinson et al., in press-b). In southern Australia, there is considerable evidence that vegetation had a strong control on gully formation (Prosser and Slade, 1994). The causes of gully erosion in Northern Australia have not been fully resolved; however, recent research suggests that, similar to southern Australia, gully erosion was either initiated, or accelerated, when cattle were introduced into these watersheds (Shellberg et al., 2010, 2012).

Bank erosion rates on the Burdekin River are relatively low (at least during drought conditions) (Bartley et al., 2007) and generally low by world standards (Bainbridge, 2004). There are, however, parts of the Burdekin watershed where alluvial gullies (see Brooks et al., 2009) are located along channel banks and it is difficult to differentiate these erosion processes. Depending on any future classification of alluvial gully systems, bank erosion may be found to be a much greater source of sediment in some areas of the Burdekin watershed (e.g. see Fig. 2b).

6.3. Sediment storage

The amount of fine sediment that reaches the outlet of large river systems varies considerably with watershed area and particle size, and is generally only a small proportion (~10%) of the sediment eroded in the watershed (Walling, 1983). Thus, any attempt to link watershed disturbance to changes in the sediment flux to the ocean must take account of the storage processes. According to Phillips (1991, p231) 'Sediment storage and transfer within a watershed may be the single most important aspect of determining how a system responds to environmental change'. Once sediment is eroded from hillslopes or channels, it may be stored temporarily (until the next event) or for long time frames (>1000 years). These storage processes can dampen or remove evidence of increased sediment flux within the watershed, and complicate the link between upstream and downstream response to human impact (Walling, 2006).

Relatively little data are available on the storage of sediment (fine or coarse) within the Burdekin channel network. There is evidence to suggest that some of the fine (silt) material is stored in-stream (within benches) and floodplain deposits in the western watersheds of the Burdekin (e.g. Belyando and Suttor), although these streams carry relatively little coarse sediment. Coarse material is trapped on top of bench and vegetated bar features in the Upper Burdekin River system (Fielding and Alexander, 1996), although, particle size analysis of bed sediments in the Upper Burdekin (data not shown) suggest that very little fine (<63 μm) sediment is stored within the channel. Similarly, little contemporary fine sediment is trapped on the lower floodplain of the Burdekin River (Alexander et al., 1999). Coarse grained sediment (>63 μm) has been found stored on the bed of gullies and channels, in constriction areas and at tributary junctions in the Burdekin and adjacent Fitzroy watersheds (Bartley et al., 2007; Fielding and Alexander, 1996; Thompson et al., 2011), particularly catchments draining granitic geologies. Dating of these coarse sediment deposits suggests that much of this material is from a phase of channel erosion triggered by changes to land use and management in the late nineteenth century (Hughes et al., 2009a). Other potential sites for sediment storage include reservoirs and weirs which are discussed below.

6.4. The impact of impoundment on sediment delivery

Although humans have significantly increased soil erosion, ~50% of the sediment eroded globally is trapped in artificial impoundments (Syvitski et al., 2005; Vorosmarty et al., 2003). Flow and sediment export is regulated in the lower reaches of the Burdekin River following the construction of the Burdekin Falls Dam in 1987. The dam captures

Fig. 2. Examples of channel erosion in the Burdekin Watershed (a) gully erosion in the Bowen Watershed. Tree height in image ~2 m (b) alluvial gully erosion along the banks of the Upper Burdekin River. Channel width in image ~200 m.

flow from ~88% of the Burdekin watershed and has a capacity of 1.86 million ML. Water has spilled over the dam wall in all but one wet season since construction. Lewis et al. (2013) determined that between 50% and 85% of fine sediment delivered annually to the dam is trapped (mean 66%); however, the proportion of sediment trapped depends on the grain size of the material and the volume of the inflow events. Lewis et al. (2013) found that 100% of <0.5 μm, 50% of 0.5–5.0 μm, 25% of 5.0–30 μm, and 5% of >30 μm passed over the dam wall. Half (50%) of the <0.5 μm fraction originates from only one of the contributing rivers, the Suttor River, while supply of the three coarser fractions is dominated by the upper Burdekin River (87–95%). Overall, it is estimated that the Burdekin Dam has reduced the total sediment load from the Burdekin River by ~35% (Lewis et al., 2009).

Much of the agricultural expansion and associated severe erosion in the Upper Burdekin occurred prior to the construction of the dam

(Burdekin Project Committee, 1977; Lewis et al., 2007), and modelling suggests that the dam has not reduced the sediment yield to the coast to levels below those in pre-European times (Kroon et al., 2012).

6.5. Summary: identifying and quantifying the erosion and sediment storage processes

In summary, sediment tracing in the Bowen and Upper Burdekin sub-watersheds demonstrated that sub-surface erosion is the dominant process contributing to sediment yields (Wilkinson et al., in press-b), and further research is required for other sub-watersheds. The particle size characteristics, timeframes of initiation and causes of gully erosion are not well understood; however, changes to the amount, and distribution of native woody vegetation are a likely cause (see Section 7 for more detail) (Shellberg et al., 2012). The Burdekin Falls Dam now traps more than

50% of the mean-annual fine sediment between 0.5 and 30 μm; however, the silt and clay sized fractions of suspended sediment, which cause high sedimentation stress in corals (Weber et al., 2006), can move through reservoirs (Lewis et al., 2013) and be transported to coastal coral reefs during flood events (Bainbridge et al., 2012). Whilst it is estimated that the Burdekin Dam has reduced the total sediment load from the Burdekin River by ~35% (Lewis et al., 2009), modelling suggests that it is still several times higher than the pre-European load (Kroon et al., 2012). To further understand the sediment storage and budget processes in catchments of this size will require a number of additional areas of research (discussed in Section 9.1).

7. The drivers of erosion and sediment loss

Following the introduction of cattle grazing and cropping in the 1800s, there are a number of factors that may have influenced watershed hydrology and sediment delivery. These include changes to cattle numbers, the amount, composition and distribution of vegetation, as well as changes to the soil condition and structure. This section reviews the literature in this area to help understand what changes have occurred so that we can develop appropriate strategies for mitigating the degradation of the past.

7.1. The effect of changing vegetation on runoff and soil erosion

Over the last 100 years it is estimated that native vegetation has reduced by ~25% in the Burdekin watershed (DSITIA, 2012; Peña-Arancibia et al., 2012). A number of studies in the Burdekin and surrounding watersheds have investigated the effect of changing ground cover (pasture) on runoff and erosion at the hillslope scale. Runoff plot trials have shown that areas with high cover have lower runoff than areas with low cover (Scanlan et al., 1996b). Runoff varies considerably with the arrangement of cover (Bartley et al., 2006), however, cover may have little effect on overland flow during very large rainfall events (>100 mm with intensities between 45 and 60 mm/h) due to Hortonian (excess) overland flow processes (McIvor et al., 1995; Scanlan et al., 1996b). Roth (2004) determined that ground cover needs to be >75% to enable infiltration during high intensity events. Even with high cover, localised infiltration varied widely, mainly as a function of macroscopically visible bioturbation by soil macrofauna such as ants, termites and earthworms. Soil loss from grazed hillslopes increases as vegetation cover decreases, with the rate decreasing sharply as cover increases beyond 40% (Bartley et al., 2010a; McIvor et al., 1995; Scanlan et al., 1996b). When cover is <40%, both fine (<63 μm) and coarse (>63 μm) sediment fractions are eroded; however, when cover is high (>70%), coarse fractions are trapped on the hillslope, and only fine fractions move off the hillslope (Scanlan et al., 1996b; Silburn et al., 2011). Ground cover is also very 'patchy' in these landscapes (Ludwig et al., 2007) and this results in large variability in sediment yields even for hillslopes under the same management regime (Bartley et al., 2006). Patchy vegetation on erodible soils within riparian zones can also lead to the initiation of alluvial gullies and scald features (Shellberg et al., 2010). Adequate ground cover, on both hillslopes and riparian zones, needs to be maintained to reduce the potential for gully formation.

Luminescent lines in corals have also been used to reconstruct the history of major freshwater flows reaching the GBR from the Burdekin River (1685–1981), and although there appears to be no overall trend toward wetter or drier conditions, the reconstructions suggest that the variability of rainfall and river flow has increased during the twentieth century with more very wet and very dry extremes than in earlier centuries (Lough, 2011). Interestingly, trend analysis of recent stream-flow records (1920–2007) using pre and post clearing river flow data in the Upper Burdekin suggest that some of this increased variability may be the result of decreases in base-flow following tree clearing, and increased event storm flow during large rainfall events (Peña-Arancibia

et al., 2012). Changes to the runoff regime are likely to be one of the factors driving increased erosion in the Burdekin watershed.

Studies in semi-arid rangeland areas outside of the Burdekin suggest that converting (Brigalow) forest to pasture can increase runoff by ~80% at sub-watershed scales (Thornton et al., 2007) and ~40% for river basin scales (Siriwardena et al., 2006). Similar average cover thresholds of 50–70% are required to reduce surface erosion in other rangeland environments (Sanjari et al., 2009; Silburn et al., 2011). Thus, changes to the vegetation type and amount appear to have changed the magnitude of runoff events in these environments, thus increasing the potential for erosion.

7.2. The influence of grazing on soil condition

Soil condition provides a link between the physical processes at the watershed scale, and biological processes at finer scales, and has a major impact on hydrological processes such as erosion. A number of smaller scale studies carried out in the Burdekin watershed have demonstrated that surface compaction was higher, and populations of macro-fauna were lower, in heavily grazed sites compared with lightly grazed areas (Holt et al., 1996). Research by Dawes (2010) in savanna woodland areas similar to those in the Burdekin determined that un-cleared areas have higher soil macrofauna and higher soil water storage than cleared sites. Overall, Trimble and Mendel (1995) emphasise that it is the heavy or severe grazing that has the most impact on soil condition and erosion, and the effect of light and moderate grazing is much less severe. The Burdekin grazing lands have had a history of heavy grazing (McKeon et al., 2004) and this is still evident in many areas that have poor soil condition, low macro-porosity, poor infiltration, low proportion of tussock grass species and associated low litter and biomass production (Bartley et al., 2010a; Roth, 2004).

7.3. Summary: the drivers of erosion and sediment loss

In summary, the key to reducing all forms of soil erosion is to reduce runoff. High rates of runoff fuel hillslope and channel erosion, and increase the risk of (the ecologically threatening) sediment (<16 μm) reaching the GBR. To reduce runoff, ground cover needs to be maintained close to ~75% to enable infiltration during high intensity events. Adequate ground cover, on both hillslopes and riparian zones, is also needed to reduce the potential for gully formation. It is heavy or severe grazing that is likely to have the greatest impact on vegetation and soil condition.

8. Linking reductions in sediment yield to changes in land management in grazed watersheds

A number of practical guides to pasture and watershed restoration have been developed specifically for the Burdekin watershed (e.g. Coughlin et al., 2008). Following implementation of such recommendations, there is evidence of improvements in terms of soil structure, vegetation productivity and land condition. There is, however, little evidence that these recommendations will actually improve downstream water quality, and reduce the amount of ecologically threatening sediment reaching offshore coral reefs. This section will outline some of the scientific approaches that have been trialled to link land management change and improved water quality delivery to marine systems.

8.1. Evidence demonstrating that changed land management will reduce erosion

Field studies of the responses to changes to land management have shown that it is possible to reduce sediment concentrations in hillslope runoff, and to reduce runoff volumes from early wet season events, through improved grazing land management within ~5 years (Bartley et al., 2010a; Hawdon et al., 2008). Improvements have been more

rapid (reducing runoff coefficients by 25% over 3 years) when cattle were excluded completely, and where pastures were dominated by more resilient tussock grasses (Connolly et al., 1997). In areas of the watershed with low erosion rates, responses are difficult to detect over short time scales (<5 years) (O'Reagain et al., 2005).

Studies in the Burdekin and adjacent Fitzroy watersheds (see Fig. 3) have found that increasing ground cover generally increases the amount of rainfall required to initiate runoff (Bartley et al., 2010a; Connolly et al., 1997) and reduces peak discharges (Ciesiolka, 1987). Extrapolation of such data using water balance modelling suggests that the most effective revegetation strategy, in terms of runoff reduction, was to increase cover levels modestly across the whole watershed rather than to revegetate small areas intensively (Connolly et al., 1997). To change or reduce runoff at the hillslope scale, average cover needs to be >75% and biomass >2000 kg/ha (Ciesiolka, 1987; Roth, 2004).

Reducing runoff and sediment yields from degraded areas at the watershed scale will take a lot longer (>10 years) because of the time lags associated with soil and pasture recovery (Colloff et al., 2010) and the geomorphic changes required to reduce the rates of channel erosion. In the semi-arid Concho River (~10,000 km^2) in the USA, an 80 year flow record has shown that annual streamflow has decreased by ~70% and stormflow (which is generated in large events) declined between 1960 and 2005. This change was attributed to a decline in grazing animal numbers over the latter half of the century resulting in improved soil infiltrability due to improved ground condition (Wilcox et al., 2008).

8.2. Management actions required to reduce runoff and sediment yields to the reef

At a global level, there are fewer than 5 rivers that have demonstrated a reduction in end of river sediment loads to coastal waters in response to improved land management (Walling and Fang, 2003; Wang et al., 2007, 2011; Zhang and Wen, 2004). Where reductions have occurred, the

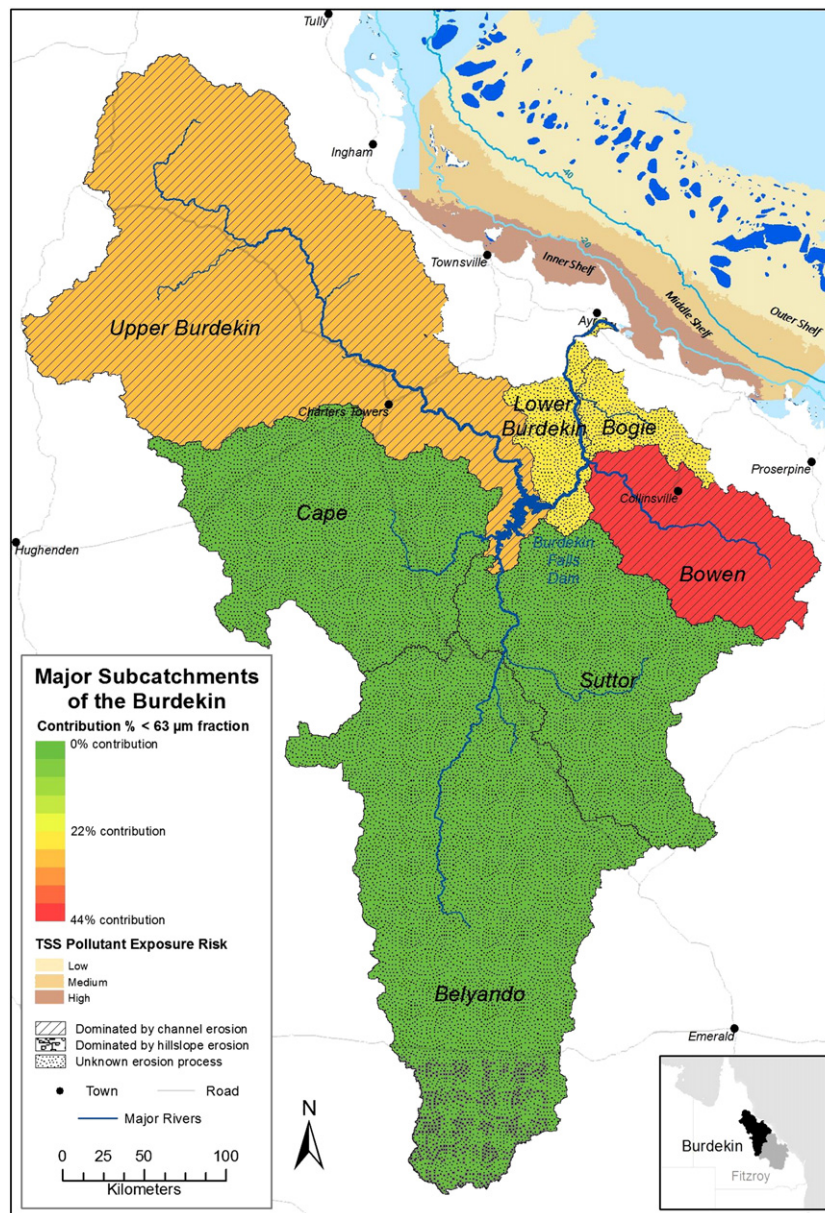**Fig. 3.** The % contribution of fine (<63 μm) sediment to the watershed outlet from each of the major sub-watersheds in the Burdekin based on monitoring data (2005–2009). The figure takes into account particle size and dam trapping of the sediment, and provides a description of the dominant erosion process delivering the sediment. The marine TSS pollutant exposure risk data is derived from Devlin et al. (2012) for the Burdekin region.

financial investments into watershed restoration have been substantial, and the land-uses that dominated these studies have been more intensive than the rangeland grazing in the Burdekin watershed. For example, a study by Garbrecht and Starks (2009) showed a reduction in sediment yields over long (~60 year) time periods due to the combined effects of activities such as conservation tillage, terracing of cropland, gully shaping, grade control structures, channel stabilization, sediment trapping by water impoundments, and road surfacing in watersheds ranging between 49 km^2 and 826 km^2. Kuhnle et al. (2008) measured reductions of about 60% in fine and total sediment concentrations over a 9 year period from a 21.3 km^2 watershed dominated by channel erosion, after highly erodible cultivated land was reduced from 26% to 8% of the watershed. This was attributed to reduced runoff from crop land and reduced channel transport capacity. These studies were conducted in headwater watersheds, and a reduction in sediment yields to coastal waters was not measured.

In grazing lands of the Burdekin watershed the principles of land management for reducing runoff and sediment loss include (i) reducing forage utilisation (which is heavily influenced by stocking rates) to increase ground cover, and (ii) redistributing grazing pressure away from areas vulnerable to erosion such as gullies and streambanks (Thorburn and Wilkinson, in press). Several studies have found that levels of livestock forage utilisation of 25–30% (of maximum annual biomass) are required to ensure that the pasture productivity and erosion control functions of rangeland vegetation are sustained (e.g., Ash et al., 2011). The marginal economics of many grazing enterprises often prevent the adoption of these principles, and long-term profitability and sustainability is frequently compromised in favour of short-term income (Landsberg et al., 1998). Targeted gully revegetation and remediation techniques may accelerate recovery in high-priority areas, although the effectiveness of these practices has yet to be locally evaluated (Thorburn et al., in press).

Monitoring changes to land management over long time scales requires considerable financial investment. Therefore, most attempts to predict the potential water quality benefit of improved land management in the Burdekin have been undertaken using models (e.g. SedNet; Kinsey-Henderson et al., 2005). More recently, a time-stepping watershed model based on SedNet (Wilkinson et al., 2014a), with corrected erosion source ratios based on new data and information (Dougall, 2013), is being coupled with one-dimensional models of forage production (e.g. GRASP, HowLeaky) (Ash et al., 2000). These coupled models are being used to estimate changes in pasture cover under different grazing and climate scenarios and their impact on fine sediment loads (Carroll et al., 2012; Dougall, 2013; The State of Queensland, 2013). Further field measurements are required to determined if and when these modelled changes will occur in measured stream loads (e.g. Bartley et al., in press), and whether reductions being modelled are sufficient to protect coral ecosystem health (Kroon, 2012).

More broadly, the water quality change following improved land management practice has been hampered in long term studies by (1) inappropriate targeting of the critical source/pathway of the sediment, (2) the dominance of channel rather than surface soil erosion; and (3) time lags, historical legacies and variable climate within the monitoring periods (Tomer and Locke, 2011). Some of the difficulties in measuring and identifying a response in sediment yield to land management change are also due to the lack of long term, well managed, statistically robust data sets (Richardson et al., 2008).

The high variability of runoff and sediment yield in the Burdekin basin will make it difficult to link changes in watershed management to end of watershed sediment yields. Statistical modelling suggests that with current monitoring programs it will take at least 50 years to detect an average 20% reduction in suspended sediment loads with reasonable (80%) confidence (Darnell et al., 2012). The role of sediment storage in large watersheds can also make linking land management changes and sediment response challenging (Walling et al., 2011). For example the Coon Creek (in the USA) work by Trimble (1981, 1983)

suggests that even after the implementation of soil conservation measures in the 1930s reduced gross erosion by ~25%, the sediment yield at the basin outlet changed very little. This was due to increased efficiency of sediment transfer through the channel system (via reduced deposition) and the remobilization of sediment that had accumulated in the valley during the preceding period of accelerated erosion.

Extending the existing field evidence on sediment source contributions across the Burdekin basin is a priority to ensure erosion control efforts target the dominant sources, to effectively reduce sediment loads. For example, the Ord River Watershed Regeneration Project (ORCRP) in Western Australia involved reducing cattle numbers and remedial works to re-establish pasture in areas where serious erosion was identified (Fitzgerald, 1976). After almost 30 years, the ORCRP has had no measurable effect on the sedimentation rate in Lake Argyle which is downstream of the restored area (Wasson et al., 2002). This is because the scheme invested a lot of money into hillslope rehabilitation yet gully erosion was the main form of erosion, and therefore sediment yields did not decline (Wasson et al., 2002).

8.3. Summary: linking reductions in sediment yield to changes in land management in grazed watersheds

In summary, due to the costs and challenges with long term monitoring, there are very few studies anywhere in the world that have demonstrated a reduction in runoff and fine sediment delivery to marine ecosystems following improved land management. For restoration to be effective, and reduce the delivery of the ecologically threatening sediment, it must target the primary erosion process. It is likely that increasing cover levels across the whole watershed will help reduce runoff and prevent or reduce further hillslope and channel erosion; however, once gullies are well established, specific remediation measures may be required. Depending on the scale and effectiveness of restoration measures, detecting reductions in end-of-river sediment loads may take years to decades using current monitoring programs (Darnell et al., 2012).

9. Synthesis

Previous reviews undertaken for the GBR watersheds have generally looked at multiple pollutants across the entire GBR region (e.g. Brodie et al., 2012). This review has shown how erosion processes in the watershed can influence the characteristics, fate, and ecological impact of sediment in the marine system (see Fig. 3). Data from the Burdekin River in north east Australia were used for this purpose due to the large amount of research that has been conducted in this area over the last 30 years. The results of this review, however, are relevant to many reefs around the globe that are under threat from increased land based runoff and resultant sediment loads (Maina et al., 2013). *in Madagascar!*

To recap the key points from the review, McCulloch et al. (2003) found that the amount of fine sediment (silt and clay) leaving the Burdekin River has increased at least 5 times over the last 150 years. The sediment of most concern to reef health is the nutrient/organic rich silt and clay sized (<63 μm) fractions (Weber et al., 2006) that have a concentration of >10 mg L^{-1} near shore (De'ath and Fabricius, 2008; Rogers, 1990). Resuspension of such sediment in windy conditions or strong tidal currents in shallow waters (<10 m) leads to conditions where TSS concentrations are above the GBR water quality guidelines (De'ath and Fabricius, 2008; Great Barrier Reef Marine Park Authority, 2009), and this threatens coral reefs (Fabricius, 2005) through reduced light for photosynthesis.

Using monitoring data, it is estimated that the annual fine sediment load delivered by the Burdekin basin is highly variable and ranges from 4 to 15,740 kt/yr (average of ~3930 kt/yr) (Kuhnert et al., 2012). Only sediment <4 μm (clay) is transported more than 5 km offshore, and sediment <16 μm is transported <3 km from the river mouth (Bainbridge et al., 2012). However, all fine sediment

fractions (<63 μm) are transported to the river mouth where they can be re-suspended (Fabricius et al., 2013) and potentially impact on marine ecosystems. Prior to the construction of the Burdekin Falls Dam (BFD), the Upper Burdekin was most likely the major source of total and fine sediment to the marine zone. The BFD now traps more than 50% of the mean-annual fine sediment between 0.5 and 30 μm. However, the fine silt and clay sized fractions of suspended sediment, which cause high sedimentation stress in corals (Weber et al., 2006), can move through reservoirs (Lewis et al., 2013) and be transported to coastal coral reefs during flood events (Bainbridge et al., 2012).

In average rainfall years, the major source of fine (<16 μm and <63 μm) sediment is the Upper Burdekin, Bowen and Lower Burdekin sub-watersheds (Table 1). Tracing data in several sub-watersheds demonstrates that sub-soil (gully, bank, rill and scald) erosion is the dominant erosion process contributing to sediment yields (Wilkinson et al., in press-b). The timeframes of initiation and causes of sub-soil erosion are not well understood; however, over-grazing has altered the amount and structure of vegetation in these landscapes, and is considered to have exacerbated the amount of runoff and erosion. It is recommended that average ground cover levels are kept at or above 75% to reduce hillslope runoff which drives sub-surface or channel erosion downstream. However, depending on the scale and effectiveness of restoration measures, detecting reductions in end-of-river sediment loads will take decades using current monitoring programs (Darnell et al., 2012). The findings from this review may inform future terrestrial and marine monitoring programs in the watersheds draining to the Great Barrier Reef and other coral reef systems around the globe.

9.1. Areas of further research

The information presented in this review will help inform watershed management processes and potentially improve water quality delivered to coral reefs. Despite the considerable progress (documented herein) linking the impact of sediment on coral reef ecosystems to the amount, source and processes contributing to the sediment generation in the watershed, there are still several outstanding areas of research. These include, but are not limited to, the following:

1. Improved sediment source characterisation
   There is considerable variation in the way in which sediment concentrations and sizes are measured and reported between terrestrial and marine systems (e.g. NTU is commonly used in marine studies and mg L^{-1} in terrestrial studies). A consistent approach between disciplines would make it easier to identify and track the ecologically threatening sediment between systems. This may involve the development (or adjustment) of new monitoring techniques. More research on the nutrient status of the source sediments (clays and silts) (e.g. Douglas et al., 2010), and how mobile the recently deposited sediments are once they reach marine waters, would also help target the sediment most likely to impact on coral reef ecology.
2. Quantifying end of watershed sediment loads under pre-European conditions
   Estimates of pre-European erosion and sediment delivery are currently predicted using 'best estimates' within models. There are, however, more sophisticated methods available to establish historical sedimentation and erosion rates (e.g. Hewawasam et al., 2003; Rustomji and Pietsch, 2007), which have yet to be applied in the Burdekin basin. More accurate (non-modelled) estimates of pre-European soil erosion and sediment yield would allow the quantitative assessment of how much soil erosion and sediment yields have changed following the introduction of agriculture. This would then allow the setting of practical and achievable soil erosion and water quality targets for particular sub-watersheds or landscape types that have taken into consideration the level of inherent natural (geological) variability between sub-watersheds.

3. Calculation of residence times and storage for different sized sediment
   It is acknowledged that many management actions undertaken today may potentially take decades before a change is measured in downstream water quality (Tesoriero et al., 2013). This is because considerable sediment, of varying size classes, is stored in the watershed. This sediment may be stabilised and remain there for many thousands of years, or it may be in temporary storage and could potentially threaten the GBR in decades to come (e.g. Dosseto et al., 2008; Madej, 1987). It is critical to understand these residence times of eroded and stored sediments so that the response time of different land management strategies time can be quantified.
4. Water quality benefits from improved land management and erosion reduction
   Sub-surface or channel erosion has been identified as a major source of sediment in a number of watersheds. Sub-surface erosion can take many forms including gully, stream bank or scald erosion. Further research to identify the dominant process (and particle size) will help with erosion management at the property scale, and techniques are available for this purpose (e.g. Hancock and Revill, 2013; Hancock et al., 2013; Polyakov et al., 2009). Differentiating between active and mature erosion areas would also be important for targeting remediation. Given that runoff drives erosion (including channel and gully erosion processes), there is also a need to quantify the effects of changing pasture type and quality on runoff at both plot and watershed scales. New techniques that can link water balance measurements with remote sensing would be useful for this purpose (e.g. Shuttleworth et al., 2010). Finally, research is also needed on the options, feasibility and cost-effectiveness of various rehabilitation techniques for eroded landscapes (e.g. Wade and Heady, 1978).
5. Assimilation of multiple information sources
   No single project or research technique will ever provide all the answers, and integrating and assimilating data and research outcomes is increasingly important for tackling large complex issues such as the one presented in this paper. Each technique has its strengths and weaknesses. For example modelling can cover large areas, but generally has high uncertainties, and tracing and monitoring techniques can be useful, but are relatively expensive. Cherry et al. (2008) suggest that 'these assessment methods should be integrated to maximise their potential usefulness and positive attributes'. Data assimilation or integration methods may provide useful insights that will help better understand the watershed-to-marine system transfer of sediment, and improve targeting of catchment management and associated monitoring programs.

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^{15}N natural abundance studies in Australian commercial sugarcane

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Abstract

The measurement of natural ^{15}N abundance is a well-established technique for the identification and quantification of biological N$_2$ fixation in plants. Associative N$_2$ fixing bacteria have been isolated from sugarcane and reported to contribute potentially significant amounts of N to plant growth and development. It has not been established whether Australian commercial sugarcane receives significant input from biological N$_2$ fixation, even though high populations of N$_2$ fixing bacteria have been isolated from Australian commercial sugarcane fields and plants. In this study, δ^{15}N measurements were used as a primary measure to identify whether Australian commercial sugarcane was obtaining significant inputs of N via biological N$_2$ fixation. Quantification of N input, via biological N$_2$ fixation, was not possible since suitable non-N$_2$ fixing reference plants were not present in commercial cane fields. The survey of Australian commercially grown sugarcane crops showed the majority had positive leaf δ^{15}N values (73% >3.00‰, 63% of which were >5.00‰), which was not indicative of biological N$_2$ fixation being the major source of N for these crops. However, a small number of sites had low or negative leaf δ^{15}N values. These crops had received high N fertiliser applications in the weeks prior to sampling. Two possible pathways that could result in low δ^{15}N values for sugarcane leaves (other than N$_2$ fixation) are proposed; high external N concentrations and foliar uptake of volatilised NH$_3$. The leaf δ^{15}N value of sugarcane grown in aerated solution culture was shown to decrease by approximately 5‰ with increasing external N concentration (0.5–8.0 m*M*), with both NO$_3^-$ and NH$_4^+$ nitrogen forms. Foliar uptake of atmospheric NH$_3$ has been shown to result in depleted leaf δ^{15}N values in many plant species. Acid traps collected atmospheric N with negative δ^{15}N value (-24.45 ± 0.90‰) from above a field recently surface fertilised with urea. The δ^{15}N of leaves of sugarcane plants either growing directly in the soil or isolated from soil in pots dropped by 3.00‰ in the same field after the fertiliser application. Both the high concentration of external N in the root zone (following the application of N-fertilisers) and/or subsequent foliar uptake of volatilised NH$_3$ could have caused the depleted leaf δ^{15}N values measured in the sugarcane crops at these sites.

Introduction

Biological N$_2$ fixation in legume crops can be reliably quantified by the ^{15}N natural abundance method (Shearer and Kohl, 1986; Unkovich and Pate, 2000). This technique can also identify N$_2$ fixing species in natural ecosystems and is based on leaf δ^{15}N signatures relative to the leaf δ^{15}N of known non-N$_2$-fixers present in that system (Boddey et al., 2000; Roggy et

al., 1999). Plants dependent on N$_2$ fixation for most of their N requirement typically have δ^{15}N values from -4.4‰ to 0.0‰ (Hobbie et al., 2000; Rowell et al., 1998). Conversely, plants assimilating predominantly soil derived N generally have positive leaf δ^{15}N values. Högberg (1997) suggests a δ^{15}N difference of 5‰ between non-N$_2$-fixers and N$_2$-fixers when calculating the percentage of plant N derived via biological N$_2$ fixation (%Ndfa). Thus the value 5‰ becomes a point where plants not solely reliant on N$_2$ fixation can

be identified. However, leaf $\delta^{15}N$ can vary significantly when environmental factors other than N source impact on the plant and leaf (Binkely et al., 1992; Domenach et al., 1989; Roggy et al., 1999).

Free-living, N_2-fixing bacteria are known to be associated with sugarcane (James and Olivares, 1997 and references within). Diazotrophic bacteria have also been isolated from Australian sugarcane fields (Chapman et al., 1994; Li and Macrae, 1991; Murphy and Macrae, 1985). Indeed Li and Macrae (1992) reported the highest population counts of *Acetobacter diazotrophicus* bacteria isolated from sugarcane in a commercial field to date. While there are reports of high N fertilisation reducing the populations of N_2-fixing bacteria (Muthukumarasamy et al., 1999), the presence of the bacteria in commercial Australian sugarcane fields (where high N fertiliser regimes are routinely employed) shows the resilience of these bacteria. Nevertheless, the relative importance of biological N_2 fixation for the nitrogen economy of Australian sugarcane remains unresolved.

Yoneyama et al. (1970) were the first to assess N_2 fixation in sugarcane using $\delta^{15}N$ techniques. These researchers measured the $\delta^{15}N$ values of sugarcane leaves from many sites (commercial fields and village gardens) in Brazil, the Philippines and Japan. All sugarcane leaves analysed in their study had positive $\delta^{15}N$ values, ranging from 1.0 to 11.0‰, with the majority having highly positive $\delta^{15}N$ values (50%>5‰). As a primary indicator of biological N_2 fixation, this suggests that most of the sugarcane crops sampled were not dependent on biological N_2-fixation.

When Yoneyama et al. (1997) calculated the %N incorporated via N_2 fixation (%Ndfa) for these sugarcane plants (using 'reference' plants growing adjacent to the sugarcane), 32% showed no (=0%) Ndfa, but the range extended as high as 76% Ndfa. These very high %Ndfa values were clearly due to the adjacent 'reference' plant having a $\delta^{15}N$ value more positive than the sugarcane. The range of $\delta^{15}N$ values for all of the 'reference' plants was greater than the range of values for sugarcane, ranging from -0.4 to 12.9‰, with the range within some species being equivalent to the $\delta^{15}N$ range seen for the sugarcane. If these 'reference' species represent soil-N $\delta^{15}N$ values then the range of soil $\delta^{15}N$ values could also explain the range of $\delta^{15}N$ values seen in the sugarcane. The 'reference' plants accessing different soil N sources could easily cause this, rather than the sugarcane having lower leaf $\delta^{15}N$ due to N_2 fixation.

Thus, selecting a suitable reference plant for comparison with sugarcane to use in a %Ndfa calculation is quite problematic. This is because a (reference) plant with roots penetrating as deeply as sugarcane, i.e. down 5–7 m (Moore, 1987) and accessing similar soil volume is difficult to find. Other problems are the long lifecycle of sugarcane crops and high fertiliser regime employed in sugarcane agriculture.

In this study, the ^{15}N natural abundance technique was used to survey commercial sugarcane crops in Australia. Due to the difficulty of finding appropriate 'reference' plants, only sugarcane leaf $\delta^{15}N$ values are presented. The value of the leaf $\delta^{15}N$ is used as a primary measure to identify possible N_2 fixing plants, i.e. low or negative leaf $\delta^{15}N$ ≤0.00‰ indicates a plant using N_2 fixation as it's primary source for plant N requirements, while a positive $\delta^{15}N$, ≥5.00‰ suggests no, or limited N_2 fixation. Other values of leaf $\delta^{15}N$ are considered indeterminate as indicators of either soil or atmospheric N sources being solely utilised by the plants. The influence of high N fertilisation on sugarcane leaf $\delta^{15}N$ values, and the potential for the incorrect identification of a plant's N source based on leaf $\delta^{15}N$, are also discussed.

Materials and methods

Plant material and culture conditions

Commercial field samplings

Commercial, field-grown sugarcane crops were sampled on single occasions from 12 sites in Australia extending from far-north Queensland to northern New South Wales in the 1993, 1994 and 1995 growing seasons. From the 12 sites, samples were taken from 15 cultivars of *Saccharum* spp. hybrids and included both plant and ratoon crops (Table 1). All crops had been fertilised according to standard commercial practice at each site. Three soil samples (0–150 mm depth) were collected from Rocky Point sites 1, 2 and 3. The $\delta^{15}N$ data for the soil samples from each site were averaged.

The data from the Yandina site are part of a crop cycle experiment established by The Bureau of Sugar Experiment Stations, Bundaberg, Queensland. The site was planted with Q110 sugarcane and had two fertiliser applications, 50 and 150 kg N ha^{-1}. The plants were sampled on four occasions, 1 day before application of urea fertiliser, followed by sampling on days 28, 141 and 279 after fertilising.

Table 1. Commercial sugarcane sites surveyed for sugarcane leaf $\delta^{15}N$ values

Site	Cultivar	Crop class	Plant or harvest date	Fertiliser treatment (kg N ha^{-1})	N form	Date last fertilised	Sample date
Gordonvale	Q152	Plant[a]	01/07/94	230	Urea	08/11/94	13/12/94
Macknade	Q117	Plant[a]	18/08/93	357	Urea	NR	05/05/94
Burdekin	Q96	Plant[a]	07/94	247	Urea	11/94	17/02/95
	Q157	Plant[a]	07/94	247		11/94	17/02/95
	CP51-21	Plant[a]	07/94	247		11/94	17/02/95
Bundaberg site 1	Q136	NR[a]	NR	180	Urea	NR	18/05/94
Bundaberg site 2	CP51-21	Plant[a]	01/09/92	170	NH_4^+	27/10/92	05/07/93
Bundaberg site 3	Q124	Plant[a]	31/08/94	285	Urea	07/12/94	16/01/95
	Q141	Plant[a]	31/08/94	285		07/12/94	16/01/95
	Q154	Plant[a]	31/08/94	285		07/12/94	16/01/95
Yandina	Q110	Plant[a]	21/06/93	150	NH_4^+	10/12/92	15/09/93
Rocky Point site 1	Q124	1st Ratoon[a]	09/94	190	NH_4^+	20/12/94	21/06/95
Rocky Point site 2	Q124	1st Ratoon[a]	09/94	190	NH_4^+	20/12/94	21/06/95
	Q138	1st Ratoon[a]	09/94	190		20/12/94	21/06/95
	Q141	1st Ratoon[a]	09/94	190		20/12/94	21/06/95
	Q153	1st Ratoon[a]	09/94	190		20/12/94	21/06/95
	Q154	1st Ratoon[a]	09/94	190		20/12/94	21/06/95
	Q155	1st Ratoon[a]	09/94	190		20/12/94	21/06/95
	CP51-21	1st Ratoon[a]	09/94	190		20/12/94	21/06/95
Rocky Point site 3	Q124	Plant[a]	08/01/95	NR	NH_4^+	NR	21/06/95
Broadwater	Q124	Plant[b]	NR	200	NR	12/94	03/95
	Q141	Plant[b]	NR	200		12/94	03/95
	TS65-28	Plant[b]	NR	200		12/94	03/95
	Dart	Plant[b]	NR	200		12/94	03/95
Harwood	Q117	Plant[b]	01/09/93	#15	NH_4^+	12/02/95	01/03/95
	TS65-21	Plant[b]	01/09/93	#15		12/02/95	01/03/95

NR=data not recorded.
[a] 1 year crop. [b] 2 year crop.
#=Fertiliser applied by fertigation, cumulative total of N applied not recorded.

Experimental field trial

A controlled, no N fertiliser, field trial was established at Samford, SE Queensland. Eleven sugarcane cultivars (Q117, Q124, Q141, Ajax, Mandalay, Coimbatore, 89B30, M1819-63, H56-752, Fiji 27, and Badilla) were germinated as set out below and then planted randomly in three blocks. At the same time a number of 'reference' plants were randomly planted, as seeds, in three blocks adjacent to the sugarcane. These plants were nodulating (*Vigna unguiculata* cv. Red Lagoona) and non-nodulating (*Glycine max* cv. Clark 63) legumes, and a variety of C$_4$ grasses (*Panicum maximum* var. trichoglume cv. Petrie, *Zea mays* cv. 8532 ex Ord 92, *Sorghum sudanense* cv. Sudan grass, *Chloris gayana* cv. Samford and *Melinis minutiflora*). The field had not previously grown sugarcane.

Superphosphate fertiliser (10% P) was applied at a rate of 200 kg/ha and the site was trickle irrigated. Five soil samples (0–150 mm depth) were collected from the site, and the soil $\delta^{15}N$ data averaged for the whole site.

Aerated solution culture

Q141 sugarcane was germinated as set out below. Polypropylene culture drums were filled with 20 L of continually aerated, minus-N nutrient solution with the following composition (μM); CaSO$_4$.2H$_2$O, 750; MgSO$_4$.7H$_2$O, 600; KCl, 600; KH$_2$PO$_4$, 40; FeNaEDTA.H$_2$O, 200; MnSO$_4$.H$_2$O, 0.5; CuSO$_4$.5H$_2$O, 0.1; ZnSO$_4$.7H$_2$O, 0.3; (NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O, 0.005; Na$_2$SiO$_3$.5H$_2$O, 250; H$_3$BO$_3$, 1; CoCl$_2$, 0.02. Germinated sets were transplanted into polystyrene cups, filled with black poly-

ethylene beads, with a mesh bottom to enable the sugarcane free access to the nutrient solution. Cups were held in position by the drum lid with three cups per culture drum. A completely randomised design with two replicates of eight N concentrations of either NO_3^- or NH_4^+ was chosen. Treatments were either $Ca(NO_3)_2.4H_2O$ or $(NH_4)_2SO_4$ at eight concentrations (μM); 500, 1000, 2000, 4000, 8000, 16 000, 32 000 and 64 000. Solution pH was checked daily and maintained at 5.5 ± 0.2. Nitrate (Sloan and Sublett 1966), ammonium (McCullough, 1967) and phosphorus (Motomizu et al., 1983) were checked and adjusted every second day. Nutrient solutions were completely replaced every 14 days.

Germination of sugarcane sets

Germination of all sugarcane plants used in glasshouse experiments or the Samford controlled experiment was conducted in the glasshouse. One eye sets were planted in washed river sand. Sets were dipped in fungicide (0.5% Benlate® WP, DuPont) and fertilised with 200 μM $CuSO_4$ solution. When 'seedlings' were at the three-leaf stage, plants were ready to transfer to experiments.

Sampling strategy

Sampling of the experimental field trial initially involved collection of whole plant including as much root material as possible. Comparison of whole plant $\delta^{15}N$ *versus* leaf $\delta^{15}N$ showed the same trend in $\delta^{15}N$ arrangement with relation of nodulated legumes to non-nodulated legumes to sugarcane and C4 grasses. As a result, only leaf samples were collected in subsequent field surveys and only leaf $\delta^{15}N$ values are presented in this study.

Experimental field trial

The youngest, fully expanded leaf samples were collected from individual plants in each block 72 days after planting. Samples were bulked from within blocks to provide three replicate samples for analysis. Many pink coloured nodules were evident on the roots of the nodulating *V. unguiculata*, indicative of active symbiotic N_2-fixation. No nodules were found on the non-nodulating *G. max*.

After 414 days growth, all sugarcane plants were harvested by cutting at ground level, and green leaf material and cane trash were removed from the site. The ratoon crop was side-dress fertilised with urea, applied twice: 50 kg N ha^{-1} immediately after harvest, followed by a second treatment of 200 kg N ha^{-1} 2

weeks later. Four days prior to harvest and the first urea fertiliser treatment, 13 Q141 sugarcane plants (germinated as described above) were planted in pots and placed randomly within the trial plot. The potted plants were prevented from accessing the soil via their roots by use of saucers. At the same time, acid traps (see below) were placed beside the potted plants.

Sugarcane cultivar leaves were sampled 12 days prior to harvest, potted Q141 plant leaves were collected 4 days prior to harvest, and then all plants were sampled 9 and 29 days after the second fertiliser application. Three C_4 grass species (*P. maximum* var. Trichoglume cv. Petrie, *C. gayana* cv. Samford, and *M. minutiflora*) were also sampled 12 days prior to harvest and 29 days after the second N-fertiliser application.

Aerated solution culture

Two plants were harvested from each replicate after 134 days in culture. The youngest, fully expanded leaf from each plant was collected. Replicate plant leaves were combined and prepared for $\delta^{15}N$ analysis.

Leaf, soil, atmospheric N sample collection

The middle 200 mm of the top visible dewlap (TVD) leaf was collected, and the main rib removed, from each of three individual sugarcane plants of each cultivar sampled. Samples were oven dried (50 °C), finely ground and analysed by continuous-flow, isotope ratio mass spectrometry (CF-IRMS, Tracer Mass, Europa Scientific, Crewe, UK) as described by Stewart et al., (1995). Reproduciblity of measurements on replicate standards (*Eucalyptus crebra* leaves) was $\pm 0.1‰$.

Acid traps for the interception of volatilised ammonia generated after the fertilisation of the controlled experiment at Samford were established as described by Erskine et al. (1998). The traps consisted of acidified filters enclosed in polytetrafluoroethylene (PTFE) tape (Sorensen and Jensen, 1991) that were hung from bamboo poles under paper cups approximately 0.5 m above the ground. Background atmospheric ammonia was not detectable, as assessed by hanging traps out for 4 days prior to fertilisation and collecting at the time of first urea application. Traps were collected from five sites within the experimental plot at 3, 5, 7 and 9 days after the 200 kgN ha^{-1} treatment. There was no difference in either the amount of N present in the acid traps or the $\delta^{15}N$ at any of these post-fertiliser application sampling times. All data were combined and a single value for volatilised $NH_3 - \delta^{15}N$ is presented.
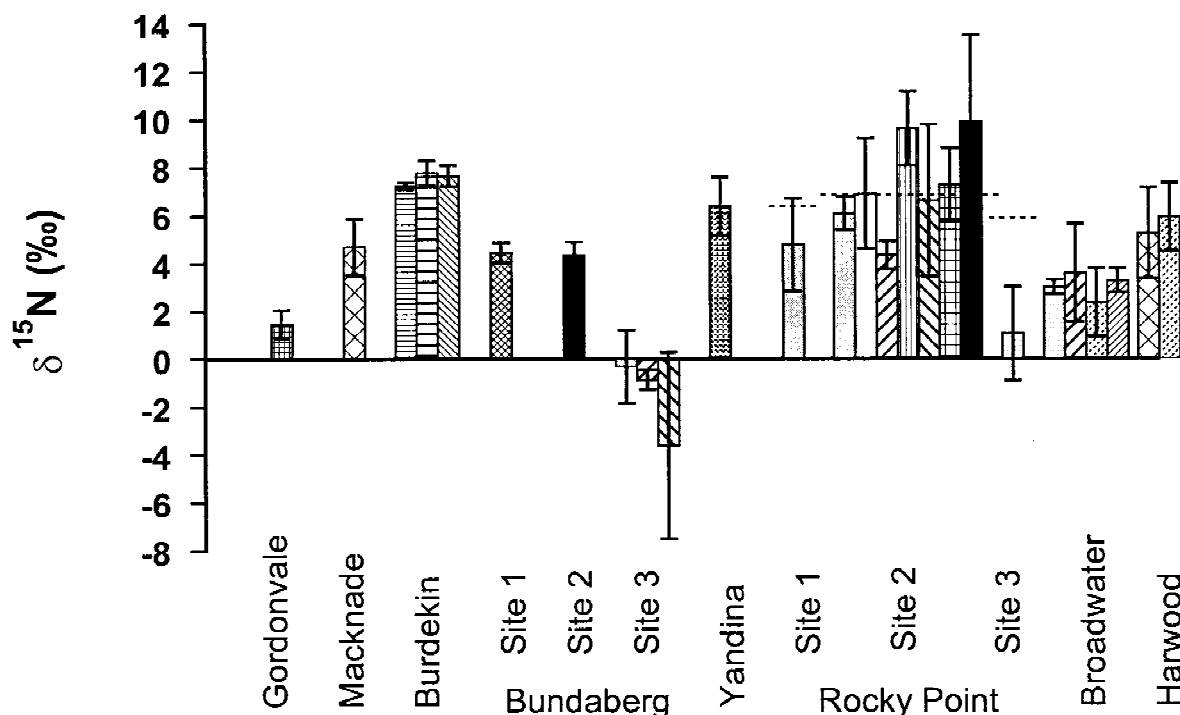
Figure 1. Leaf δ^{15}N values for sugarcane cultivars sampled from 12 commercial sugarcane sites in Queensland and northern New South Wales. Sites are arranged geographically from the northern most site (Gordonvale) to the southern most site (Harwood). Bulk soil δ^{15}N values for the three Rocky Point sites represented by dashed line. Values represent the mean of three plants; error bars equal 1 SD. Sugarcane cultivars: ▤ Q96, ▨ Q117, ☐ Q124, ▦ Q136, ☐ Q138, ▨ Q141, ▦ 152, ▥ 153, ▧ Q154, ⊞ Q155, ▤ Q157, ■ CP51-21, ▨ TS65-28, ▨ H56-21, ▨ Dart.

Results

A survey of leaf δ^{15}N values of commercial sugarcane crops from sites ranging from far north Queensland to northern New South Wales is shown in Figure 1. Different crops were sampled during the growing seasons in 1993, 1994 and 1995 and included both plant and ratoon crop classes (Table 1). Fifteen sugarcane cultivars from 12 sites are represented in this survey. The sugarcane δ^{15}N values measured ranged from −3.65‰ to 9.86‰. At one site (Bundaberg site 3), negative δ^{15}N signatures were measured for three sugarcane cultivars (Q124, Q141, Q154), while at Gordonvale (Q152), Rocky Point site 3 (Q124) and Broadwater (Q124, TS65-28) a low positive δ^{15}N values were recorded.

Within cultivars, similar ranges of variation in leaf δ^{15}N signature were seen. For example, ranges of −0.35‰ to 6.06‰, and −0.90‰ to 4.30‰ were measured for Q124 and Q141, respectively (Figure 1).

No trends were seen either for crop class or year of sampling (data not shown).

Figure 2 presents leaf δ^{15}N for sugarcane, C_4 grasses, as well as nodulating and non-nodulating legumes grown at Samford, SE Queensland. Conditions were controlled with respect to plant age, sugarcane crop class and nitrogen fertilisation. The nodulating *V. unguiculata* had a typical N_2-fixation leaf δ^{15}N value (−0.25‰) compared to the non-nodulating *G. max* with a positive δ^{15}N value (4.70‰). Alongside these plants were grown a range of sugarcane cultivars and C_4 grasses, and these all had positive δ^{15}N signatures similar to, or higher than, the non-nodulating *G. max*.

At ratoon, sugarcane crops are typically fertilised with either a single or split dose of urea fertiliser. The controlled Samford experiment was taken to a simulated harvest (Figure 3A). Leaf δ^{15}N values for the sugarcane were 3.25‰ 16 days before the first fertiliser application, then dropped to −0.18‰ 25 days after the first (and 10 days after the second) fertil-
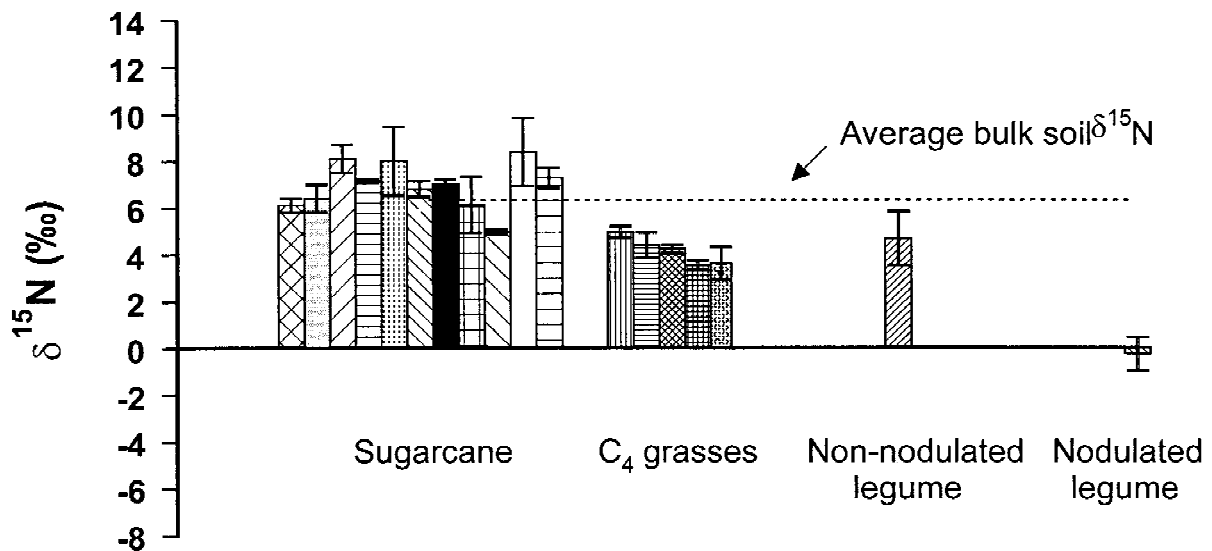
Figure 2. Leaf δ^{15}N values for sugarcane cultivars, C$_4$ grasses, a non-nodulating legume, and a nodulating legume grown in a controlled experiment, no N-fertilisers used, at Samford, Queensland. The δ^{15}N values represent the mean of three leaf samples, error bars are 1 SD. Bulk soil δ^{15}N value is represented by dashed line. Sugarcane cultivars: ▨ Q117, ☐ Q124, ▨ Q141, ▤ 89B30, ▨ M1819-63, ▨ H56-752, ■ Fiji 27, ⊞ Badilla, ◺ Ajax, ☐ Coimbatore, ▤ Mandalay. C$_4$ grasses: ▥ *Zea mays* cv. 8532 ex Ord 92, ▤ *Sorghum sudanense* cv. Sudan grass, ▨ *Panicum maximum* var. Trichoglume cv. Petrie, ⊞ *Chloris gayana* cv. Samford, ▨ *Melnis minutiflora*. Other plants: ▨ *Glycine max* cv. Clark 63 (non-nodulating, Q15963 ex. Lawes 90). ▨ *Vigna unguiculata* cv. Red Lagoona (nodulating).

iser application and recovered to 3.72‰ 20 days later on day 45. C$_4$ grasses were sampled 16 days before (1.30‰) and 45 days after (2.19‰) fertiliser application but, unfortunately, were not sampled at 25 days after the first fertiliser application as occurred with the sugarcane.

Positioned amongst the plants in the trial plot were potted Q141 sugarcane plants (Figure 3B). These potted plants did not have access to the soil and were not fertilised with the other trial plants. These plants also showed a drop in leaf δ^{15}N after the application of urea fertiliser (3.24–0.30‰) but did not recover (1.12‰) to their pre-fertiliser δ^{15}N value by day 45. Similar decreases in sugarcane leaf δ^{15}N values were seen in commercial sugarcane after N-fertiliser applications, followed by recovery (Figure 3C), and when sugarcane was grown in aerated solution culture on increasing N concentrations (Figure 4).

Discussion

In this survey of Australian commercial sugarcane fields, 73% of leaves sampled had highly positive δ^{15}N values and of this group, 63% had δ^{15}N values higher than 5‰. This suggests that these sugarcane crops were not obtaining the majority of their N require-

ments via biological N$_2$ fixation. Of the remainder, 15% had low positive δ^{15}N values. These samples are considered indeterminate in terms of their N source using the technique applied in this study. Plants with mixed N sources tend to have δ^{15}N values ranging from 0.0 to 3.00‰ (Roggy et al., 1999). Only three cultivars at a single site, Bundaberg site 3, had δ^{15}N signatures more typical of plants solely dependent on N$_2$ fixation for their N requirements, i.e. leaf δ^{15}N values from −4.4‰ to 0.0‰ (Hobbie et al., 2000; Rowell et al., 1998).

The variation in the leaf δ^{15}N values observed in our study was probably due to factors intrinsic to the site where the leaves were sampled. The survey data (Figure 1) showed no trends with geographic location of site, crop class or cultivar. There was, however, a trend of higher leaf δ^{15}N value with increasing time since the last N-fertiliser application. The sites from which the lowest δ^{15}N values were recorded, Bundaberg site 3 and Gordonvale, were both sampled within 6 weeks of their last N-fertiliser treatment. The Rocky Point site 3 also recorded a low δ^{15}N value. This was the youngest crop in the survey (only 5 months old) and was probably fertilised in the period before sampling, though this was not officially recorded. Eight weeks after the last N-fertiliser application,
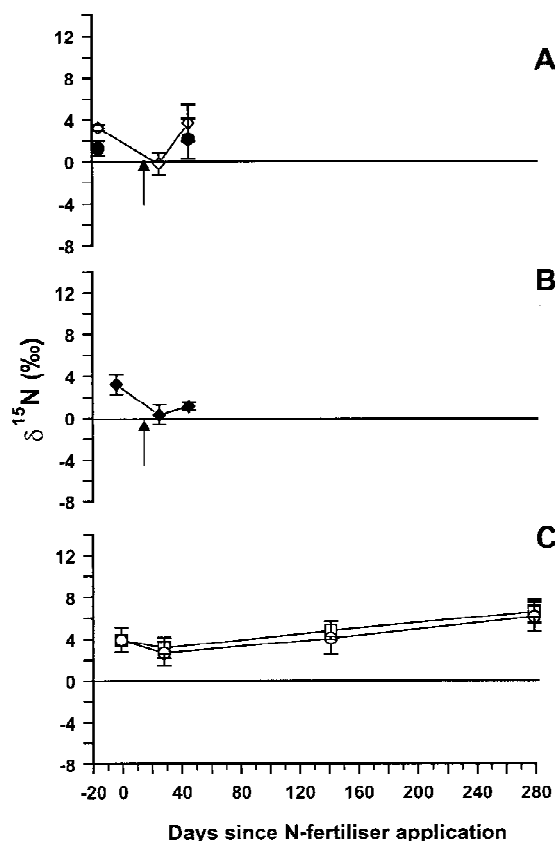
Figure 3. Influence of N fertiliser application on sugarcane leaf δ^{15}N. (A) Simulated ratoon crop at Samford. Two fertiliser applications; time 0 days–50 kgN ha^{-1}, and (↑) 200 kgN ha^{-1} at time 14 days. Sugarcane cultivars (◇), non-N$_2$ fixing species (●). (B) Potted Q141 sugarcane reference plants (◆). Two fertiliser applications; time 0–50 kgN ha^{-1}, and (↑) 200 kgN ha^{-1}. (C) Commercial Q110 sugarcane at Yandina, Queensland. Application of 50 kgN ha^{-1} (◯), 150 kgN ha^{-1} (□) at time 0.

Table 2. Soil-NH_4^+ and $-NO_3^-$ in N-fertilised sugarcane fields. Data from Ian Vallis Pers. comm. Urea fertiliser applied – Red Earth=40.90 mM and Podzolic=39.00 mM. 2 M KCl extraction, colorimetric assay

Weeks after fertilisation	Red Earth		Podzolic	
	NH_4^+-N (mM)	NO_3^--N (mM)	NH_4^+-N (mM)	NO_3^--N (mM)
Pre-fertilisation	1.50	0.85	0.30	0.80
4	45.90	4.70	0.60	13.00
12	5.60	2.20	1.70	6.10
24	0.10	0.90		

C$_4$ grasses or non-nodulating legume (Figure 2). The *V. unguiculata* (nodulating legume) at the site had a typical (for an N$_2$-fixing plant) negative δ^{15}N value (-0.25 ± 0.73‰). All measured δ^{15}N values were typical of literature values for plants dependent on either soil N (sugarcane, C$_4$ grasses and non-nodulating *G. max*) or N$_2$ fixation (*V. unguiculata*). The indication from this controlled experiment was that under the experimental conditions, sugarcane did not depend on N$_2$ fixation for its N requirements.

The survey of Australian commercial sugarcane, the controlled field experiment at Samford, and the survey of Yoneyama et al. (1997) show the majority of sugarcane leaf δ^{15}N values to be highly positive and thus not directly indicative of N$_2$ fixation. The isolated negative or low-positive sugarcane leaf δ^{15}N values suggest the possibility of alternative N sources, perhaps a mixed N source (possibly including N$_2$ fixation), the possible influence of mycorrhiza on N uptake, or the influence of the high N-fertiliser applications used in sugarcane agriculture.

Recommended N-fertiliser rates for the Australian sugarcane industry (150–250 kg N ha^{-1} year^{-1}) are high by world standards and growers often exceed these rates (Keating et al., 1997). Soon after the application of N fertilisers to sugarcane crops, there is usually an increase in the soil solution N concentration that dissipates with time (pers. comm. Ian Vallis, Table 2). Keating et al. (1994) also showed a similar trend for the NO_3^- pool below a sugarcane crop. At the two sites sampled by Vallis, the NH_4^+ levels peaked at 45.9 mM at the red earth site and NO_3^- levels peaked at 4.7 mM for the red earth site and 13.0 mM at a podzolic soil site. These concentrations are well in excess of the concentrations used in the aerated solution cultures. This pulse of N could temporally influence plant δ^{15}N

leaf δ^{15}N values were uniformly positive regardless of site or cultivar. Only at the Broadwater site, where 12 weeks after the last application of fertiliser two low δ^{15}N values were measured, did the leaf δ^{15}N values not fit this observation.

To test leaf δ^{15}N in a controlled situation, where sugarcane could be directly compared with other plant species and to remove N fertiliser effects, plants were grown in a no N-fertiliser field trial at Samford, SE Queensland. The leaf δ^{15}Ns from this trial showed sugarcane to have positive δ^{15}N values (5.0–8.4‰) similar to, or higher than, those of C$_4$ grasses (3.6–5.0‰) and a non-nodulating legume (*G. max*) (4.7$\pm$1.16‰). The δ^{15}N averaged for total soil N sampled from this site was also similar to the δ^{15}N of the sugarcane but more positive than either the
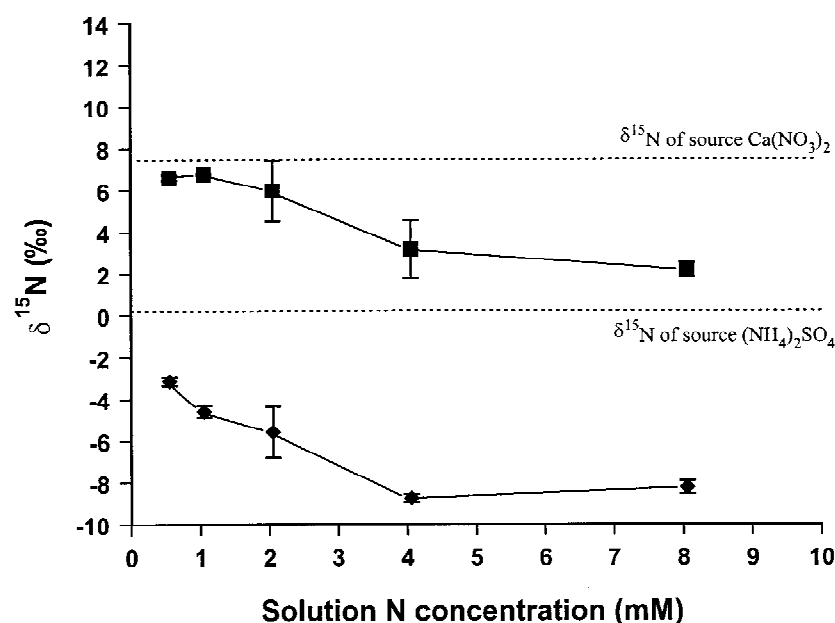
Figure 4. Leaf δ^{15}N of Q141 sugarcane grown in aerated solution culture on either NO_3^- (■) or NH_4^+ (♦) nitrogen forms. Values represent the mean of two plants and error bars are 1 SD.

Table 3. δ^{15}N of commercial N-fertilisers (typically used in sugarcane agriculture) and laboratory chemicals (used aerated solution culture, see Figure 4). $(NH_4)_2SO_4$ and $Ca(NO_3)_2$ are analytical grade reagents. Values represent the mean of three replicates, figures in parenthesis are SD

N-fertiliser	N form	%N	δ^{15}N
Urea (Crop King, Incitec)	Urea	41.6 (0.8)	−1.35 (0.09)
'Granam' (Crop King, Incitec)	NH_4^+	19.0 (0.0)	−1.23 (0.31)
'140S' (Crop King, Incitec)	Urea	28.4 (0.7)	−0.99 (0.10)
$(NH_4)_2SO_4$ (Fisons, AR grade)	NH_4^+	20.9	0.17
$Ca(NO_3)_2$ (Ajax, AR grade)	NO_3^-	11.4	7.45

in a manner similar to that seen in the solution culture experiment presented here (Figure 4).

When external N supply saturates high affinity N uptake systems, increased discrimination against uptake of ^{15}N results in a depleted δ^{15}N value, especially in any NO_3^- pools in leaves (Evans et al., 1996). As the external N supply increases, leaf δ^{15}N decreases (Bergersen et al., 1988; Yoneyama et al., 1991). This trend was seen in the aerated solution culture Q141 sugarcane (Figure 4) and the negative δ^{15}N of the $(NH_4)_2SO_4$ further depleted the leaf δ^{15}N compared to the $CaNO_3$ source. Our data show that the inorganic N fertilisers commonly used in sugarcane agriculture in Australia ('Granam' or urea-based blends) have negative δ^{15}N values (Table 3). These two factors (either individually or together) could result in depleted leaf

δ^{15}N values in periods soon after high N-fertiliser applications.

A second potential cause for depleted leaf δ^{15}N values after N fertilisation is the uptake of volatilised NH_3. There is a potential for up to 20% of applied N to be lost via volatilisation in the first 3 weeks after the surface application of urea (Freeney et al., 1994). This volatilised N source has a negative δ^{15}N value and is accessible to plants via their leaves, resulting in plants with depleted leaf δ^{15}N values (Erskine et al., 1998). The evolution of volatilised NH_3 from the soil would leave a more enriched soil N source behind and this possibly explains the relatively quick recovery of plant δ^{15}N several weeks after the fertiliser application. Thus, there are two potential mechanisms for N fertilisers to influence the δ^{15}N value of sugarcane leaves.

After the simulated harvest and ratooning of the controlled field experiment (including the application of 250 kg N ha^{-1}), there was a dramatic drop in the leaf δ^{15}N of the potted sugarcane placed amongst the field sugarcane in this experiment (Figure 3B). This mirrored the decrease in leaf δ^{15}N of the planted sugarcane (Figure 3A). The potted sugarcane had no access to the soil N pools and so was not influenced by increased external N source concentration. This indicates that foliar uptake of volatilised NH$_3$, from the surface applied urea fertiliser resulted in the change in leaf δ^{15}N. Acid traps placed among the sugarcane plants to intercept atmospheric N showed a pulse of N with an extremely depleted δ^{15}N value ($-24.45\pm0.90\permil$) in the period just after the application of the urea fertiliser. A similar trend is seen in a commercial sugarcane field (Figure 3C). The recovery of the δ^{15}N value, which follows the pulse of N fertiliser application, could be due to a shift in the N source of the sugarcane from the volatilised pulse of NH$_3$ to the available soil N sources which would have a more enriched δ^{15}N signature due to the loss of the ^{15}N depleted NH$_3$.

Either the foliar uptake of volatilised NH$_3$-N or fractionation due to high external N sources can decrease sugarcane leaf δ^{15}N values and could explain the low δ^{15}N values seen at some sites in this survey. These effects could also explain some of the low positive δ^{15}N values seen by Yoneyama et al. (1997). The low positive δ^{15}N values at the Broadwater site, however, do not fit with these explanations. The isolated nature of this result may suggest biological N$_2$ fixation. However, the site is relatively wet with high soil organic matter and it is possible that other factors such as mycorrihizal influences on N uptake have influenced the plant leaf δ^{15}N values.

Our study indicates that in Australian commercial sugarcane fields N$_2$ fixation is not a major source of N for sugarcane plants. While the power of the δ^{15}N technique is further highlighted, this study shows that fertiliser management practices peculiar to intensive cropping agriculture can have dramatic effects on the δ^{15}N value of sugarcane leaves. These effects are temporary but must be considered if accurate interpretation of the δ^{15}N data is to be made.

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Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement

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Abstract

^{15}N isotope and N balance studies performed over the last few years have shown that several Brazilian varieties of sugarcane are capable of obtaining over 60% of their nitrogen (>150 kg N ha^{-1} year^{-1}) from biological nitrogen fixation (BNF). This may be due to the fact that this crop in Brazil has been systematically bred for high yields with low fertilizer N inputs. In the case of wetland rice, N balance experiments performed both in the field and in pots suggest that 30 to 60 N ha^{-1} crop^{-1} may be obtained from plant-associated BNF and that different varieties have different capacities to obtain N from this source. ^{15}N$_2$ incorporation studies have proved that wetland rice can obtain at least some N from BNF and acetylene reduction (AR) assays also indicate differences in N$_2$-fixing ability between different rice varieties. However in situ AR field estimates suggest plant-associated BNF inputs to be less than 8 kg N ha^{-1} crop^{-1}. The problems associated with the use of the ^{15}N dilution technique for BNF quantification are discussed and illustrated with data from a recent study performed at EMBRAPA–CNPAB. Although many species of diazotrophs have been isolated from the rhizosphere of both sugarcane and wetland rice, the recent discovery of endophytic N$_2$-fixing bacteria within roots, shoots and leaves of both crops suggests, at least in the case of sugarcane, that these bacteria may be the most important contributors to the observed BNF

contributions. In sugarcane both *Acetobacter diazotrophicus* and *Herbaspirillum* spp. have been found within roots and aerial tissues and these microorganisms, unlike *Azospirillum* spp. and other rhizospheric diazotrophs, have been shown to survive poorly in soil. *Herbaspirillum* spp. are found in many graminaceous crops, including rice (in roots and aerial tissue), and are able to survive and pass from crop to crop in the seeds. The physiology, ecology and infection of plants by these endophytes are fully discussed in this paper. The sugarcane/endophytic diazotroph association is the first efficient N_2-fixing system to be discovered associated with any member of the gramineae. As yet the individual roles of the different diazotrophs in this system have not been elucidated and far more work on the physiology and anatomy of this system is required. However, the understanding gained in these studies should serve as a foundation for the improvement/development of similar N_2-fixing systems in wetland rice and other cereal crops.

Introduction

The "green revolution" in agriculture of the developing world which resulted in large increases in cereal grain production since the 1960s, has been a result of the development of plant genotypes highly responsive to chemical fertilizers, particularly nitrogen. It requires approximately 18.5 Mcal of fossil energy to produce one kg of fertilizer nitrogen and even though, unlike other fertilizers, there is an unlimited supply of this element in the air, this is more than 6 times the energy required to produce either phosphate or potassium fertilizers (Da Silva et al., 1978). With the inevitable price rises of fossil fuels (not to mention proposed carbon taxes) that must occur over the next few decades due to the depletion of petroleum reserves and increased production costs of other fuels, now is the time that alternative strategies for nitrogen supply should be developed before these increased costs force farmers to cut N inputs which will result in drastic yield reductions in the staple cereal crops which feed the burgeoning human population of the Third World.

In traditional wetland rice culture yields of 2 to 3 t grain ha^{-1} (either one or two crops year^{-1}) seem to be sustainable indefinitely, even where no N fertilizer is applied, if flood water is well controlled. For such yields to be sustained between 60 and 80 kg of nitrogen are required for each crop (Bennett and Ladha, 1992) and while some of this input may be supplied in rainfall and irrigation water, several field N balance studies suggest that N is supplied in part by nitrogen-fixing organisms (Firth et al., 1973; Koyama and App, 1979; Walcott et al., 1977).

Virtually all of the varieties of sugar cane planted in Brazil were bred under conditions of low N fertilizer inputs. Probably for this reason, the plant-crop rarely responds to nitrogen fertilizer (Azeredo et al., 1981), and while ratoon crops do often respond to N application, quantities applied rarely exceed 100 kg N

ha^{-1} and fertilizer use efficency is usually less than 35% (Oliveira et al., 1994; Sampaio et al., 1984). A sugar cane crop yielding 100 t cane ha^{-1} accumulates between 180 and 250 kg N ha^{-1} (Orlando-Filho et al., 1980; Stanford and Ayres, 1964). The mean Brazilian yield is 65 to 70 t cane ha^{-1} and average whole crop N accumulation is between 100 to 120 kg N. Of this approximately two thirds is transported to the mill in the cane stems and a further 25% is in the senescent leaves (trash), which in Brazil, as in most countries, is burned off before harvest (Oliveira et al., 1994). Less than 10% of the N in the form of flag leaves remains in the field. It is apparent from these data that continuous cropping of sugar cane should deplete soil N reserves such that cane yields eventually decline. However, such decline in yields or soil N reserves are not normally observed even after many decades, or even centuries, of cane cropping. Such observations have led several authors to suggest that sugar cane may benefit significantly from inputs from biological nitrogen fixation (BNF) (Döbereiner, 1961; Purchase, 1980; Ruschel et al., 1978).

Quantification of biological nitrogen fixation

Sugar cane

Only a few studies have been published on the quantification of the BNF contribution to sugar cane and all of them were performed in Brazil. Experiments using ^{15}N-labelled N_2 gas conducted at the Centro de Energia Nuclear na Agricultura (CENA) in Piracicaba (São Paulo) showed that 90 day-old sugar cane plants obtained considerable N from BNF (Ruschel et al., 1975). However, because of the difficulties of exposing plants grown in the field to controlled atmospheres, the agronomic significance of these N inputs could not be evaluated (Matsui et al., 1981). In a subsequent

[15]N-aided N balance study performed at CNPAB, sugar cane was grown in pots containing 64 kg soil (Lima et al., 1987). Both the N balance and ^{15}N enrichment data indicated that between 40 and 60% of plant N was derived from plant-associated BNF and extrapolation to the field (15,000 plants ha^{-1}) suggested inputs of over 150 kg N ha^{-1} $year^{-1}$.

Our group has recently completed a three-year ^{15}N isotope dilution and N balance study on 10 sugar cane varieties grown in a concrete tank (20 × 6 × 0.8m) filled with soil amended with ^{15}N-labelled organic matter, and using *Brachiaria arrecta* as a non-N_2-fixing control plant (Urquiaga et al., 1992). The soil had a low N content (0.108% N) and was fertilized with phosphorus, potassium and micronutrients and well irrigated throughout the experiment, but no N fertilizer was added. In the first year yields of fresh cane of the commercial varieties were high, ranging from the equivalent of 175 to 230 t ha^{-1}, and in the varieties CB 45–3 and SP 70–1143 these high yields were maintained during the subsequent two ratoon crops. In these same varieties and the *Saccharum spontaneum* variety, Krakatau, the nitrogen accumulation also continued to be high and stable over the three years. However, other varieties (e.g., CB 47–89, NA 56–79, SP 71–799, Chunee) showed a decline in total N content after the first year as would be expected from the observed decline in the availability of soil N. Over the whole three years, the weighted mean ^{15}N enrichments of all of the sugar cane varieties were much lower than that of the non-N_2-fixing *B. arrecta* control, indicating large contributions of plant associated BNF (Table 1).

At the second and third annual harvests (first and second ratoon crops) there were only small difference in the ^{15}N enrichments between the different varieties and that of the control crop, which was due to the carry-over of labelled nitrogen from one harvest to the next in the stem bases and roots of cane varieties, which did not occur in the case of the *B. arrecta*. The interpretation of the ^{15}N data was further complicated by the fact that the uptake of soil N by the *B. arrecta* was almost certainly inhibited towards the end of each growing season due to shading of this crop by the tall sugar cane plants, and this probably resulted in a somewhat higher ^{15}N enrichment in the control crop than otherwise would have occurred.

These difficulties are fully discussed in the original paper (Urquiaga et al., 1992), and because of them it was decided to perform a total N balance on the whole tank by the careful analysis of the N content of soil samples taken at plant emergence in the first

year in comparison with samples taken at the final harvest. These data showed that there were significantly ($p<0.05$) positive N balances associated with the varieties CB 45–3, SP 70–1143, SP 79–2312 and Krakatau, and that there was a good agreement between the ^{15}N dilution and the total N balance estimates of the contributions of BNF to the sugar cane varieties (Table 1).

These results were recently confirmed in a long-term nitrogen balance experiment conducted on a sugar cane plantation in Pernambuco, NE Brazil (Oliveira et al., 1994). In this experiment the effect of pre-harvest burning of the cane (to remove the senescent leaves) on the yield and N accumulation of the crop, and N balance of the cropping system, were investigated. At the end of the 9 year study the total N accumulated in the system was found to be between 300 and 620 kg ha^{-1} greater than the initial N (Table 2). This extra N was attributed to a mean annual BNF input to the crop of between 38 and 77 kg N ha^{-1}, this being a minimum estimate as gaseous or leaching losses were not quantified.

Wetland rice

Several field N balance studies on lowland rice have been reported from studies in Thailand (Firth et al., 1973; Walcott et al., 1977), Japan (Koyama and App, 1979) and at the experimental fields of the International Rice Research Institute (IRRI) in the Philippines (App et al., 1984; Ventura et al., 1986). All studies report a positive balance even when N from rainfall and irrigation water were discounted indicating inputs of between 30 and 60 kg N ha^{-1} $crop^{-1}$, but in these studies no data are available to determine what proportion of this N may be derived from free-living N_2-fixing cyanobacteria in the flood water, heterotrophic N_2 fixers in the soil or those associated with the plant.

Various nitrogen balance experiments have been performed in pots which indicate that the plant/soil system can benefit from biological N_2 fixation (BNF) even when the activity of cyanobacteria on the soil surface is inhibited by shading (De and Sulaiman, 1950; Willis and Green, 1948). In a very careful N balance study performed in pots by App et al. (1980) on 4 to 6 consecutive crops, the contribution of plant-associated BNF was estimated to be equivalent to 18% of plant N. In a further N balance study on 83 wild and cultivated rice cultivars (in 6 separate experiments each with 3 consecutive crops) reported by App et al. (1986), large and significant differences between cultivars were found.

Table 1. ^{15}N enrichment and total nitrogen accumulation of sugar cane and *Brachiaria arrecta* and estimates of nitrogen derived from BNF using N balance and ^{15}N isotope dilution techniques (g N m^{-2}). Means of 4 replicates. After Urquiaga et al. (1992)

Variety / Species	Weighted mean atom % ^{15}N excess	Final N content of soil	N accum. whole plant 3 years	Estimates of BNF contribution			
				All three years		Annual mean	
				N balance[z]	^{15}N[y]	N balance	^{15}N
				(g N m^{-2})			
CB 47–89	0.191bcd	835	61.4bc	39.7	34.8c	13.2	11.6
CB 45–3	0.166cde	864	84.3ab	62.6	52.6b	20.9	17.5
NA 56–79	0.198bc	884	57.8c	36.1	32.6c	12.0	10.9
IAC 52–150	0.188bcd	924	59.6bc	37.9	33.8c	12.6	11.3
SP 70–1143	0.146de	852	77.5bc	55.8	51.9b	18.6	17.3
SP 71–799	0.183bcd	860	56.9c	35.2	33.3c	11.7	11.1
SP 79–2312	0.198bc	845	63.6c	41.9	35.4c	14.0	11.8
Chunee	0.227b	826	33.0d	11.3	16.9d	3.8	5.6
Caiana	0.190bcd	857	11.6d	-10.1	6.7d	- 3.4	2.2
Krakatau	0.133e	857	102.8a	81.1	71.8a	27.0	23.9
B. arrecta	0.443a	830	24.9	3.2	-	1.1	-
CV (%)	13.6	5.1	25.0	29.2		29.2	

[z] N balance estimate of BNF contribution = total N accumulated by crop + mean total N content of soil in tank at final harvest - mean total N content of soil in tank at emergence. Mean change in soil N content from emergence until final harvest = 27.1 g N m^{-2} with a standard error of the difference between the means of 22.0 g N m^{-2}. N balances greater than 37.7 g N m^{-2} (12.4 g N m^{-2} year^{-1}) were significantly greater than zero (p=0.05, Student t test).

[y] ^{15}N isotope dilution estimate of BNF contribution = (total N accumulated by the crop) × (1 - (weighted mean atom % ^{15}N excess of sugar cane)/(weighted mean atom % ^{15}N excess of *B. arrecta*).

The positive N balances were equivalent to between 16 and 70 kg N ha^{-1} crop^{-1} assuming 25 plants m^{-2} and, although in all 6 experiments there were significant correlations between N balance and plant N uptake, because of the nature of this technique it cannot necessarily be assumed that the N was fixed and immediately incorporated into the plants.

Direct evidence that heterotrophic diazotrophs can contribute significant quantities of N to rice plants was obtained by the short-term exposure of individual plants to ^{15}N enriched N$_2$ gas (Ito et al., 1980; Yoshida and Yoneyama, 1980; Eskew et al., 1981; Nayak et al., 1986), but most of the labelled nitrogen fixed remained in the rhizosphere soil. However, these data do not permit estimation of BNF contributions over the entire plant growth cycle.

There are many studies which have used the acetylene reduction (AR) assay to study BNF associated with rice. The early studies (e.g. Rinaudo and Dommergues, 1971; Yoshida and Ancajas, 1970, 1973)

utilized an assay on excised roots. Later studies on rice and other grasses and cereals suggested that these techniques were unreliable and perhaps overestimated actual N$_2$ fixing activity (Barber et al., 1976; Koch, 1977; Tjepkema and Van Berkum, 1977), and subsequently in situ assays were developed (Balandreau and Dommergues, 1971; Boddey et al., 1978; Lee et al., 1977). The use of these in situ techniques in the field showed considerable AR activity associated with field grown plants (Watanabe et al., 1978a, 1981) but this technique suffers from several disadvantages for the estimation of actual BNF contributions to the plants: Firstly, the AR technique measures nitrogenase activity and not incorporation of fixed N into the plant, secondly much of the evolved ethylene may be retained in the waterlogged soil and not diffuse into the atmosphere which is sampled, and finally the measure is instantaneous and requires many assays throughout the growing season if overall contributions of BNF to the crop are to be assessed (Boddey, 1987; Roger and Watan-

Table 2. Effect of pre-harvest burning on total nitrogen balance (g N m^{-2}) of the soil/plant system of field grown sugar cane over a sequence of the plant crop followed by 7 ratoon crops. Means of 16 replicates

Treatment	N accumulated by crop in over 8 cuts 1983–1992	Total N in soil/plant system Considering soil N content in the layer:					
		0–20cm			0–60cm		
		N[a] Initial	N[b] Final	Balance	N[a] Initial	N[b] Final	Balance
				(g N m^{-2})			
Burned	58.3	365.9	354.1	-11.8	789.0	744.6	-44.4
Unburned	73.6	369.1	400.6	+31.5	774.3	828.7	+54.4
HSD[c] *p*=0.05	7.0	24.2	29.7	30.6	74.5	64.1	61.9
CV (%)[d]	14.2	8.9	10.6	*(10.3)*[e]	12.8	11.0	*(20.9)*[e]

[a] Initial N in soil plant/system = total N in soil at planting + added fertilizer N.
[b] Final N in soil/plant system = total N in soil at final harvest + N accumulated by crop over 8 harvests (1983 to 1992).
[c] Honest significant difference (Tukey).
[d] Coefficient of variation.
[e] Value in *italics* = Standard error of mean.

abe, 1986). In studies where many in situ AR assays were taken, the estimates of total "acetylene reduced" thoughout the whole crop cycle were approximately 40 to 60 m mol ethylene m^{-2} (Boddey and Ahmad, 1981; Watanabe et al., 1978b) which extrapolate to only 5 to 8 kg N$_2$ fixed ha^{-1}. Results from the excised root and in situ AR assays on wetland rice were of similar magnitude (Boddey et al., 1978; Boddey, 1981) and it is generally considered that this technique over-estimates N$_2$-fixing activity (Berkum and Bohlool, 1980; Giller, 1987).

It seems therefore that there is a considerable disparity between the N balance and AR estimates of plant-associated BNF to wetland rice. Some of the field and pot N balance studies suggest contributions of more than 30 kg N ha^{-1} crop^{-1} whereas the acetylene reduction studies suggest inputs not higher than 8 kg ha^{-1}.

The ^{15}N isotope dilution technique has the potential to estimate contributions of BNF to the plants over the whole growth season and unlike the N balance and acetylene reduction techniques, it estimates fixed N actually incorporated into the plant tissue (Chalk, 1985; McAuliffe et al., 1958). The main problem with this technique lies in labelling the soil with ^{15}N. If the enrichment varies with area, depth or time, different plants (the control and different rice varieties) may have different N uptake patterns and do not obtain the same ^{15}N enrichment in the soil derived N, an assumption essential to the application of the technique (Boddey, 1987; Witty, 1983). In the studies reported so far the soil N was not stable with time and no suitable non-N$_2$-fixing control plant was found that would grow in waterlogged soil (Nayak et al., 1986; Ventura and Watanabe, 1983).

A recent study was conducted at our institute (CNPAB) near Rio de Janeiro (Oliveira, 1994) and at the first planting 40 rice varieties were planted in a tank (20 × 6 × 0.6m) filled with waterlogged soil amended with ^{15}N-labelled compost (Urquiaga et al., 1992) and inoculated with soil taken from a long-established rice paddy in the Paraíba valley of São Paulo State. Analyses of leaf samples showed that there was a considerable decline in ^{15}N enrichment in the plant tissue during plant growth and earlier maturing varieties showed higher ^{15}N enrichments than later maturing varieties (Table 3). There were considerable differences in total N accumulation and ^{15}N enrichment between different varieties but higher N accumulation was not well correlated with lower ^{15}N enrichment even within each maturity group (Table 4).

Subsequently 20 of these rice cultivars were replanted in the same tank. Again ^{15}N enrichment in plant tissue decreased with time and the varieties

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Table 3. Grain production, N accumulation and ^{15}N enrichment of leaf samples at 40 days after emergence (DAE) and of whole plant at final harvest of 5 rice varieties from each of 3 maturity groups. Plants grown in tank of soil labelled with ^{15}N. Means of 4 replicates. After Oliveira (1994)

Rice variety	Grain yield[z] (g m^{-2})	N accumulation (g N m^{-2})	^{15}N enrichment Atom % ^{15}N exc. Final[y] harvest
Maturity group 1 (60–85 DAE)			
Labelle	396 d	7.37 d	0.2074a
CNA 6837	814 ab	9.28 bc	0.2305a
Bluebelle	633 c	8.55 cd	0.1984a
BR-IRGA-410	766 ab	9.25 bc	0.2301a
BR-IRGA-409	759 ab	10.54 ab	0.2134a
C.V. (%)	10.3	9.0	11.7
Mean for whole group			
7 varieties	711	94.9	0.2160
Maturity group 2 (80–110 DAE)			
IR 4432–28–5	942 b	17.82 a	0.1475 c
MG-1	1097 a	15.83 ab	0.1559 bc
IR-841	701 c	11.46 c	0.1586 bc
CICA-9	930 b	14.43 b	0.1618 bc
CNA 4215	698 c	8.87 d	0.1822 ab
C.V. (%)	11.0	11.0	11.2
Mean for whole group			
18 varieties	906	13.3	0.1608
Maturity group 3 (110–140 DAE)			
Metica-1	1130 ab	16.19 b	0.1557 cd
De-Abril	1070 ab	22.18 a	0.1421 d
IAC-4440	1100 ab	15.30 b	0.1973 a
CICA-8	1070 ab	14.32 b	0.1758 abc
IR-42	799 c	15.68 b	0.1388 d
C.V. (%)	10.8	12.0	10.5
Mean for whole group			
15 varieties	1060	15.0	0.1621

[z] Grain at 14% humidity.
[y] Weighted mean ^{15}N enrichment of whole plant.

Table 4. Regressions of total nitrogen accumulation and ^{15}N enrichment
at final harvest of 40 rice varieties divided into 3 maturity groups planted
in waterlogged ^{15}N-labelled soil. First crop (1989/90). After Oliveira
(1994)

Maturity group	Days after emergence	Correlation coefficient	Probability	No. of data points
1	60–85	+ 0.281	0.147	28
2	85–110	- 0.320	0.006	72
3	110–140	- 0. 201	0.124	60

IR 42 and IR 4432–28–5 showed significantly lower ^{15}N enrichment and higher N accumulation than the variety IAC 4440 and the non-N_2-fixing control plant, *Brachiaria arrecta* (data not shown). Data from the N balance study of App et al. (1986) as well as acetylene reduction assays and a natural abundance (delta) ^{15}N study both performed at IRRI in the Philippines also suggest that the variety IR 42 is able to obtain significant contributions from plant associated BNF (Barraquio et al., 1986; Ladha et al., 1987a, b; Watanabe et al., 1987a).

Results from the third planting of this ^{15}N experiment were lost due to a fire in the drying oven but at the fourth planting just these 3 varieties were planted with the same control plant and harvests were taken at six times during plant growth (Table 5). The acetylene reduction activity of the 4 crops was evaluated by incubating the plant/soil system at constant temperature in the dark as described by Barraquio et al,. (1986). No significant differences were found between varieties but the rice varieties were far higher in AR activity than the *B. arrecta* control (data not shown). After the 3rd harvest (86 DAE) the ^{15}N enrichment of the *B. arrecta* control was lower than that of the rice varieties (significantly so at the final harvest) but this result could not be due to a soil N uptake pattern different from the rice varieties as the data indicate that the ^{15}N enrichment of the soil mineral N was virtually stable during crop growth. Furthermore, while the variety IR 4432–28–5 had a lower ^{15}N enrichment than the other two rice varieties the total N accumulation of this cultivar showed a tendency to be lower.

Hence, the data obtained in this study do not confirm significant BNF contributions to any of the 3 varieties of wetland rice even though two of them were pre-selected for high N accumulation and low ^{15}N enrichment. Whether this is due to adverse soil fertility factors or indicates that BNF inputs are generally very

low requires further investigation. The results illustrate the difficulties involved in the application of this technique for quantifying BNF contributions to wetland rice and the necessity to use soil with a uniform and stable ^{15}N enrichment.

Plant-associated N_2-fixing bacteria

Sugar cane

In the 1950s Döbereiner (1961) found N_2-fixing bacteria of the genus *Beijerinckia* in high numbers in sugar cane fields, with selective enrichment in the rhizosphere and especially on the root surface. At the same time a new species of *Beijerinckia* was discovered (*B. fluminense*) associated with this crop (Döbereiner and Ruschel, 1958). Subsequently, other authors (Graciolli et al., 1983; Purchase, 1980) isolated a wide range of N_2-fixing bacteria from the roots, stems and even leaves of sugar cane including species of *Erwinia*, *Azotobacter*, *Derxia*, *Azospirillum* and *Enterobacter*. None of these bacteria seemed to occur in large enough numbers to account for the extremely high rates of N_2 fixation reported above.

More recently, a new species of N_2-fixing bacteria, *Acetobacter diazotrophicus*, was found to occur in large numbers in the roots and stems of sugar cane (Cavalcante and Döbereiner, 1988; Gillis et al., 1989). This most extraordinary diazotroph was originally isolated from semi-solid sugar cane juice inoculated with dilutions of sugar cane roots and stems which showed acetylene reduction (nitrogenase) activity in dilutions up to 10^{-6} to 10^{-7} (fresh weight). A more specific medium (LGIP) has now been developed (Reis et al., 1994).

The bacteria is a small, Gram-negative, aerobic rod showing pellicle formation in N-free semi-solid medi-

Table 5. Total nitrogen accumulation and ^{15}N enrichment of 3 rice varieties and *Brachiaria arrecta* planted in waterlogged ^{15}N labelled soil during the plant growth cycle. Fourth crop (1992/3). Harvested area 0.5 m^2. Means of 4 replicates. After Oliveira (1994)

Variety	Days after emergence of rice					
	36	52	86	94	108	130
Total N accumulation (g N m^{-2})						
IR 42	0.728ab	0.692ab	1.951ab	2.389a	3.837a	4.449a
IAC 4440	0.902a	0.767a	3.101a	3.299a	4.093a	4.799a
IR 4432–28–5	0.792a	0.774a	2.013ab	2.476a	3.757a	4.055a
B. arrecta	0.166b	0.321b	0.867b	0.601b	1.609b	1.009b
C.V. (%)	41.37	27.61	44.61	54.5	27.01	18.63
^{15}N enrichment (Atom % ^{15}N excess)						
IR 42	0.0549c	0.0558a	0.0552a	0.0527a	0.0536a	0.0553ab
IAC 4440	0.0680a	0.0558a	0.0482a	0.0553a	0.0497a	0.0606a
IR 4432–28–5	0.0643ab	0.0561a	0.0552a	0.0553a	0.0484a	0.0484bc
B. arrecta	0.0582bc	0.0549a	0.0517a	0.0482a	0.0428a	0.0419c
C.V. (%)	5.69	9.36	10.9	8.38	16.11	8.65

Means in the same column followed by the same letter are not significantly different at $p=0.05$ (Tukey).

um with 100 g L^{-1} sucrose but without cane juice, forming a thick surface pellicle after 7 to 10 days. Best growth occurs with high sucrose or glucose concentrations (100 g L^{-1}) and strong acid production results in a final pH of 3.0 or less. Growth and N_2 fixation (more than 100 n moles C_2H_2 mL^{-1} h^{-1}) continues at this pH for several days (Stephan et al., 1991). Ethanol is also used as a C source for growth and is oxidized to CO_2 and H_2O. Dark brown colonies form on potato agar with 100 g L^{-1} sucrose, and dark orange colonies on N-poor (0.02 g L^{-1} yeast extract) mineral agar medium with 100 g L^{-1} sucrose and bromothymol blue. The bacterium possesses no nitrate reductase and N_2 fixation is not affected by high levels (25mM) of NO_3^-. Also NH_4^+ causes only partial inhibition of nitrogenase, especially when grown on 100 g L^{-1} sucrose (Boddey et al., 1991; Teixeira et al., 1987).

Another interesting aspect is that *A. diazotrophicus* growing in 10% sucrose showed an optimum dissolved oxygen concentration for acetylene reduction in equilibrium with 0.2 kPa O_2 in the atmosphere, but continued to fix N_2 up to 4.0 kPa, showing a much higher O_2 tolerance than *Azospirillum* spp. (Reis et al., 1990).

Experiments on mixed cultures of *A. diazotrophicus* with an amylolytic yeast (*Lypomyces*

kononenkoae), used as a model system for plant/bacteria interactions, showed that 48% of the total nitrogen fixed by the bacteria was transferred to the yeast, starting right from the beginning of the culture (Cojho et al., 1993). These results are important in that until now the lack of evidence for efficient transfer of fixed N from diazotrophs to plants has been a source of scepticism that such associations could be of agronomic importance.

This bacterium has been found in many sugar cane varieties in several regions of Brazil as well as in Mexico, Cuba and Australia (Fuentes-Martinez et al., 1993, Li and Macrae, 1992) and numbers were in the range of 10^3 to 10^7 in roots, basal and apical stems, leaves and in sugar cane trash (Döbereiner et al., 1988). It was not found in soil between rows of sugar cane plants or roots from 12 different weed species taken from cane fields. It was also not found in grain or sugar sorghum, but was isolated from a few samples of washed roots and aerial parts of *Pennisetum purpureum* cv Cameroon, and from sweet potatoes (Döbereiner et al., 1988, 1994; Paula et al., 1989).

Sterile micropropagated sugar cane seedlings were not infected by *A. diazotrophicus* by traditional root inoculation methods, and generally infection of cane

plants by this bacterium is rare except when inoculated "in vitro". However, under these conditions *A. diazotrophicus* was found to colonize extensively the exterior and interior of the shoot and root (James et al., 1994). This study was performed using immuno-gold labelling with both optical and electron microscopic techniques. On the root surface the bacteria was found especially in cavities in lateral root junctions and these junctions and the root tips appeared to be preferred sites of bacterial entry. Within the roots *A. diazotrophicus* was observed in apparently intact, enlarged epidermal cells, and at the base of the stem within xylem vessels through which the bacteria appear to migrate upwards in the transpiration stream so that all shoot tissues become infected. The difficulty of infection of plants grown in soil or vermiculite can be overcome by co-inoculation with VA mycorrhizal fungi, especially originating from fungal spores infected by the bacteria (Paula et al., 1991). This technique of introduction of a N_2-fixing bacteria into sugar cane plants may be important for introducing selected, or genetically improved, strains into plants for further propagation in the field via stem cuttings.

Bacterial taxonomists working in Belgium found that the bacteria known as *Pseudomonas rubrisubalbicans*, a sugar cane endophyte which causes mottled stripe disease in some varieties from the USA and other countries, but not in Brazilian varieties, was closely related genetically to a N_2-fixing bacterium called *Herbaspirillum seropedicae* (Gillis et al., 1991). *Herbaspirillum* was first isolated from the roots of maize and other cereals at our Centre (Baldani et al., 1986). Most of the isolates of *P. rubrisubalbicans* were found to be able to fix nitrogen and were identical in most other respects to *Herbaspirillum* (Pimentel et al., 1991). Recently results from DNA/rRNA hybridization and computer-assisted auxanographic tests have established that this generically-misnamed plant endophyte, *"Pseudomonas" rubrisubalbicans*, must now be included in the genus *Herbaspirillum* (Gillis et al., 1991).

Recently a more specific culture medium (JNFb) for this organism has been developed and $^{15}N_2$ gas incorporation confirmed, not only in strains of the original *H. seropedicae*, but also in isolates from different culture collections of *H. rubrisubalbicans* identified as the causitive organism of mottled stripe disease (Table 6). *Herbaspirillum* spp. have been isolated from sugarcane leaves, stems and roots and are other N_2-fixing bacteria which do not survive well in the soil but only within plants (Baldani et al., 1992a).

Table 6. $^{15}N_2$ incorporation into cells of *P. rubrisubalbicans* and *H. seropedicae* strains grown in semi-solid JNFb medium. Means of three replicates. After Baldani et al. (1992)

Strains	Atom % ^{15}N excess
P. rubrisubalbicans	
M1 (LMG[a] 1278)	0.5271
M4 (ATCC[b] 19308)	0.4891
M5 (LMG 6415)	0.5681
M6 (LMG 6420)	0.5172
IBSBF 175 (LMG 10462)	0.3881
H. seropedicae	
Z67 (ATCC 35892)	0.5881
Z78 (ATCC 35893)	0.4405
ZM 176	0.4891
Controls	
M4 + 20mM NH$_4^+$	0.0002
Z67 + 20mM NH$_4^+$	0.0000

[a] LMG Belgian type culture collection.
[b] ATCC American type culture collection.

When non-sterile soil was inoculated with 10^8 cells g^{-1} of either species of *Herbaspirillum*, the number of viable cells decreased until the bacteria was undetectable after 21 days with *H. rubrisubalbicans* and 28 days with *H. seropedicae* (Olivares et al., 1993). However, 50 days after *Herbaspirillum* spp. were undetectable, surface-sterilized, sorghum seeds were planted in these pots and *Herbaspirillum* spp. were detected in the roots and rhizosphere soil when the plants were 30 days old.

In both monoxenic sugarcane and sorghum plants inoculated with *Herbaspirillum* spp. the bacteria have been localized, using the immunogold technique and both electron and optical microscopy, within the meta and protoxylem (Olivares et al., in preparation). In the case of a sugar cane variety (B–4362), susceptible to mottled stripe disease, *H. rubrisubalbicans* was found to completely block some of the xylem vessels, whereas in a resistant variety the bacteria were encapsulated by membranes probably of plant origin.

Wetland rice

As long ago as 1929, an Indian research worker suggested that wetland rice plants were able to obtain some contribution of nitrogen from N_2-fixing bacte-

ria associated with the plant roots (Sen, 1929). His evidence was based on the isolation of *Azotobacter* spp. from rice roots. Since this time many diazotrophs have been isolated from the rhizosphere and roots of rice including species of *Beijerinckia* (Dobereiner and Ruschel, 1962), *Azospirillum* (Baldani and Dobereiner, 1980; Baldani et al., 1981; Ladha et al., 1982), *Alicagenes* (Qui et al., 1981), *Pseudomonas*, (Barraquio et al., 1983), *Klebsiella* and *Enterobacter* (Ladha et al., 1983); *Flavobacterium* (Bally et al., 1983), and *Agromonas* (Ohta and Hattori, 1983). However, the presence of N_2 fixing bacteria associated with rice roots does not necessarily mean that the plants obtain significant contributions from biological nitrogen fixation (BNF). For example, in a study of the inoculation of wheat plant grown in ^{15}N-labelled soil numbers of *Azospirillum brasilense* above 10^6 cells g fresh root^{-1} were counted on washed/surface sterilized roots and plant N uptake was significantly increased by *Azospirillum* inoculation, but ^{15}N enrichment data showed that the response was not due to BNF inputs (Boddey et al., 1986).

Azospirillum spp. have been isolated in considerable numbers from the rhizosphere and histosphere of wetland rice (Baldani et al., 1981; Ladha et al., 1982, 1987b; Omar et al., 1989) and a new species of *Pseudomonas* (*P. diazotrophicus*) was reported to dominate the rhizosphere bacterial population (Barraquio et al., 1982; Watanabe et al., 1987b). However, as has been pointed out by several authors, N_2-fixing bacteria are distant from the main sources of carbon substrates in the root (the vascular tissue) and are in competition with other soil microorganisms for these substrates (Barber and Lynch, 1977; Berkum and Bohlool, 1980; Kennedy and Tchan, 1992). On the other hand N_2-fixing bacteria found within rice roots or aerial tissue are unlikely to suffer from these disadvantages, and in view of the discovery of endophytic diazotrophs in sugar cane, research at our Centre has focussed on the search for such bacteria in lowland rice.

In the first report of the discovery of *Herbaspirillum seropedicae*, this N_2-fixing bacteria was isolated from washed roots of upland rice as well as from those of wheat, maize and sorghum (Baldani et al., 1986). Further studies have shown that this bacteria can be found in seeds, stems and leaves of rice as well as roots. Roots, stems and leaves of rice plants grown from seeds which were surface sterilized using hydrogen peroxide followed by acidified hypochlorite, were found to be infected with *H. seropedicae*, and only careful surface sterilization of dehulled seeds prevented this (Baldani

et al., 1992b; V L D Baldani, unpubl. data). In the experiment described above to quantify BNF contributions to rice plants grown in the tank of ^{15}N-labelled soil (Oliveira, 1994) counts of *Herbaspirillum* spp. were made using the selective medium described by Baldani et al. (1992). The results showed that numbers of *Herbaspirillum* in washed roots, shoots and leaves were as high as 10^7, 10^5 and 10^4 cells g fresh weight^{-1}, respectively, and the ontogenic variation in numbers varied in a similar manner to the acetylene reduction activity associated with the plants (Fig. 1).

A further N_2-fixing bacteria has been found to be present in rice roots, shoots and leaves in numbers similar to those reported in Figure 1. As was suggested before, for the first attempts to isolate N_2-fixing bacteria from plants it is desirable to base isolation media on the carbon substrates known to be available within the plants (Boddey and Döbereiner, 1988). This was why malate was chosen for the semi-solid media first used to isolate *Azospirillum* as it was known to be an important constituent of maize sap (Döbereiner and Day, 1976). For the same reason cane juice was used for the first attempts to isolate diazotrophs from sugar cane (Cavalcante and Döbereiner, 1988). Boreau (1977) investigated the root exudates of 20 day-old sterile rice plants and discovered that glucose was the single most important carbon source and that in the organic acid fraction oxalate and citrate were quantitatively most important. Based on these results, a N-free semi-solid medium containing glucose, oxalate and citrate (medium 'M') was inoculated with dilutions of washed rice roots and rice stems (Oliveira, 1992). Slow but significant growth with initial acid production was observed, indicating the consumption of glucose. The medium was later alkalinized, indicating subsequent use of the dicarboxylic acids. Maximal AR activity was observed after 10 days incubation in N-free medium and AR activity continued until the 18th day of growth. The bacteria are small motile rods, but have not yet been identified as any of the known diazotrophs. The isolates grow best at pH between 5 and 6 and growth is very slow at pH 7. They use glucose, mannitol, celobiose, maltose, sucrose or trehalose as sole carbon sources and will hydrolyse Tween 80. This bacteria is most closely related phenotypically to *Herbaspirillum seropedicae* and *H. rubrisubalbicans* but whether it is a member of this genus awaits further investigation using DNA/rRNA homology tests etc.

A further possible candidate for an endophytic diazotroph which will infect rice plants are bacteria of the newly denominated genus *Azoarcus* (Reinhold-Hurek
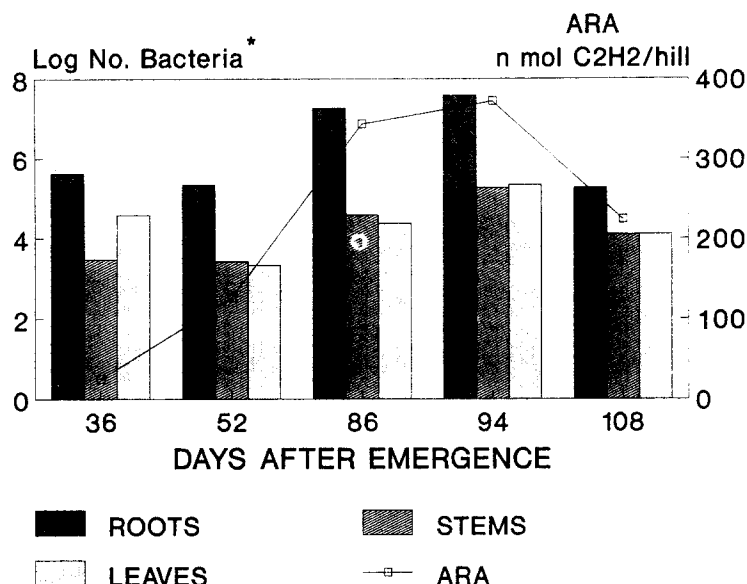
Fig. 1. Counts of *Herbaspirillum* spp. in roots, stems and leaves, and acetylene reduction activity (plant soil system, Barraquio et al., 1986 of the wetland rice variety IR 42 grown in a tank of ^{15}N-labelled waterlogged soil. * Bacterial numbers expressed per g fresh weight plant tissue (after Oliveira et al., 1994).

et al., 1993). The bacteria (labelled with the beta-glucuronidase reporter gene) were found to be able to penetrate rice roots, forming large inter- and intra-cellular colonies in the root cortex and just occasionally within the stele and were also found within the stem bases and shoots (Desomer et al., 1992; Hurek et al., 1991).

Prospects for the future

Brazilian sugar cane varieties are known to be capable of obtaining very considerable contributions of biologically fixed N under field conditions. Recent data suggest that water supply is critical to the maintenance of high BNF activity. A recent trial (16 areas totalling 900 ha) at a sugar cane plantation in Campos (NE Rio de Janeiro State) showed that where year round irrigation was used there was no response of ratoon cane to 200 kg ha^{-1} of urea fertilizer and yields of ratoon crop cane averaged 95 t ha^{-1}. As a result of this trial the plantation managers abandoned N fertilization on 4000 ha of irrigated cane making an annual economy of US $ 250,000 (Boddey, 1995). All attempts to isolate *Acetobacter diazotrophicus* from sugar cane from anywhere in the world have been successful except where high N fertilizer additions have been made (J Caballero

Mellado, pers. commun.). Apart from Brazil no data are yet available for the occurrence of *Herbaspirillum* spp. in this crop.

The complete absence of *A. diazotrophicus* in soil and the restricted occurrence of *Herbaspirillum* spp., suggest that once selected (or even genetically manipulated) strains of these bacteria are established in cane plants in the field, the chances are slight that wild type strains will contaminate the plants to compete with them. For phytosanitary reasons the use of direct planting of monoxenic micropropagated cane plantlets is now being tested at several cane plantations in São Paulo state and this may soon offer an economically viable opportunity to propagate cane plants infected by superior strains of endophytic diazotrophs.

With regard to wetland rice it is evident that for BNF to contribute to high rice yields a great improvement in its efficiency is required. A meeting held at IRRI (Philippines) in 1992 was dedicated solely to this subject. Three possible strategies to increase BNF contributions to wetland rice were discussed (Bennett and Ladha, 1992):

1. Induction of "nodulation" of rice using hydrolytic enzymes (Al-Mallah et al., 1989), 2,4-D (Kennedy and Tchan, 1992) or other means (Rolfe and Bender, 1990) and subsequent infection with *Rhizobium, Azospirillum* or other diazotrophs. True

N_2-fixing legume nodules are complicated structures equipped with vascular tissue to supply C substrate and export fixed N. They possess a sophisticated oxygen protection mechanism with leghaemoglobin and both fixed and variable physical barriers to O_2 diffusion, and an array of specific enzyme systems and feedback controls. The induction of deformations on the root to house bacteria only constitutes a tiny fraction of the symbiotic system and the remaining parameters are dictated pricipally by the plant genome, the *Rhizobium* being mainly responsible for "switching on" the plant nodulation program (Dénarié and Roche, 1991). It thus seems that the induced nodulation strategy has little chance of success especially as true legume nodules serve to protect the nitrogenase system from external oxygen flux from the soil and in wetland rice the soil is anaerobic and oxygen flow to the root is via the aerenchyma (expanded cortex) of the root.

2. Direct integration of *nif* genes into the plant genome. Attempts to introduce just 2 of these genes into tobacco chloroplasts has met with some success although expression was found to be at extremely low levels (Dowson-Day et al., 1991). So far it is not known exactly how many, or which, *Rhizobium* genes will be necessary to make an active N_2-fixing system nor what levels of activity could be achieved.

3. Improvement/modification of existing associations of N_2-fixing bacteria with rice plants. Little enthusiasm has been expressed for this strategy as almost all attention has been focussed on diazotrophs found in the rice rhizosphere (Kennedy and Tchan, 1992). However, the recent discovery that some sugarcane varieties can obtain very large contributions of BNF under field conditions, and the existence of abundant populations of endophytic diazotrophs (*A. diazotrophicus* and *Herbaspirillum* spp.) in this crop which are probably responsible for this activity, opens up entirely new avenues for developing a similar system for rice or other cereal crops. Already one of these endophytic diazotrophs, *Herbaspirillum* spp., has been isolated in moderately high numbers from within roots and aerial tissue of rice, although evidence is lacking that these organisms contribute any significant quantities of fixed N to the plants. However, when more knowledge is accumulated concerning how the N_2-fixing system in sugarcane functions, it should be a much smaller step to try to introduce this into a plant which already can be infected by similar diazotrophs than trying to build a whole N_2-fixing system from scratch.

Acknowledgements

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Use of the ^{15}N natural abundance technique for the quantification of the contribution of N$_2$ fixation to sugar cane and other grasses

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Abstract.   The use of the ^{15}N natural abundance technique to quantify contribution of biological nitrogen fixation (BNF) to any plant is based on the observation that N derived from soil is generally slightly different [usually higher in ^{15}N abundance (δ^{15}N‰)] than that of the air. Plants or micro-organisms growing solely on BNF generally accumulate N with ^{15}N isotopic abundance lower than that of the air (i.e. δ^{15}N‰ is negative), while plants obtaining all N from the soil generally show a positive δ^{15}N‰ signal. The technique is applied by estimating the ^{15}N abundance of the putative 'N$_2$-fixing' crop and analysing the ^{15}N abundance of neighbouring non-N$_2$-fixing reference plants. However, often there are such large variations in the N derived from the soil by different non-N$_2$-fixing plants that in natural ecosystems it is often impossible to even distinguish plants that are benefiting form BNF, let alone quantity this contribution. The reasons why soil derived N can vary so widely, especially in natural ecosystems, are briefly discussed and a sampling strategy is described to assess possible BNF inputs to sugar cane plants in commercial plantations in Brazil. The results suggest that in nine of the 11 sites studied, BNF inputs were significant ranging from 25 to 60% of N assimilated.

Introduction

Only techniques based on the use of the ^{15}N isotope of nitrogen are able to give an integrated measure of the contribution of biological nitrogen fixation (BNF) to plant nutrition from a single sampling of the 'N$_2$-fixing' crop. The use of ^{15}N-labelled N$_2$ gas is only suitable for short-term evaluations of N$_2$ fixation and then usually only applicable to one or two plants maintained under carefully controlled conditions (e.g. Eskew *et al.* 1981). The other options are to use soil naturally or artificially enriched with the ^{15}N isotope relative to atmospheric N$_2$. These, so called '^{15}N dilution' techniques, are based on the premise that N derived from the soil and that derived from the air are isotopically distinct, and the isotopic abundance of the 'N$_2$-fixing' plant represents the net result of the mixture of N derived from the two sources.

In the case of the ^{15}N enrichment technique where soils are artificially enriched with ^{15}N, the ^{15}N enrichment of plant-available N varies in depth and with time, even if the labelled fertiliser is distributed uniformly over the soil surface. Hence, different plants that have different spatial or temporal N uptake patterns will accumulate N with different

^{15}N enrichments. To overcome this, soils may be amended with enriched N (applied as ^{15}N enrichment fertiliser or organic matter), and left for many months or years for the added labelled N to become equilibrated with the native soil N, such that the ^{15}N enrichment of the soil mineral N is uniformly distributed with regard to area and depth, and remains essentially constant during the growth of the target ('N$_2$-fixing') crop, and the reference crops. The latter are necessary to evaluate the ^{15}N enrichment of the N derived from the soil by the 'N$_2$-fixing' crop. These constraints make the application of this technique in the open field situation very difficult, especially where the proportional contribution of BNF is small. The problems associated with the use of this technique to quantify BNF with legumes have been previously discussed by many authors (Witty 1983; Danso 1986; Rennie 1986; Boddey *et al.* 1995; Chalk and Ladha 1999) and specifically for non-legumes by Boddey (1987), Chalk (1991) and Boddey *et al.* (1998).

With the development of automated high-precision continuous-flow isotope-ratio mass spectrometers (Barrie *et al.* 1995), the use of the ^{15}N natural abundance version of the isotope ratio has become within the capabilities of many

Abbreviations used: BNF, biological nitrogen fixation.

more laboratories. Atmospheric N_2 shows a natural abundance of 0.3663 atom% ^{15}N and does not vary perceptibly anywhere on the planet (Mariotti 1983). In soils, the ^{15}N natural abundance of many N forms including plant-available N may deviate by as much as 0.005 atom% from this value. To deal with these small values, the units used are called delta ^{15}N units expressed in parts per thousand (‰) and determined by the equation:

$$\delta^{15}N\ (‰) = 1000 \times (atom\%\ ^{15}N\ sample - 0.3663)/0.3663\ (1)$$

such that the ^{15}N natural abundance of the air = 0.00 ‰.

Usually, although not always, plant-available soil N is naturally slightly enriched with ^{15}N and the reasons for this were discussed in two recent reviews (Högberg 1997; Boddey *et al.* 2000).

There are three values of ^{15}N abundance that must be established in order to estimate the proportion of N derived from biological nitrogen fixation (BNF) in any crop. First, the ^{15}N abundance of the N in the plant derived from the air via N_2 fixation (this value is termed 'B'); second, the ^{15}N abundance of N derived from the soil $\delta^{15}N_{ref}$; and third, the measure of the ^{15}N abundance of the putative N_2-fixing plant ($\delta^{15}N_{fixing\ plant}$). Once these values are known their substitution in the equation,

$$\%Ndfa = 100\ (\delta^{15}N_{ref} - \delta^{15}N_{fixing\ plant})/(\delta^{15}N_{ref} - B),\quad (2)$$

will allow the computation of the proportion of plant N derived from the atmosphere via BNF (%Ndfa). The determination of these three values for the estimation of BNF contributions to non-nodulated plants will be considered in sequence.

'B' — the ^{15}N natural abundance of N in the plant derived from BNF

For most nodulating legumes, it is possible to grow the plants to maturity solely on N_2 fixation in N-free hydroponic or sand culture. If the mass, N content and ^{15}N abundance of the seeds are known then the ^{15}N abundance of the N derived from BNF can be calculated. This has been done for a wide variety of legumes, and while there are some doubts concerning some of the earlier values in the literature, in general shoot tissue of such plants is slightly negative in ^{15}N abundance (0 to –2‰), although nodules are frequently positive, sometimes above +10.0‰ (see Table 4 in Boddey *et al.* 2000). The value of B varies from species to species and with growth, generally decreasing as fixed N accumulates. Even different varieties of the same species can show a considerable range in B value (Table 1). The exceptionally high value for the Hunter river variety of lucerne reported by Turner and Bergersen (1983) may be an analytical artefact (Boddey *et al.* 2000).

To estimate the value of B for a non-nodulating crop would require growing the crop in the total absence of fixed N. So far this has not been achieved for any such crop, so at present we must conservatively assume that $B = 0.0‰$, equal to that of air. There is evidence that N fixed and incorporated into free-living bacteria is depleted with respect to air. Delwiche and Steyn reported that cells grown on BNF showed a ^{15}N natural abundance of –3.9‰, and similar negative values ranging from –0.74 to –4.44‰ were recently published by Rowell *et al.* (1998), the differences in the values being due to the type of nitrogenase; whether Mo, V or Fe based. Other data are given by Handley and Raven (1992) for other species of *Azotobacter* and for various cyanobacteria and in all cases the N derived from N_2 fixation was slightly depleted in ^{15}N. Thus, it seems likely that the N derived from BNF in any non-nodulating crop will be of negative ^{15}N natural abundance and if N derived from soil is of positive isotopic abundance then to assume B is zero will yield underestimates of the contribution of BNF to plant nutrition.

The ^{15}N natural abundance of plant N derived from soil

Recent evidence has been accumulated which shows that many plants are able to absorb N from the soil, or decaying organic matter, not only as NO_3^- or NH_4^+ but also as amino acids and other forms of N (Abuzinadah and Read 1988; Chapin *et al.* 1993; Turnbull *et al.* 1995). There is strong evidence that ericaceous-, ecto- or even endomycorrhizae may play a role in this uptake (Stribley and Read 1980; Handley *et al.* 1993; Näsholm *et al.* 1998) but exact mechanisms have not been elucidated. For the purposes of the application of the ^{15}N natural abundance to quantify BNF, these observations may have serious consequences. It is known that different N forms in the soil often differ widely in their ^{15}N abundance, both spatially and with time (see reviews of Handley and Scrimgeour 1997; Högberg 1997; Boddey *et al.* 2000). The result of this may be that different plants, even those incapable of obtaining N from BNF, may show widely different values of ^{15}N abundance some even lower than 0.0‰. This makes impossible the determination of what value of ^{15}N abundance to use for the N derived from the soil in the putative N_2-fixing plants and the technique

Table 1. Values of the ^{15}N natural abundance of different lucerne varieties grown entirely on biological nitrogen fixation
NS: not specified

Lucerne variety	Plant part	B value (‰)	Reference
NS	Whole plant	–0.92	Mariotti *et al.* (1980*b*)
Du puits	Whole plant	–0.98	Yoneyama *et al.* (1986)
Natsuwakaba	Whole plant	–0.98	Yoneyama *et al.* (1986)
Sirivar	Shoot only	–0.44	Brockwell *et al.* (1995)
Trifecta	Shoot only	–3.18	Hossain *et al.* (1995)
NS	Shoot only	0.00	Steele *et al.* (1983)
Mireille	Shoot only	–0.92	Ledgard (1989)
Hunter River	Whole plant	+0.97	Ledgard (1989)
Hunter River	Whole plant	+3.56	Turner and Bergersen (1983)

cannot be applied. Examples of such sites were reported by Högberg (1990) and Pate *et al.* (1993). The natural eco-system studied by Hansen and Pate (1987), a eucalypt forest (predominantly *E. marginata*) in south-western Australia, is typical of one where the ^{15}N natural abundance technique was not even able to distinguish 'N₂-fixing' from non-N₂-fixing species, let alone quantify BNF contributions. The ^{15}N abundance of total soil N (0–15 cm) ranged from –2.15 to +5.4‰ with a mean of +2.1‰, and non-N₂-fixing non-leg-umes ranged from –1.02 to +1.79‰, which was a smaller range than that of the values for the understorey legumes (principally *Acacia* spp.) of –1.56 to +3.42‰. However, these sites were in natural ecosystems where there is a large number of potential sources of N in plant litter, dead plants and decaying roots. Man-made agricultural ecosystems are generally more uniform, with cultivation and residues often burned off before of after cropping. Lime and fertiliser additions, as well as cultivation, normally stimulate mineral-isation and nitrification, leaving the greatest proportion of plant-available N in the form of NO_3^- and, sometimes, lesser quantities of ammonium.

It is probably because of this less complex matrix of N forms of different ^{15}N abundance that the application of the ^{15}N natural abundance technique to quantify BNF contri-butions to legume crops has met with such success. There have been many comparisons, mostly in agricultural set-tings, of the use of the natural abundance technique with others, particularly the isotope dilution technique using ^{15}N-enriched N. Studies where comparisons have been made between the ^{15}N natural abundance technique and other independent techniques have been discussed thoroughly in earlier papers (Shearer and Kohl 1986; Peoples and Herridge 1990; Doughton *et al.* 1995; Högberg 1997). In general, there has been good agreement between the methods, vindicating the use of the natural abundance for estimating BNF contributions to grain and forage legumes.

However, from the above discussion it is apparent that even in cultivated areas different 'reference' plants may vary considerably in the ^{15}N abundance of the N they accumulate. Which of these values, if any, matches the ^{15}N abundance of the N absorbed from the soil by the N₂-fixing crop is not possible to ascertain. The strategy that we recommend (see details in section 5) is to sample a number of neighbouring different, preferably botanically diverse, reference plants. If all of these non-N₂-fixing reference plants exhibit values of ^{15}N abundance significantly above that of the target 'N₂-fixing' crop, it is considered possible to conclude that the target crop has obtained some N from associated BNF and estimates of this contribution can be made.

Sampling of the 'N₂-fixing' and reference plants

As is the case with trees and shrubs (Boddey *et al.* 2000), it may not always be convenient or possible to quantitatively sample the entire plant. Data from work on trees suggests

that there are very considerable variations in ^{15}N abundance between different plant organs. An extreme situation was that reported by Shearer *et al.* (1983), where below- and above-ground tissue of a deep-rooting leguminous tree (*Prosopis gladulosa*) was sampled in the Sonoran desert in California (Table 2). The ^{15}N abundance of leaves averaged +1.2‰ and, as in other studies, twigs and branch wood were lower than this by 0.3 and 1.1‰, respectively. Trunk wood showed ^{15}N abundance of –3.5‰ and roots were almost as low in [15]N (–2.5‰). The low ^{15}N abundance in the trunks was attributed to the fact that the trunks were probably sinks for N. However, because of the relatively small proportion of total N of the tree in the trunk wood, the weighted average of all above-ground tissues, excluding trunk wood, was +0.4‰, which was statistically indistinguishable from the weighted mean of all tissue, including trunk wood (–0.1‰). Other studies on trees suggest that this species was exceptional in the large variation between different tissues (Peoples *et al.* 1991, 1996).

To investigate this matter for sugar cane, a single plant growing at our field station was intensively sampled. All leaves and nodes of the stem were sampled from an 8-month-old sugar cane plant (plant crop) growing in an infertile sandy soil (80% sand, Arenic Hapludult). The ^{15}N abundance of neighbouring sorghum plants (eight repli-cates) was 4.35‰, and of maize 5.43‰. As can be seen from the results (Fig. 1), the highest ^{15}N abundance was recorded in the emerging shoot (+5.0‰) and the lowest value in the internode in the middle of the plant (+3.4‰). The weighted mean ^{15}N abundance of the whole plants was 3.9‰ and the arithmetic mean almost the same at +4.0‰. The third emergent leaves are normally used by sugar cane agrono-mists for 'crop logging' (monitoring crop nutrient status during crop growth) and these leaves showed a ^{15}N abun-dance of 4.0‰. From this preliminary study it appears that the ^{15}N abundance of the third emergent leaf is a good

Table 2. Distribution of N and ^{15}N abundance in aboveground tissue of *Prosopis gladulosa* growing in the Sonoran Desert (California, USA)

After Shearer *et al.* (1983). For all plant parts except trunkwood, values are means of five trees samples at eight occasions during the 1980 growing season. Trunkwood sampled only on one occasion.

Plant part	Mean (± s.e) relative N content (%)	Mean (± s.e.) ^{15}N abundance ($\delta^{15}N$ ‰)
Leaves	27.3 ± 1.0	+1.2 ± 0.2
Flowers	5.0 ± 0.6	+1.7 ± 0.6
Fruit	12.6 ± 3.6	+0.9 ± 0.3
Juvenile twigs	1.0 ± 0.2	+0.9 ± 0.4
Branch wood	38.3 ± 2.6	+0.1 ± 0.2
Trunk wood	15.8 ± 1.4	–3.5 ± 0.1
Total (excluding trunk wood)	84.2 ± 1.4	+0.4 ± 0.4
Total (including trunk wood)	100.0 ± 1.4	–0.1 ± 0.4

indicator of the weighted mean ^{15}N abundance of the whole plant.

However, it should be pointed out that if large quantities of N fertiliser have been added to the crops shortly before sampling, or if any other major change in N nutrition of the plant may have occurred soon before sampling, this might stimulate sudden changes in ^{15}N abundance in the plants that may not be evenly distributed.

Sampling of sugar cane fields in Brazil

In this study, sugar cane fields in four different regions of Brazil were sampled, a total of 11 cane fields (Table 3). These were established cane fields on commercial plantations, as is usual practice in Brazil, plant crops were not fertilised with nitrogen, and on these farms ratoon crops were supplied with 40–60 kg N at full leaf cover. Samples were taken towards maturity of the cane crop, usually 6–7 months after the N-fertiliser addition. The fields were

divided in to four strips, 50 m long and 10 m wide, to act as replicate blocks. In each block, third emergent leaf samples were taken from 30 cane plants and the samples bulked for each block. Through the blocks (non-N$_2$-fixing), weeds were procured and, where there were several samples of the same weed species, the whole shoots of the weeds were collected and the samples again bulked by block. In some fields, as many as five different weed species were found and sampled (e.g. field of variety RB 72-454, UFRRJ-Campos, RJ), in others only two. Bulked samples were dried (65°C for >72 h), ground with a Wiley mill to <0.85 mm followed by fine grinding with a roller mill based on that of Smith and Myung (1990). ^{15}N abundance was determined using a continuous-flow isotope-ratio mass spectrometer (Finnigan Matt DeltaPlus, Bremen, Germany).

The data were analysed by comparing the four replicate values of the ^{15}N abundance of each weed with those of the cane samples, by using the Student's *t*-test. As can be seen
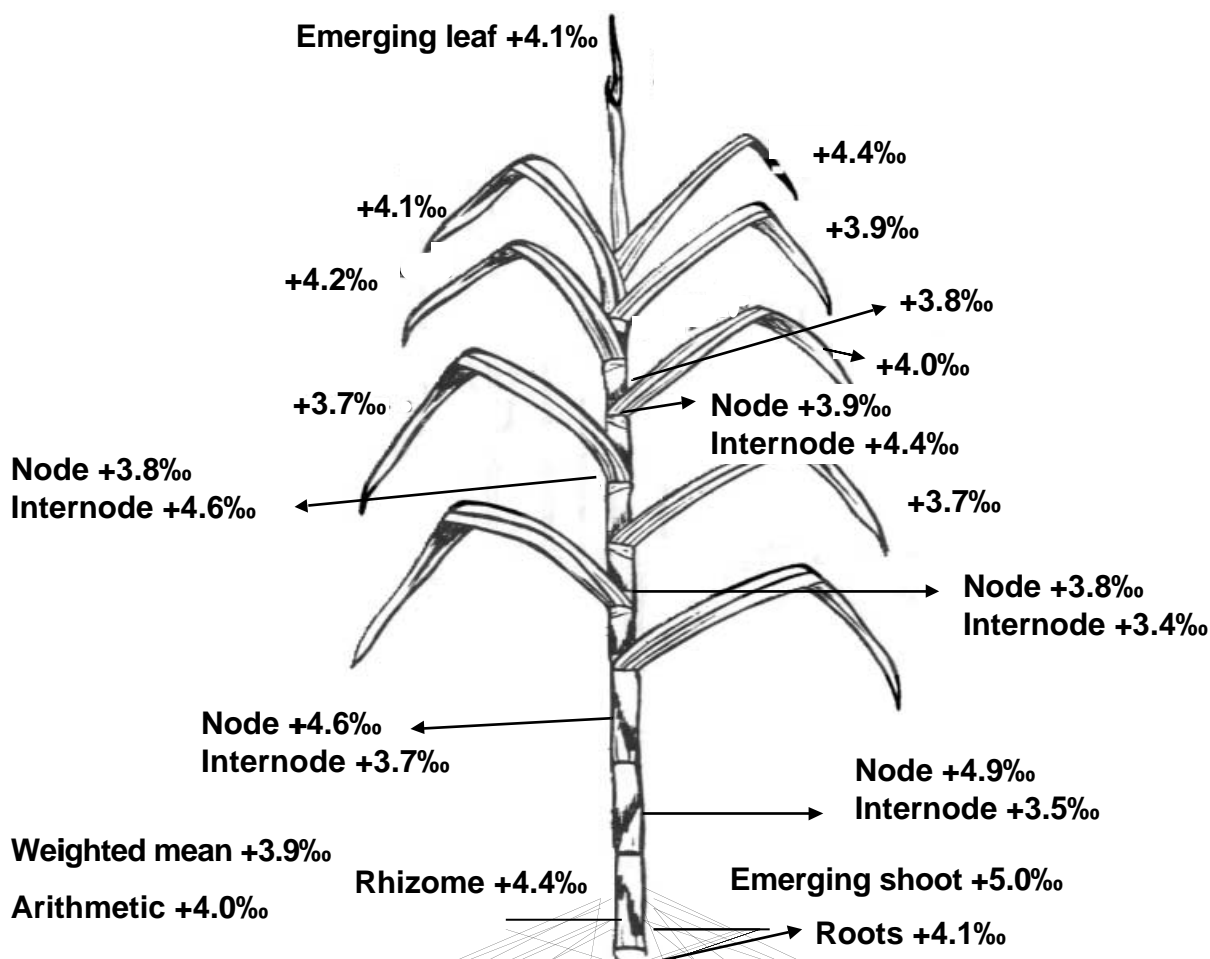

Fig. 1. ^{15}N natural abundance of leaves, stem (nodes and internodes) roots and emerging shoots of a sugarcane plant (cv. SP 70-1143) growing in the field on a sandy Planosol (Arenic Hapludult). Data from Resende AS (1999), MSc thesis, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro.

from the results, in some cases (sites 1 and 6) the ^{15}N abundance of one or more of the reference plants was not significantly higher than that of the sugar cane. In this case, we feel that it is not possible to conclude that there were any contributions of BNF to the cane plants. At site 6, planted to the variety SP 80-1842, the two reference weed species were marginally, but not significantly, lower in ^{15}N abundance than the cane leaves.

For all other sites, the ^{15}N abundances of the weed species were significantly higher than the sugar cane leaves. It is possible, that the N acquisition strategies of some of these weeds could lead them to remove N from sources in the soil that had higher ^{15}N abundance than those available to the cane plants, which could then be wrongly interpreted as an input of BNF to the cane. However, in the cases where four or five different weeds were chosen (e.g. sites 4, 5 and

Table 3. ^{15}**N natural abundance of 3rd emergent cane leaves and whole weed plants taken from 11 different sugar cane plantations in four different states in Brazil (SP, São Paulo; MG, Minas Gerais; RJ, Rio de Janeiro; PE, Pernambuco)**

Using these data estimates have been made of the proportion of N derived from BNF by the cane plants (% Ndfa). *, ^{15}N natural abundance of the reference plants was significantly different from that of the 3rd emergent leaves (bulked samples for each block) using the Student's t-test at $P = 0.05$; ns, mean ^{15}N abundance of reference plant not significantly different to that of sugar cane plants using the Student's t-test at $P = 0.05$. Data from J. C. Polidoro (unpublished PhD thesis), Universidade Federal Rural do Rio de Janeiro, Itaguaí, Rio de Janeiro

Usina/town/ state	Cane variety — growth cycle	Reference plant	δ^{15}N (‰)	% Ndfa
São José/Macatuba/SP	SP 80-1842 — 2nd ratoon	—	6.80 ± 0.23	—
		Sonchus espontaneum	9.17 ± 0.30	25.9*
		Amaranthus sp.	12.92 ± 0.07	47.4*
		Erechites heracifolia	7.2 ± 0.22	5.6^{ns}
São José/Macatuba/SP	RB 72-454 — plant crop	—	5.24 ± 0.37	—
		Eragrostis pilosa	7.59 ± 0.69	31.0*
		Sida rhobifolia	7.86 ± 0.97	33.3*
São José/Macatuba/SP	RB 72-454 — 2nd ratoon	—	3.59 ± 0.07	—
		Emilia sonchifolia	6.10 ± 0.46	41.2*
		Panicum maximum	5.35 ± 0.32	32.9*
São José/Macatuba/SP	SP 80-1842 — plant crop		3.34 ± 0.12	—
		Partenium histerophorus	7.22 ± 1.02	53.7*
		Lepidium virginicum	6.13 ± 1.24	45.5*
		Panicum maximum	11.08 ± 0.21	69.9*
		Melinus minutifolia	11.79 ± 0.06	71.7*
Sítio Pedreira/Oratórios/MG	RB 86-7515 — plant crop	—	5.20 ± 0.97	—
		Sida rhobifolia	8.60 ± 0.03	39.5*
		Melinus minutifolia	7.57 ± 0.06	31.3*
		Eleusine indica	7.66 ± 0.05	32.1*
		Emilia sonchifolia	7.11 ± 0.02	26.9*
Sítio Pedreira/Oratórios/MG	SP 80-1842 — plant crop	—	8.87 ± 0.07	—
		Lepidium virginium	7.90 ± 0.06	−12.3^{ns}
		Bidens pilosa	8.20 ± 0.03	−8.1^{ns}
UFRRJ/Campos/RJ	CB 45-3 — 1st ratoon	—	5.33 ± 0.22	—
		Sidrastum sp.	7.85 ± 0.03	32.0*
UFRRJ/Campos/RJ	RB 72-454 — 1st ratoon		5.34 ± 0.24	
		Acamthopurpureum australe	7.96 ± 1.07	32.9*
		Bidens pilosa	8.06 ± 0.27	33.8*
		Croton lobatus	9.82 ± 0.14	45.6*
		Commelina benghalensis	6.90 ± 0.33	22.6*
		Sida rhombifolia	8.02 ± 0.07	33.4*
Usina Barcelos/Campos/RJ	RB 74-454	—	7.07 ± 0.19	—
		Eclipta alba	9.85 ± 0.001	28.2*
		Ni^{B}	9.63 ± 0.22	26.6*
Usina Cruangi/Timbaúba/PE	RB 78-4764 — 1st ratoon	—	6.20 ± 0.56	—
		Panicum maximum	7.88 ± 0.05	21.3*
		Brachiaria mutica	9.31 ± 0.21	24.1*
		Capim achó^{A}	12.82 ± 0.05	51.6*
Usina Cruangi/Timbaúba/PE	RB 83-102 — 1st ratoon	—	13.20 ± 1.36	—
		Monordica charantia	26.48 ± 0.12	49.1*

^{A}Common name in Portuguese, species not yet identified. ^{B}Ni, weed species not identified.

8) and where some of the weeds were dicots and other monocots, the fact that all weed species showed ^{15}N abundance values statistically significantly higher than the ^{15}N abundance of the cane leaves, is extremely strong evidence that there was a BNF input into the cane. Only if sugar cane has some almost unique N acquisition strategy that allows it to tap sources of N with lower ^{15}N abundance than any of the weed crops, would the above conclusion be false. Further studies are required to examine the effective depth of rooting of sugar cane and whether in most of the sugar cane fields sampled plant available N (e.g. NO_3^-) was more depleted than the mineral N acquired by spontaneous vegetation. The study here (more results are still being gathered for other sites in Brazil) suggest that in commercial plantations up to 60% of plant N is being derived from plant-associated BNF.

Conclusions

As has been pointed out by Handley and Scrimgeour (1997), the ^{15}N natural abundance technique for estimating the contribution of associated BNF to plants essentially relies on the concept of two distinct sources of N with discreetly different values of ^{15}N abundance. In the real world, this seldom occurs and while plant N derived from BNF is generally close to a δ^{15}N value 0.0‰, or a unit or two below this, plant N derived from the soil can be highly variable, both negative and positive, sometimes for different non-N_2-fixing plants growing at the same site (Hansen and Pate 1987; Pate *et al.* 1993). In an agricultural situation, where plant diversity has been drastically reduced, most litter buried or burned, the results of applying the ^{15}N natural abundance technique indicate that the number of different N sources with different ^{15}N abundances are reduced, such that an approximation to the two-source model can function. The test of the validity of this assumption is based on a strategy of using as many non-N_2-fixing reference plants as possible, and as diverse as possible, and utilising a statistical comparison between their ^{15}N abundance and that of the target non-nodulating 'N_2-fixing' plant to determine whether their δ^{15}N values are significantly different from the target 'N_2-fixing' plant.

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NITROGEN MANAGEMENT
IN THE QUEENSLAND SUGARCANE INDUSTRY

THE ECONOMIC RISKS OF POLICIES THAT PRESCRIBE
NITROGEN RATES BELOW INDUSTRY GUIDELINES

CANEGROWERS

CONTENTS

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DISCLAIMER

EXECUTIVE SUMMARY

Background

Cane farming generates wealth, drives economic growth and supports the jobs, wages and livelihoods of thousands of residents in communities along the Queensland coast. However, there are concerns over the contributions of cane farms to catchment loads of dissolved inorganic nitrogen (DIN) and the possible effects on the health of inshore marine ecosystems within the lagoon of the Great Barrier Reef (GBR).

Nutrient management is a key component of sugarcane agronomy, and the SIX EASY STEPS™ (6ES) Program provides growers with evidence-based, block-specific recommendations for meeting the nitrogen (N) and other nutrient requirements of the crop for each year of the crop cycle. However, there is a persistent belief within parts of the Federal and State governments that 6ES recommendations exceed crop requirements. This belief has permeated the design and justification of voluntary incentive programs, regulations, and the evaluation framework used to measure progress towards practice and water quality targets. For example, the Queensland Government regulations are based on growers moving to what is termed the 'B' risk category for water quality, and this requires growers to apply N rates that are 15 to 30% below 6ES guidelines.

This report quantified the impacts of such blanket reductions in N rates, relative to the 6ES recommendations, on cane farms and mills, and on the economic value of the industry to regional communities and the State as a whole. It used a generalised nitrogen response function for each cane region, derived from N response trials within each region.

Main findings

The main finding is that the blanket use of N rates below those recommended by the SIX EASY STEPS program would markedly reduce the production and incomes of farms, the profitability of mills, and the economic and social health of regional economies. For example, a 30% reduction in N rate would cause reductions of 5.0 -7.5 tonne/ha in cane yields and 0.7 to 1.2 tonne/ha in sugar yields, depending on district. This, in turn, would reduce crop partial net returns by $142 to $266/ha, again depending on district.

These on-farm impacts would lead to reductions in the industry's direct economic benefits for regional economies, ranging from approximately $11 million per annum in the Wide Bay-Burnett to $44 million per annum in north Queensland. The collective direct regional impacts would penalise the Queensland economy by up to $110 million each year. When the indirect economic impacts of the reduction in cane farming are accounted for, the annual cost to the State's economy is approximately $160 million or nearly $1.3 billion over ten years (in present value terms).

The industry recognises that it should continue efforts to research, develop and implement cost-effective options for improving nitrogen use efficiency and reducing losses of DIN. That ongoing work is important. However, this study has clearly demonstrated the economic risk to industry and regional communities of seeking reduced DIN through widespread reductions in N application rates below those recommended by the 6ES program.

Current reef water quality policies, regulations and programs are based on unrealistic expectations of what growers can afford to do in reducing N application rates. The report also highlights the associated risks from these policies and regulations to the viability of mills and the health of regional economies. All such policies, regulations and programs need urgent review and revision with strong industry input.

1. INTRODUCTION

Cane farming generates wealth, drives economic growth and supports the jobs, wages and livelihoods of thousands of residents in communities along the Queensland coast. A recent report (QEAS 2019) quantified its importance to Queensland, including its $4 billion in economic activity, over 22,000 jobs and over 10,000 businesses. Cane farming benefits the community through employment and stimulus across the value chain, with one dollar in economic activity in cane growing supporting an additional $6.42 elsewhere in the Queensland economy.

$1 = **$6.42**

of sugarcane grown | generated in the QLD economy

$0.58
of value add in own supply chain (fertiliser, herbicide, etc.)

$4.69
of value add in downstream industries (mills, refineries & sugar terminals)

$1.15
of value add via induced consumption stimulated by whole value chain
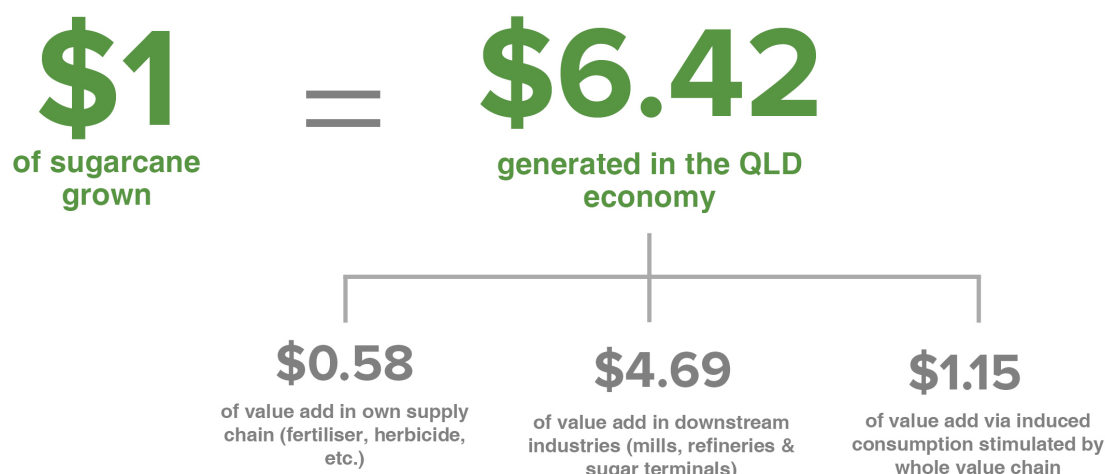
Cane farming in Queensland occurs predominantly in the lower reaches of catchments that flow into the lagoon of the Great Barrier Reef (GBR). The influence of cane and other agriculture on catchment water quality, and the possible implications of this for the health of inshore marine ecosystems, has led to Federal and State governments setting targets for improved water quality and implementing various interventions to achieve these. For cane farming, the primary focus has been on anthropogenic dissolved inorganic nitrogen (DIN) in catchment discharge. The current target for DIN is a 60% reduction in catchment loads by 2025 (relative to modelled 2009 loads) (Queensland Government 2018).

Nutrient management is a key component of sugarcane agronomy, and the SIX EASY STEPS™ (6ES) program provides growers with evidence-based and reliable recommendations for rates of nitrogen (N) and other essential nutrients for each cane block (Wood *et al.* 2003, Schroeder *et al.* 2009, Skocaj *et al.* 2012). These recommendations are based on soil tests taken at the end of each crop cycle and, for nitrogen recommendations, account for potentially mineralisable N and for other sources of available N such as fallow legume crops and mill by-products. The recommendations are district and block specific, tailored to the plant and subsequent ratoon crops, and include any ameliorants required to manage soil constraints such as sodicity and acidity. In effect, the 6ES recommendations provide a complete nutrient management plan for each cane block for the crop cycle.

The 6ES recommendations are guidelines – they are the best estimate of the optimal rates of N and other nutrient based on each block's soil types, soil testing and management history. Field trials and demonstration sites have shown these recommendations to be reliable and robust. For N recommendations, this reflects the particularly strong data sets on which they are based, which includes multi-site, long-term field trials in various production regions (see Appendix A for a description of the data sources under-pinning the 6ES program).

In addition to providing nutrient recommendations, the program includes steps for reviewing the adequacy of nutrient supply during the crop cycle (e.g. through leaf analysis, on-farm trials). However, growers using the 6ES program typically follow the recommendations closely throughout the crop cycle. Some growers may deviate from the recommendations for specific situations, such as final-year ratoons or late-harvested crops, that they consider may be less responsive to nutrients. However, there is limited data to support such situation-specific refinements. The recent launch of the SIX EASY STEPS Toolbox by Sugar Research Australia (SRA 2020) provides guidance to growers and their advisors for possible refinements to N rates for specific circumstances.

Increasing scrutiny of nutrient management by Federal and State governments has led to interventions including a sequence of voluntary programs (e.g. Reef Rescue and Reef Trust) and increasing levels of regulation (e.g. the *Environmental Protection (Great Barrier Reef Protection Measures) and Other Legislation Amendment Act 2019*). While early interventions generally focused on increasing adoption of the 6ES recommendations, especially for N, more recent programs and regulations seek the use of even lower N rates.

This arises from a persistent belief, within parts of the Federal and State governments, that crop productivity is generally too low to justify use of the 6ES recommendations and that the latter rates are only needed when and where yields are exceptionally high.

This belief has spawned alternative mechanisms for calculating optimal N rate, using calculations based on growers' yield expectations or yield history (e.g. Anon 2013, Rust *et al.* 2017, Bramley *et al.* 2019). Such yield-based calculations have been the basis for the Paddock to Reef (P2R) practice framework used to assess growers' practices in terms of the P2R perception of 'best management'. For example, the 2013 version states that best practice is a calculation of N based on applying certain multipliers to 'grower's own yield expectations' (Queensland Government 2013). The most recent version (Queensland Government 2019) states that best practice is the 'optimal amount' calculated from yield history.

A simple inspection of the field data that underpins the 6ES program shows these approaches can be spurious. Examples provided in Appendix A show that 6ES recommendations for N are derived from numerous field experiments with different sites and years covering a wide range in yields. This depth of investigation has resulted in guidelines that enable decisions on when or where yield responses to applied fertiliser N are likely. In contrast, the alternative approaches generally rely on crop response to N being related to crop size. However, Thorburn *et al.* (2018) analysed data from all such field experiments in Queensland and found there was little correlation between cane yield and optimal N rate. Despite this, the belief that 6ES recommendations are excessive has permeated the design and justification of voluntary programs, regulations, and the P2R framework used to measure growers' progress towards practice and water quality targets (e.g. Qld Gov 2013, Alluvium 2016, Office of Great Barrier Reef 2017).

Consequently, policies and programs are based on unrealistic expectations of what growers can afford to do in reducing N application rates. For example, the current Queensland Government regulations are based on growers moving to what is termed the 'best practice', or 'B', risk category for water quality (Office of Great Barrier Reef 2017), which assumes optimal N rates are generally 15 to 30% below the 6ES recommendations.

To highlight the economic risk of this approach, this report quantifies the impacts of blanket reductions in N rates, relative to the 6ES guidelines, on the profits of cane farms and mills, and on the economic value of the industry to regional communities and the State as a whole. A whole supply chain analysis provides a real world understanding of the consequences of the scenarios for surrounding communities.
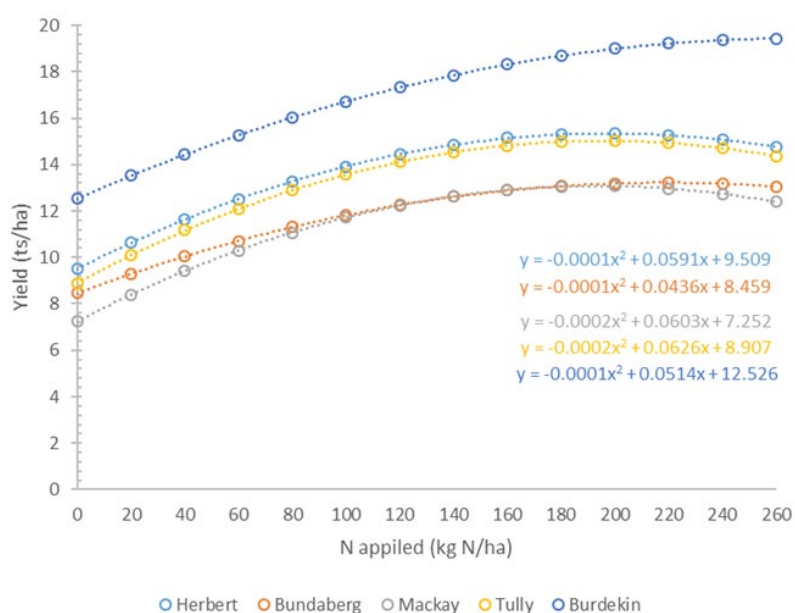
2. METHODOLOGY

There were three main steps in quantifying the economic impacts of reductions in N rates on cane farms, mills and regional economies:

1. Development and utilisation of representative N response functions for ratoon cane for each of five districts, based on field data. The method is described in Appendix A. Based on the availability of relevant field data and the need to be representative of each economic region of interest, the districts selected were Tully, Herbert River, Burdekin, Mackay and Bundaberg.

2. The generalised N response function for each district was used to estimate impacts on farm production (cane and sugar tonnes per ha) and partial net return ($/per ha) from various reductions in N rates (10%, 15%, 20%, 25%, and 30%) relative to an appropriate generalised estimate of the 6ES recommended rate (taken to be the N rate that produced 95% of maximum yield).

3. The enterprise impacts were scaled up for each of five regional economies and to the Queensland economy as a whole. The five regional economies, and the representative response functions used for each, were:

   i. Far north Queensland, from Mossman in the north to Tully in the south, but excluding the Tableland (impacts derived from Tully district response function)

   ii. North Queensland (weighted impact from the individual Herbert and Burdekin response functions)

   iii. Mackay region, including Mackay, Proserpine, and Plane Creek districts (Mackay response function)

   iv. Bundaberg and Wide Bay Burnett (WBB) region, including Bundaberg, Isis, and Maryborough districts (Bundaberg response function)

   v. Remainder of Qld (indirect economic impacts only)

2.1 NITROGEN-YIELD RESPONSE FUNCTIONS

These relationships were derived from numerous replicated and randomised N field trials located in the different districts (indicated above). In each case, yield [tonnes of cane harvested per hectare (TCH)] and the commercial cane sugar (CCS) values were determined relative to increasing rates of N applied as urea. Cane yield and CCS were used to estimate the tonnes of sugar harvested per hectare (TSH). The relationship between TSH and N applied (kg N/ha) are presented in Figure 1. See Appendix A for the methods used to derive the generalised district functions.

Figure 1. Relationship between yield (tonnes of sugar harvested per hectare (ts/ha)) and N applied (kg N/ha) for each representative district.

$$y = -0.0001x^2 + 0.0591x + 9.509$$
$$y = -0.0001x^2 + 0.0436x + 8.459$$
$$y = -0.0002x^2 + 0.0603x + 7.252$$
$$y = -0.0002x^2 + 0.0626x + 8.907$$
$$y = -0.0001x^2 + 0.0514x + 12.526$$

○ Herbert ○ Bundaberg ○ Mackay ○ Tully ○ Burdekin

2.2 IMPACTS OF REDUCED N RATES ON PARTIAL NET RETURNS OF FARMS AND MILLS

The reductions in TSH (relative to TSH at the 6ES rate) as a result of reduced N rates are shown in Table 1. For the purpose of this study, the reference 6ES rate for each region was derived from the corresponding regional response curve, and was defined as the N rate at which 95% of maximum yield was achieved.

Table 1. Reductions (% relative to TSH at 6ES rate) in TSH from reduced rates of N for each district.

N rate relative to 6ES	Bundaberg	Burdekin	Herbert	Mackay	Tully
-10%	-1.9%	-1.9%	-1.8%	-2.1%	-1.9%
-15%	-3.0%	-3.0%	-2.9%	-3.3%	-3.0%
-20%	-4.1%	-4.1%	-4.1%	-4.7%	-4.2%
-25%	-5.4%	-5.3%	-5.4%	-6.2%	-5.6%
-30%	-6.7%	-6.6%	-6.7%	-7.8%	-7.0%

Farm partial net returns

The partial net return at each level of N, as per the yield response functions (Appendix A), was calculated using the cane price formula with the CCS at each level of N application multiplied by the tonnes of cane/ha less the costs of the N applied per ha and the harvesting and levies costs per ha. This gave the partial net return per ha. The difference between the partial net return per ha for the change in N application rate provided a marginal analysis.

The formula for calculating the farm partial net return per hectare of cane is as follows:

$$PNR_{Farm}/ha = [\{ P_s \times 0.009 (CCS - 4) \} + C - H] \times TCH - P_N N$$

PNR stands for partial net return, P_s stands for the sugar price per tonne, C is the constant in the cane price ($/tonne cane), H stands for harvesting and levies costs ($/tonne cane), P_N stands for the price of nitrogen ($/kg), and N stands for nitrogen (kg) applied per hectare. Parameters used in the equation, based on district consultations and desktop review, are as follows:

- P_s of $450;
- P_N of $1.52/kg;
- C of $0.60/tonne; and
- H of $9.00/tonne.

CCS and TCH are calculated using the response functions provided for each region relating CCS and TCH to nitrogen applied (Appendix A). Note these functions are for ratoon cane, and the calculation of PNR accounts only for the variable nitrogen fertiliser and harvesting costs associated with this cane. All other costs are fixed.

Mill partial net returns

The partial net return per ha to milling is the total value of sugar per ha at each level N applied based on CCS less the payment made to growers in the CCS formula, less the marginal milling costs per tonne of cane. This was calculated using the formula:

$$PNR_{Mill}/ha = [P_s \times (CCS/100) - \{ P_s \times 0.009 (CCS - 4) \} + C] \times TCH - MC \times TCH$$

In this formula, MC stands for milling costs per tonne of cane which were assumed to be $5/tonne across the industry.

2.3 SCALING FARM IMPACTS TO REGIONAL ECONOMIES

Estimates of hectares of cane harvested by region (Table 2) were sourced from the CANEGROWERS 2019 Annual Report. The estimated impacts on TCH/ha for each region were converted into regional level impacts, using the economic model developed in QEAS's (2019) study of the economic contribution of the sugarcane industry, and which was partly based on the model used by Lawrence Consulting (2019). The economic contributions estimated in the QEAS (2019) study are summarised in Appendix B.

Table 2. Hectares of cane harvested by region, Queensland

Region	2017	2018	Average
Bundaberg & rest of WBB	42,834	44,388	43,611
Far North Queensland	77,574	76,146	76,860
Mackay	106,200	108,001	107,101
North Queensland	125,765	125,908	125,837
Rest of Queensland*	7,754	8,062	7,908
Queensland	**360,127**	**362,505**	**361,316**

Rest of Queensland in this table includes the Tableland and the area around Rocky Point. Note that direct economic impacts are not calculated for the Rest of Queensland in this report.

The correspondence between the economic regions used in the QEAS (2019) report and the representative districts for which nitrogen-response functions were derived, is shown in Table 3.

Table 3. Correspondence between economic regions in the QEAS (2019) report and districts for which nitrogen-response functions were derived.

QEAS (2019) regions	District with generalised nitrogen-response function
Wide-Bay Burnett (WBB)	Bundaberg
Mackay	Mackay
North Queensland	Burdekin, Herbert
Far North Queensland (FNQ)	Tully

Note that for the North Queensland economic region there were two N-response functions available: Burdekin and Herbert. For this region, the outputs were a combination of the individual nitrogen-response functions for Burdekin and Herbert using respective weightings of 0.6 and 0.4, the latter based on their relative tonnes of cane crushed (CANEGROWERS 2019).

To estimate the economic impacts of reduced N rates beyond the farm gate, specific shocks were formulated that corresponded to reductions in N rate of 10%, 15%, 20%, 25%, and 30% (relative to the reference 6ES recommendation). The logic underlying the economics is set out in Figure 3. The estimated reduction in TSH was assumed to have the same proportional impact on gross value added (GVA) by cane growers and sugar mills.

Figure 3. Economic model for scaling up farm impacts
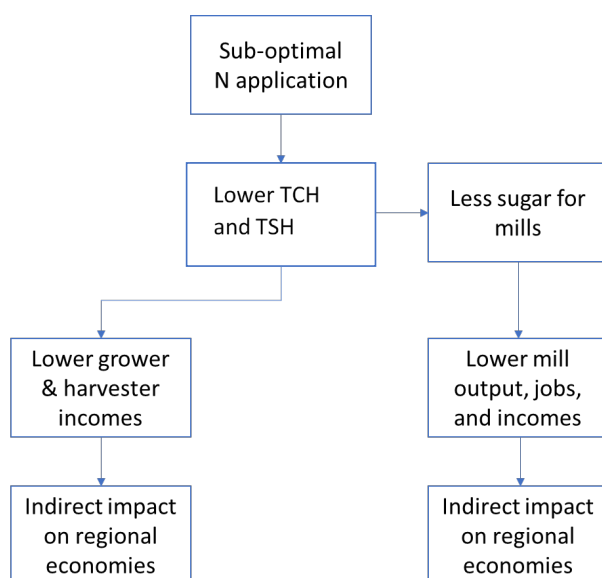
Table 4 shows the impacts of reduced N rates on regional cane tonnages, which seeded the estimates of regional economic impacts.

Table 4. Reduction in the annual tonnes of cane crushed in each region as a result of reduced N rates.

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Wide-Bay Burnett	-65,451	-101,703	-140,305	-181,258	-224,562
Far North Queensland	-142,037	-224,919	-315,710	-414,410	-521,019
Mackay	-187,156	-294,588	-411,257	-537,163	-672,304
North Queensland	-253,688	-396,076	-548,825	-711,937	-885,412
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	-648,333	-1,017,286	-1,416,098	-1,844,768	-2,303,296

The estimates of indirect impacts from this study should be taken as upper bounds of potential economic impacts - cautious interpretation of indirect/multiplier effects is required when these are generated by Input-Output models (Gretton 2013).

3. RESULTS

3.1 EFFECTS ON CROP PARTIAL NET RETURN

The translation of the N-yield response functions for each district into impacts of changing N rates on partial net return per ha for ratoon cane is shown in Figure 4.

Figure 4. Impacts of N application rates on farm partial net return for each representative district
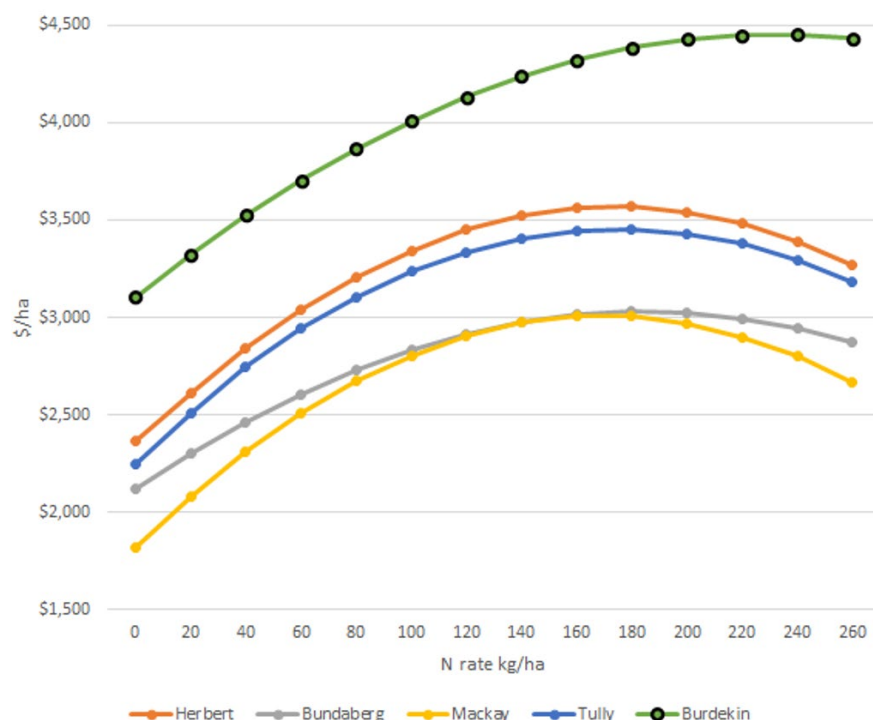
Table 5 shows the impacts of reduced N rates on partial net return of ratoon cane for each district. The impact on PNR of a 30% reduction in N rate ranged from $151 per ha for Bundaberg to $228 per ha for Burdekin (Figure 5).

For a Herbert River grower with 130 ha under cane each year, a 15% reduction in N rate would create a loss in net revenue of approximately $10,000 per year, while a 30% reduction in N rate would mean a loss of $24,830 per year. For all growers in the Herbert, this would be a total loss of $4.4 M (15% less N) or $10.9 M (30% less N), given the area harvested is around 57,000 ha. For a Burdekin grower with the same area of cane, 15% and 30% less N would result in annual losses of $12,250 and $28,500, respectively. The corresponding losses for all Burdekin growers would be $6.7 M and $15.7 M, respectively.

Table 5. Impacts of reduced N rates on partial net returns ($/ha) for farms in each representative district

N application	Bundaberg	Burdekin	Herbert	Mackay	Tully
6ES rate	$2,975	$4,356	$3,521	$2,973	$3,404
-10% (below 6ES)	$2,935	$4,294	$3,474	$2,930	$3,359
-15%	$2,911	$4,258	$3,444	$2,902	$3,331
-20%	$2,885	$4,218	$3,410	$2,870	$3,299
-25%	$2,855	$4,175	$3,372	$2,833	$3,264
-30%	$2,824	$4,128	$3,330	$2,793	$3,224

Figure 5. Reductions in farm partial net returns from reduced N rates, for each representative district
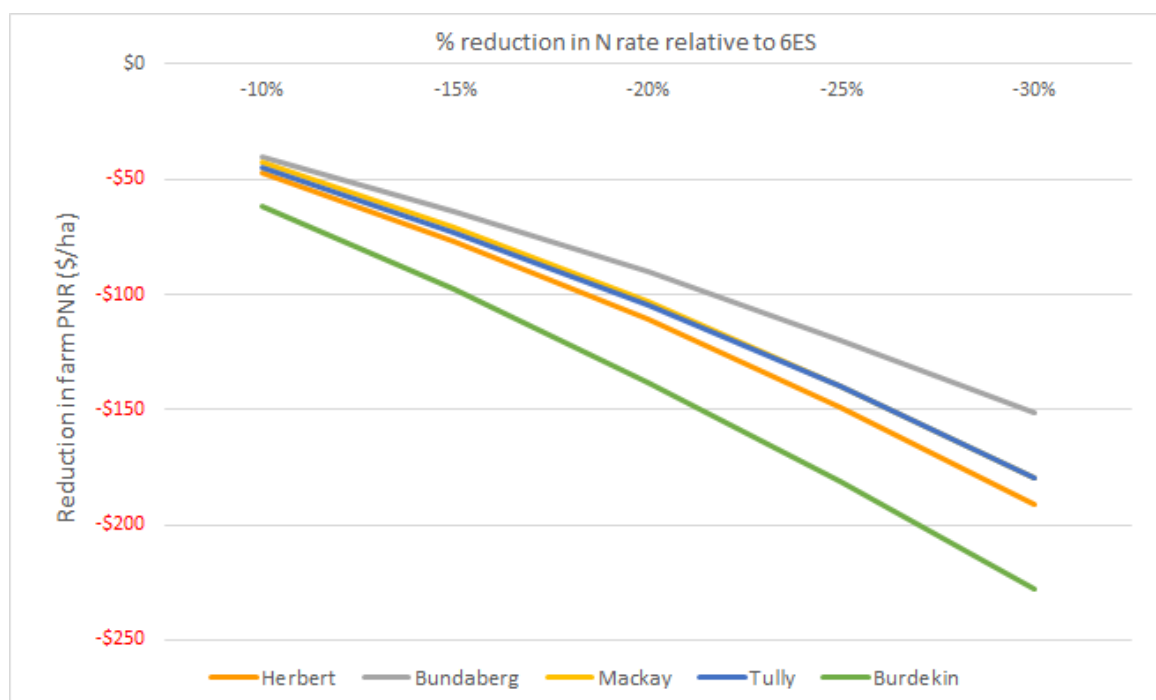
3.2 EFFECTS ON PARTIAL NET RETURN OF MILLS

Table 6 shows the impacts of reduced N rates on partial net returns per ha for sugar mills in each district. Reductions in partial net returns from a 30% reduction in N rate range from $92 to $136/ha, which is a 7 to 8% reduction for each mill. For the Burdekin mills, this would be a total annual loss of $9.4 M (68,800 ha harvested) while for the Tully mill, this would be an annual loss of $3.4 M (29,700 ha harvested).

Table 6. Impacts of reduced N rates on mill partial net return ($/ha) for mills in each representative district

N application	Bundaberg	Burdekin	Herbert	Mackay	Tully
6ES rate	$1,395	$2,075	$1,653	$1,403	$1,630
-10% (below optimal)	$1,368	$2,035	$1,622	$1,372	$1,598
-15%	$1,354	$2,013	$1,605	$1,355	$1,579
-20%	$1,338	$1,990	$1,586	$1,335	$1,559
-25%	$1,321	$1,965	$1,565	$1,314	$1,537
-30%	$1,303	$1,939	$1,543	$1,292	$1,513

3.3 EFFECTS ON INDUSTRY (FARM PLUS MILLS) PARTIAL NET RETURN

Adding the estimated impacts for cane farms in (Table 5) with the corresponding mill impacts (Table 6) produces industry impacts of reduced N rates (Table 7). Reductions in partial net returns, from a 30% reduction in N rate, range from $244 to $363/ha, which is a 5.6 to 6.6 % reduction in industry partial net return for each district. For the Tully district, this means a loss of $8.8 M per year while the Burdekin district would lose $25 M per year.

Table 7 Impacts of reduced N management on partial net return ($/ha) for the industry (farms and mills) in each representative district

N application	Bundaberg	Burdekin	Herbert	Mackay	Tully
6ES rate	$4,370	$6,430	$5,174	$4,376	$5,034
-10% (below optimal)	$4,304	$6,330	$5,096	$4,302	$4,957
-15%	$4,265	$6,272	$5,049	$4,256	$4,911
-20%	$4,222	$6,208	$4,996	$4,205	$4,858
-25%	$4,176	$6,140	$4,937	$4,148	$4,800
-30%	$4,126	$6,067	$4,873	$4,085	$4,737

3.4 DIRECT IMPACTS OF REDUCED N RATES ON REGIONAL ECONOMIES

Direct impacts from effects on farm incomes

Table 8 shows the estimated reduction in regional farm income from sugarcane production under the different nitrogen scenarios. State-wide, the annual impacts range from a reduction in farm incomes of $16.9 million for a 10% reduction in N rates relative to 6ES, to $66.5 million for a 30% reduction in N rate.

There would also be direct negative effects via lower expenditure on harvesting (Table 9) and fertiliser (Table 10). The state-wide impact of these would be lost economic value of close to $45 million for a 30% reduction in N application rates.

Table 8. Reductions in regional farm incomes, $ million, per annum.

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	-15%	-20%	-25%	-30%
Wide-Bay Burnett	-1.7	-2.8	-3.9	-5.2	-6.6
Far North Queensland	-3.5	-5.6	-8.1	-10.8	-13.9
Mackay	-4.6	-7.6	-11.1	-14.9	-19.3
North Queensland	-7.0	-11.3	-16.0	-21.2	-26.8
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	**-16.9**	**-27.3**	**-39.1**	**-52.1**	**-66.5**

Table 9. Reduction in purchases of harvesting services, $ million per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	-15%	-20%	-25%	-30%
Wide-Bay Burnett	-0.6	-0.9	-1.3	-1.6	-2.0
Far North Queensland	-1.3	-2.0	-2.8	-3.7	-4.7
Mackay	-1.7	-2.7	-3.7	-4.8	-6.1
North Queensland	-2.3	-3.6	-4.9	-6.4	-8.0
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	**-5.8**	**-9.2**	**-12.7**	**-16.6**	**-20.7**

Table 10. Reduction in purchases of fertiliser, $ million per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Wide-Bay Burnett	-0.9	-1.4	-1.9	-2.3	-2.8
Far North Queensland	-1.6	-2.5	-3.3	-4.1	-4.9
Mackay	-2.3	-3.4	-4.6	-5.7	-6.8
North Queensland	-3.0	-4.5	-6.0	-7.6	-9.1
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	**-7.9**	**-11.8**	**-15.7**	**-19.7**	**-23.6**

Direct impacts from effects on mill incomes

Table 11 shows the estimated reduction in regional mill incomes under the different nitrogen scenarios. State wide, the annual impacts to mills range from a reduction in gross income of $11.4 million for 10% less N applied, to $40.7 million for 30% less N.

Table 11. Reductions in regional mill incomes, $ million per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Wide-Bay Burnett	-1.2	-1.8	-2.5	-3.3	-4.0
Far North Queensland	-2.5	-3.9	-5.5	-7.2	-9.0
Mackay	-3.3	-5.2	-7.2	-9.5	-11.9
North Queensland	-4.5	-7.0	-9.8	-12.7	-15.8
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	**-11.4**	**-17.9**	**-25.0**	**-32.6**	**-40.7**

This in turn has a direct impact on wages of mill workers (Table 12), assuming 20% of the variable cost estimate of $5/tonne is related to labour, which is broadly consistent with IBISWorld (2019) estimates of the industry cost structure. The state-wide impact of reduced N rates ranges from an annual loss of wages of $0.6 million for 10% sub-optimal application to $2.3 million for 30% sub-optimal application.

Table 12. Reductions in sugar mill wages, $ million per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Wide-Bay Burnett	-0.1	-0.1	-0.1	-0.2	-0.2
Far North Queensland	-0.1	-0.2	-0.3	-0.4	-0.5
Mackay	-0.2	-0.3	-0.4	-0.5	-0.7
North Queensland	-0.3	-0.4	-0.5	-0.7	-0.9
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	**-0.6**	**-1.0**	**-1.4**	**-1.8**	**-2.3**

There are also direct effects on purchases of intermediate goods and services used by mills, with the state-wide impact ranging from -$2.6 million per annum for 10% reduction in N rates, to -$9.2 million per annum for 30% reduced N (Table 13).

Table 13. Reductions in purchases of intermediate goods and services by sugar mills, $ million, per annum

| | *Reduction in N (% decrease relative to 6ES rate)* | | | | |
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Wide-Bay Burnett	-0.3	-0.4	-0.6	-0.7	-0.9
Far North Queensland	-0.6	-0.9	-1.3	-1.7	-2.1
Mackay	-0.7	-1.2	-1.6	-2.1	-2.7
North Queensland	-1.0	-1.6	-2.2	-2.8	-3.5
Rest of Queensland	n.a.	n.a.	n.a.	n.a.	n.a.
Queensland	**-2.6**	**-4.1**	**-5.7**	**-7.4**	**-9.2**

Total direct impacts

Table 14 shows the estimates for total direct impacts, being the sum of tables 8 (Farm incomes), 11 (Mill incomes), and 12 (Wages). The reductions in the industry's direct economic benefits to the State ranges from $29 million per annum for 10% sub-optimal application to $110 million for 30% sub-optimal application.

Note that it does not include impacts on harvesting services, fertiliser, or intermediate goods and services because these are inputs. The value added associated with these inputs is picked up in the indirect impacts (see next section).

Table 14. Total direct economic losses from reduced N rates, $ million per annum

| | *Reduction in N (% decrease relative to 6ES rate)* | | | | |
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Wide-Bay Burnett	-3.0	-4.7	-6.6	-8.6	-10.9
FNQ	-6.1	-9.7	-13.9	-18.4	-23.4
Mackay	-8.1	-13.1	-18.7	-24.9	-31.8
North Queensland	-11.8	-18.7	-26.3	-34.6	-43.5
Rest of Queensland	0.0	0.0	0.0	0.0	0.0
Queensland	**-28.9**	**-46.2**	**-65.4**	**-86.6**	**-109.6**

3.5 INDIRECT IMPACTS OF REDUCED N MANAGEMENT

Using QEAS's (2019) economic contributions model, the potential indirect economic impacts of reduced farm incomes, as a result of blanket reductions in N rates, are provided in Table 15. Indirect impacts are up to $40.5 million state-wide. Note that the pattern of the indirect impacts across the state reflects the high leakage of expenditure from regional economies that typically occurs due to purchases of goods and services from other parts of the State, such as South East Queensland.

Table 15. Estimates of indirect economic impacts via reduced supply-chain purchases and lower incomes on cane farms, $ million, per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Bundaberg & rest of WBB	-0.7	-1.1	-1.6	-2.0	-2.6
Far North Queensland	-1.5	-2.4	-3.4	-4.5	-5.7
Mackay	-2.0	-3.2	-4.5	-6.0	-7.6
North Queensland	-2.8	-4.5	-6.2	-8.2	-10.2
Rest of Queensland	-3.8	-6.1	-8.6	-11.4	-14.4
Queensland	**-10.9**	**-17.3**	**-24.4**	**-32.1**	**-40.5**

NB. In calculating indirect impacts it is assumed that direct regional gross value added is 50% of regional purchases of harvesting services, which appears a reasonable assumption in the absence of specific data. Indirect impacts via fertiliser purchases are excluded as regional value added impacts are small.

We have calculated the potential indirect economic impacts due to lower production at sugar mills (Table 16). The indirect impacts range from -$2.7 million through to -$9.7 million.

Aggregating these estimates shows the indirect economic penalties to the State range from -$13.6 million for 10% sub-optimal application to -$50.2 million for 30% sub-optimal application (Table 17).

Table 16. Estimates of indirect economic impacts via reduced supply chain purchases and lower labour incomes at sugar mills, $ million per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Bundaberg & rest of WBB	-0.2	-0.4	-0.5	-0.7	-0.9
Far North Queensland	-0.5	-0.9	-1.2	-1.6	-2.0
Mackay	-0.7	-1.1	-1.6	-2.0	-2.6
North Queensland	-1.0	-1.5	-2.1	-2.7	-3.4
Rest of Queensland	-0.3	-0.4	-0.6	-0.7	-0.9
Queensland	**-2.7**	**-4.3**	**-5.9**	**-7.7**	**-9.7**

Table 17. Total indirect impacts from reduced N application rates, $ million per annum

	Reduction in N (% decrease relative to 6ES rate)				
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Bundaberg & rest of WBB	-1.0	-1.5	-2.1	-2.7	-3.4
Far North Queensland	-2.1	-3.3	-4.6	-6.1	-7.7
Mackay	-2.7	-4.3	-6.1	-8.0	-10.1
North Queensland	-3.8	-6.0	-8.3	-10.9	-13.6
Rest of Queensland	-4.0	-6.5	-9.1	-12.1	-15.3
Queensland	**-13.6**	**-21.5**	**-30.3**	**-39.8**	**-50.2**

3.6 TOTAL IMPACTS OF REDUCED NITROGEN APPLICATION

The combined direct and indirect impacts of blanket reductions in nitrogen application of up to 30% are presented in Table 18. The adverse impacts on the State economy from reduced yields are up to $160 million per annum. The total impacts, including indirect impacts, can be considered conservative, bearing in mind the finding of the QEAS (2019) report which demonstrated $1 in cane farming generates over $6 in value elsewhere in the value chain. The findings in this report are consistent with that finding and are based on the same economic model. A specific shock to the production process is modelled, and we have used evidence on the expected impacts on specific purchases from the supply-chain, rather than assuming reductions in the purchases of all inputs by cane growers and mills.

Table 18. Estimated total economic impacts of sub-optimal nitrogen application, $ million per annum

| | Reduction in N (% decrease relative to 6ES rate) | | | | |
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Bundaberg & WBB	-3.9	-6.2	-8.7	-11.4	-14.3
FNQ	-8.1	-13.0	-18.5	-24.5	-31.1
Mackay	-10.8	-17.4	-24.8	-33.0	-41.9
North Queensland	-15.6	-24.7	-34.6	-45.4	-57.1
Rest of Queensland	-4.0	-6.5	-9.1	-12.1	-15.3
Queensland	**-42.5**	**-67.8**	**-95.7**	**-126.4**	**-159.7**

Over a ten-year period, the loss of value added to the Queensland economy would amount to nearly $1.3 billion (Table 19).

Table 19. Estimated total economic impacts over ten years (assuming 4% real discount rate), $ million per annum

| | Reduction in N (% decrease relative to 6ES rate) | | | | |
	-10%	*-15%*	*-20%*	*-25%*	*-30%*
Bundaberg & WBB	-31.9	-50.4	-70.5	-92.3	-115.7
FNQ	-65.8	-105.5	-149.9	-198.8	-252.4
Mackay	-87.7	-141.2	-201.1	-267.5	-340.2
North Queensland	-126.4	-200.2	-280.8	-368.4	-462.9
Rest of Queensland	-32.7	-52.4	-74.2	-98.2	-124.3
Queensland	**-344.6**	**-549.6**	**-776.5**	**-1025.2**	**-1295.5**

4. DISCUSSION

This study has demonstrated the economic risk to industry and regional economies of seeking reduced losses of DIN through blanket use of N rates below the 6ES recommendations. Such reductions would clearly reduce the production and incomes of farms, the profitability of mills, and the economic and social health of regional economies. A 30% reduction in N rate would cause a 0.7 to 1.2 tonne/ha reduction in sugar yields, depending on district. This, in turn, would reduce crop partial net returns by $142 to $266/ha, again depending on district. These impacts would lead to reductions in the industry's direct economic benefits for regional economies, ranging from approximately $11 million per annum in the Wide-Bay Burnett to $44 million per annum in North Queensland. The collective regional impacts would penalise the Queensland economy by approximately $110 million each year. When we account for impacts on both the direct and indirect economic benefits of cane farming, the annual penalty to the State would be $160 million.

The report demonstrates that current reef water quality policies, regulations and programs are based on unrealistic expectations of what growers can afford to do in reducing N application rates, and also highlights the risk to the viability of mills and regional economies. All such policies, regulations and programs should therefore be urgently reviewed and revised with input from industry.

Confidence that the estimates of economic impacts are realistic is based on:
1. The considerable field data that underpin both the 6ES program and the generalised district relationships between crop production and nitrogen rate used in this study.
2. The availability of a proven model for scaling up farm and mill impacts to the regional scale, including the implications for both direct and indirect economic benefits.

Two aspects of the analysis may have led to a slight overestimate of the economic impacts. Plant crops were not considered in the analysis due to the relatively small data set. Plant crops that follow a fallow period can be less responsive to N than ratoon crops, although this is reflected in the recommendations from the 6ES program. Also, CCS values from field plots were used in the analysis, and these values are typically higher than that from commercial harvests as the latter includes extraneous matter. The CCS levels in commercial harvests may also be affected by higher rates of N if this leads to the crop lodging, so that the optimal N rate under commercial conditions may, at times, be a little lower than that from trial work.

On the other hand, the medium to long-term economic impacts of reduced N rates may be higher than estimated, due to:
1. The potential for the effects of reduced N rates to amplify over time, due to mining of soil nitrogen reserves.
2. The conservative approach to measuring indirect impacts. The QEAS (2019) report found $1 in cane farming generates over $6 in value elsewhere in the value chain but, rather than assuming reductions in the purchases of all inputs by cane growers and mills, only the impacts on specific purchases from the supply-chain were included.
3. Reduced farm profits increasing the likelihood of some cane land being abandoned or being used for less productive purposes.
4. Reduced tonnages leading to fewer harvest contractors, which would extend the harvest season.
5. Reduced tonnages through mills, compromising the viability of at least some mills.

On balance, the estimated impacts of reduced N rates are likely to be a reasonable approximation of the real economic risks to farms, districts and regional economies.

Industry should continue to research and adopt cost-effective options for improving nitrogen use efficiency and reducing losses of DIN. That ongoing work is important for both productivity and reducing risk of possible downstream impacts. However, any blanket reductions of N rates below the 6ES recommendations will damage the industry and the State. Therefore, all parties interested in reducing DIN losses from cane farms should focus on:
- Better adoption of existing cost-effective options, including use of the complete set of nutrient and ameliorant recommendations from 6ES, and better placement and timing of fertiliser applications.
- Research to identify new cost-effective technologies - one current example is the use of fertiliser coatings or other formulations to achieve better synchrony between crop demand and soil availability. This may overcome some of the inefficiencies associated with the use of highly soluble forms of N such as urea. Some products show promise but adoption will require their use to be cost-effective.
- Research to guide further refinement of current nutrient management guidelines, including identification of situations (soil properties × seasonal conditions × management factors) which are likely to be less, or more, responsive to nutrients.

5. CONCLUSIONS

This report has identified the potential economic losses from any blanket reductions of N rates below those recommended by the SIX EASY STEPS™ (6ES) Program. The collective regional impacts would penalise the Queensland economy by up to $160 million each year. Current reef water quality policies and programs, including the Queensland Government regulations, are based on unrealistic expectations of what growers can afford to do in reducing N application rates. All such policies, regulations and programs need urgent review and revision with strong input from industry.

Industry should continue to research, develop and implement cost-effective options for improving nitrogen use efficiency and reducing losses of DIN. That ongoing work is important. However, this report highlights the economic risk to industry and regional communities of seeking reduced DIN through widespread use of N application rates below industry guidelines.

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Appendix A. Mean regional nitrogen (N) response curves for sugarcane ratoon crops in Queensland

Prepared by Bernard Schroeder (University of Southern Queensland, Toowoomba) for CANEGROWERS, 100 Edward Street, Brisbane

Sustainable sugarcane production is dependent on planting productive and disease-resistant sugarcane cultivars (Cox *et al*. 2005) and using appropriate farming systems (Garside and Bell 2006, Schroeder *et al*. 2013). The latter includes sustainable nutrient management practices based on well-considered and scientifically derived guidelines (Schroeder *et al*. 2006, 2008). In Australia, this is provided by the SIX EASY STEPS[TM] program that is recognised as current best nutrient management practice (Schroeder *et al*. 2018a) in all cane-growing areas in Queensland and New South Wales. In particular, the N management guidelines are based on results of numerous field trials conducted across the industry over several decades (e.g. Chapman 1968, 1971; Schroeder *et al*. 1998, 2005, 2010a, 2018a).

The appropriate N application rates for agricultural crops are traditionally determined from response curves based on yield data from field trials that include different rates of the applied N. In sugarcane, we have done this by fitting quadratic functions to the mean yield (tc/ha) data points plotted against N applied for each crop in each trial. An example is shown in Figure 1. The most appropriate agronomic rate of applied N (optimum N rate) corresponds to 95% of the maximum yield predicted by the quadratic function (shown by the downward pointing arrows in Figure 1).
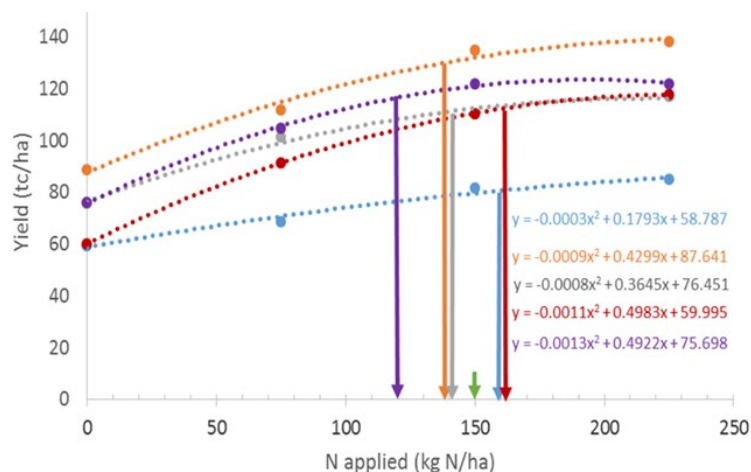

The equations shown in the figure are:
$y = -0.0003x^2 + 0.1793x + 58.787$
$y = -0.0009x^2 + 0.4299x + 87.641$
$y = -0.0008x^2 + 0.3645x + 76.451$
$y = -0.0011x^2 + 0.4983x + 59.995$
$y = -0.0013x^2 + 0.4922x + 75.698$

Figure 1. Yield response curves resulting from N applied to a trial conducted in the Herbert district over a crop cycle (after Schroeder et al., 2005) consisting of a plant crop (blue), first ratoon (orange), second ratoon (grey), third ratoon (red) and fourth ratoon (purple). The downward pointing arrows indicate the Optimum N application rate (corresponding to 95% of the maximum yield predicted by the quadratic functions fitted to the data points). The small green arrow shows the SIX EASY STEPS[TM] N rate for this soil.

The availability of data from N trials conducted at various sites across the industry provided the opportunity to determine mean N response curves for the different sugarcane regions in Queensland – Herbert, Bundaberg (Southern), Mackay (Central), Tully (Wet Tropics) and Burdekin. These data were obtained from various sources and included published papers (e.g. Hurney and Schroeder 2012; Salter *et al*. 2010; Schroeder *et al*. 2005, 2009; Skocaj *et al*. 2012, 2019), project milestone and final reports (Schroeder *et al*. 2003, 2010b, 2018b) , BSES technical (Chapman, 1976) and SRA research (Schroeder *et al*. 2015) reports, and some unpublished (Anon 1979) or yet to be published sources (Skocaj 2015).

Quadratic functions were fitted to the ratoon yield data [tonnes cane per ha (TCH)] from each of the rates of N trials included in the study [Herbert (n=16), Bundaberg (n=15), Mackay (n=16), Tully (n=16) and the Burdekin (n=7)]. The resulting quadratic coefficients (a, b and c) were used to calculate mean quadratic coefficient values for each region/district. These were then used to construct mean regional response curves (Figures 2). Variability is represented in Figure 2 by the quadratic functions obtained from the mean values, plus and minus the standard errors of the means (SEMs). Quadratic functions fitted to the commercial cane sugar (CCS) values plotted against N applied for each trial were used to determine mean regional response curves for CCS values (Figure 3). The resulting quadratic coefficients were used to calculate mean CCS for assumed N rates (0 – 260 kg N/ha) for each region (Table 1). Sugar yield [tonnes sugar per ha (TSH)] were determined from the mean TCH and CCS values and plotted against N applied (Figure 4). As the trials often had different sets of N rate treatments, the N rates shown in Table 1 and Figure 4 are not the actual N

rates applied, but rather provided a means of constructing the response curves from the calculated quadratic coefficients.
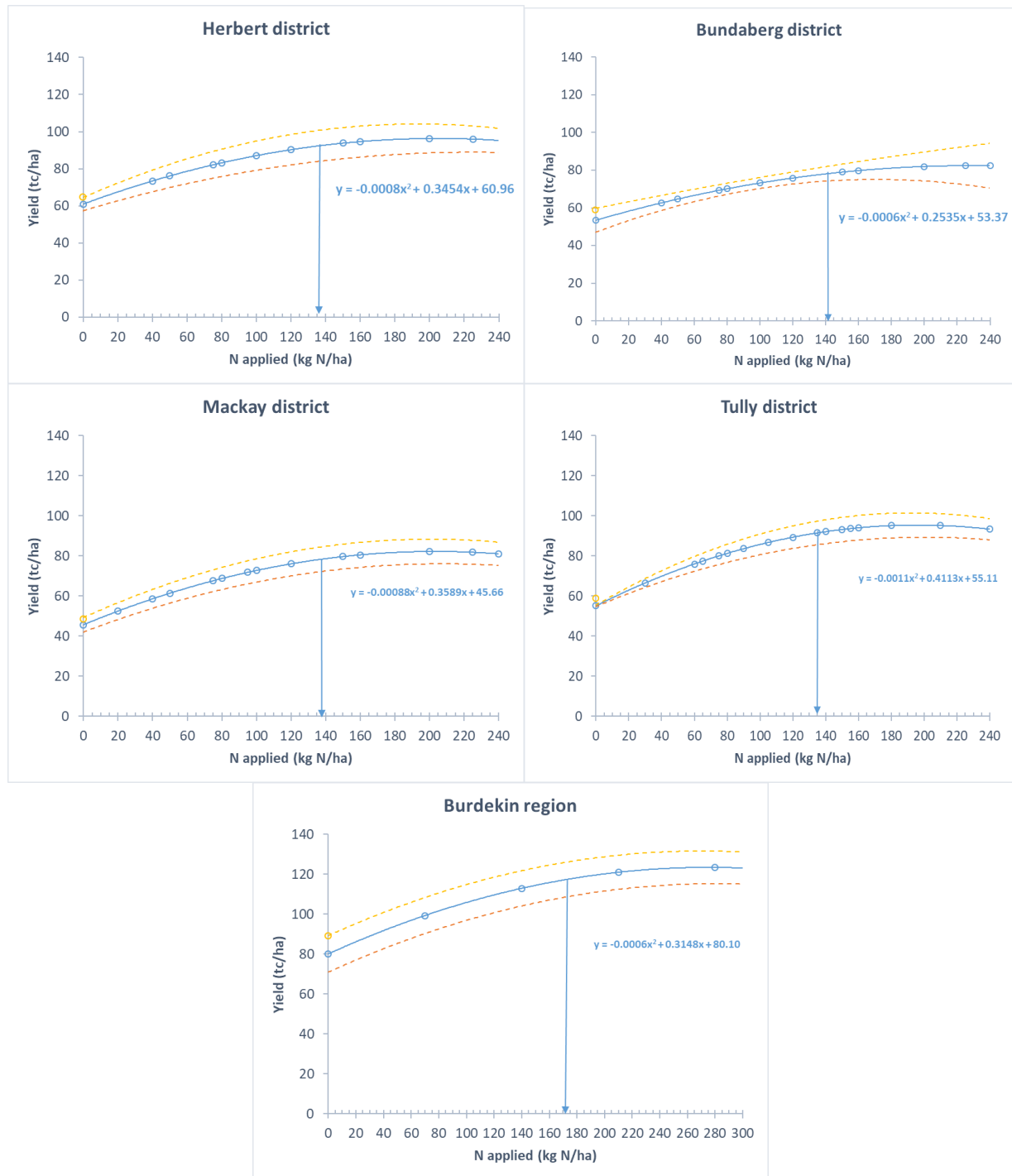

Figure 2. Mean district/regional N response curves (TCH plotted against N applied) predicted by the quadratic functions fitted to the data points for ratoon crops in each of the districts/regions (Herbert, Bundaberg, Mackay, Tully and Burdekin). The dotted lines in each graph represent the standard errors of the means (SEMs) above and below each of the response curves. The downward pointing arrows indicate the Optimum N application rate (corresponding to 95% of the maximum yield predicted by the quadratic functions).
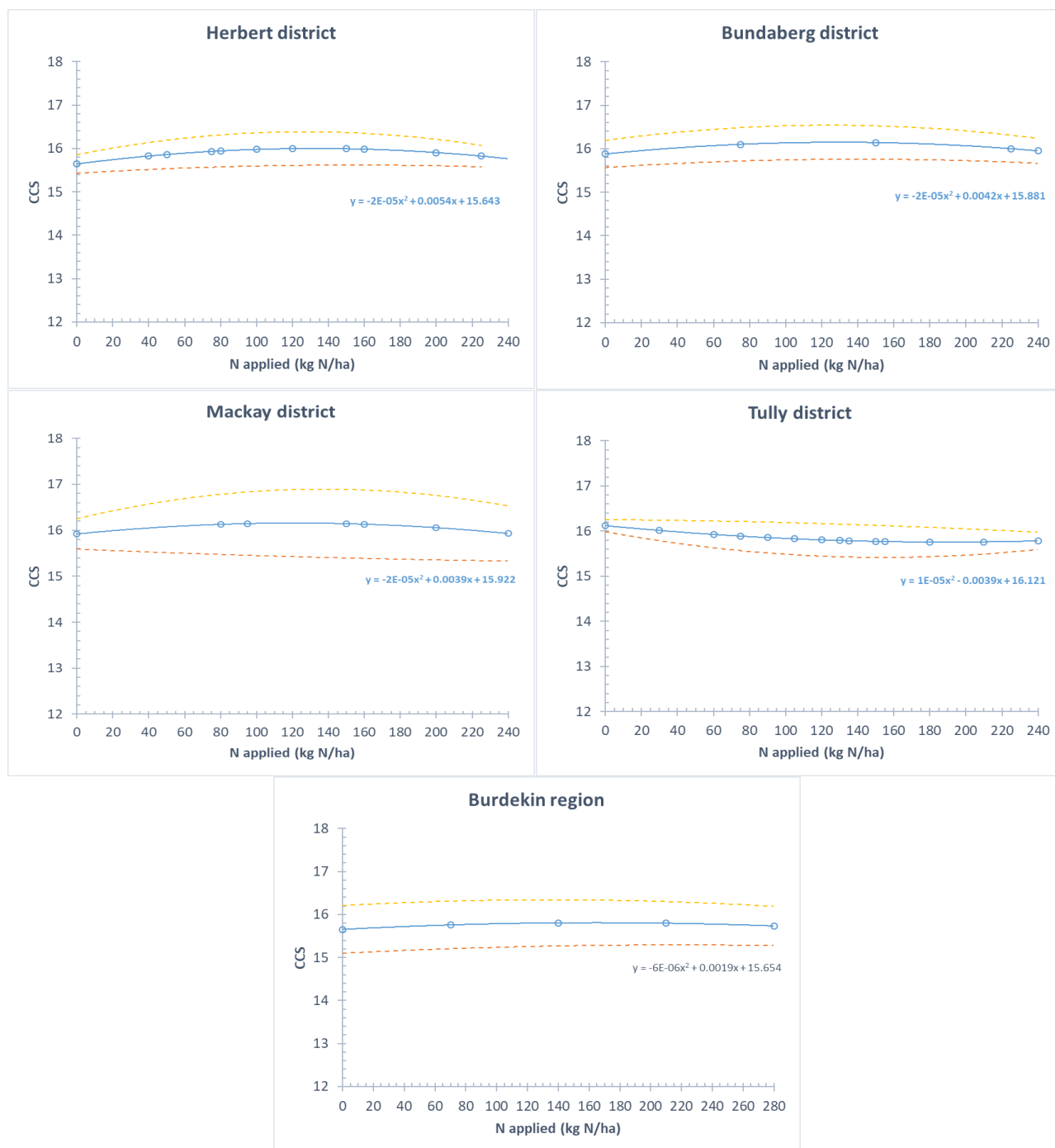
Figure 3. Mean district/regional CCS curves (CCS plotted against N applied) predicted by the quadratic functions fitted to the data points for ratoon crops in each of the districts/regions (Herbert, Bundaberg, Mackay, Tully and Burdekin). The dotted lines in each graph represent the standard errors of the means (SEMs) above and below each of the response curves.

Table 1 – Calculated mean regional CCS values for ratoon crops corresponding to the range of the assumed N application rates.

N application rate (kg N/ha)	Region/district				
	Herbert	Bundaberg	Mackay	Tully	Burdekin
	Calculated CCS (%)				
0	15.64	15.88	15.92	16.12	15.65
20	15.74	15.96	15.99	16.05	15.69
40	15.82	16.02	16.04	15.98	15.72
60	15.89	16.07	16.08	15.92	15.74
80	15.94	16.11	16.10	15.87	15.76
100	15.98	16.14	16.11	15.83	15.78
120	16.00	16.15	16.10	15.80	15.79
140	16.00	16.15	16.07	15.77	15.80
160	15.99	16.14	16.03	15.75	15.80
180	15.96	16.12	15.97	15.74	15.80
200	15.92	16.08	15.90	15.74	15.79
220	15.86	16.03	15.81	15.75	15.78
240	15.78	15.97	15.70	15.76	15.76
260	15.69	15.89	15.58	15.78	15.74

$$y = -0.0001x^2 + 0.0591x + 9.509$$
$$y = -0.0001x^2 + 0.0436x + 8.459$$
$$y = -0.0002x^2 + 0.0603x + 7.252$$
$$y = -0.0002x^2 + 0.0626x + 8.907$$
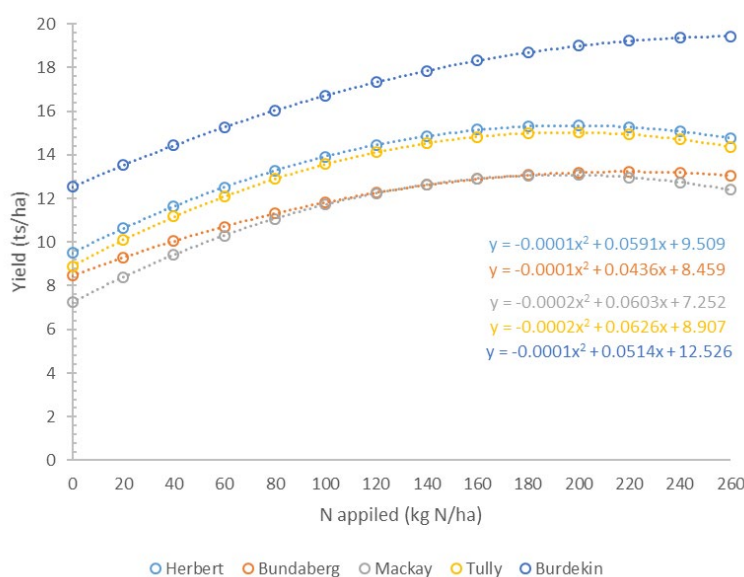$$y = -0.0001x^2 + 0.0514x + 12.526$$

Figure 4. Mean regional N response curves (TSH plotted against N applied) for ratoon crops as determined from the relevant TCH and CCS values.

The overall trends indicated that regional mean response curves (TCH and TSH) were similar for the Herbert and Tully districts, and for Mackay and Bundaberg (Figures 2 and 3). As expected, the Burdekin was different from the other regions.

The methodology associated with the study and results provided above is being developed into a scientific paper to be published in an appropriate journal. This intended paper, with the probable title of 'Mean regional nitrogen response curves for sugarcane production in Australia' (BL Schroeder, AW Wood, DM Skocaj, B Salter, JH Panitz G Park, ED Kok), will provide a summary of available data, document the process used to compile the data and information, and report on the development of the mean N response curves for the various districts/regions. This will provide a record of the mechanism that was used to develop appropriate mean response curves based on a relatively large number of trials. The process will also serve as an example for similar uses in other circumstances. It will also enable the SIX EASY STEPS™ to expand the process of capturing and processing additional trial data (past, present and future).

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Appendix B. Economic contributions estimated in QEAS (2019)

Contribution to Queensland GSP of sugarcane growing and manufacturing, 2017-18

	Sugarcane growing $M	Whole sugar value chain $M	Sugarcane growing % of GSP	Whole sugar value chain % of GSP
Total Sales	1,204.7	3196.8	0.35%	0.92%
Value added				
Direct	544.4	2,243.6	0.16%	0.64%
Indirect–supply chain	317.6	1,174.6	0.09%	0.34%
Indirect–consumption induced	249.5	631.3	0.07%	0.18%
Indirect–total	567.1	1,805.9	0.16%	0.52%
Total value added	**1,111.5**	**4,049.5**	**0.32%**	**1.16%**

Source: QEAS, 2019

Contribution to Queensland employment of sugarcane growing and manufacturing, 2017-18

	Sugarcane growing FTEs	Whole sugar value chain FTEs	Sugarcane growing % of total FTEs	Whole sugar value chain % of total FTEs
Direct	4,554	9,145	0.22%	0.44%
Indirect–supply chain	3,154	8,174	0.15%	0.39%
Indirect–consumption induced	2,126	5,337	0.10%	0.26%
Indirect–total	5,280	13,511	0.25%	0.65%
Total	**9,834**	**22,657**	**0.47%**	**1.09%**

Source: QEAS, 2019

Contribution to wages and salaries of sugarcane growing and manufacturing, 2017-18

	Sugarcane growing $ millions	Whole sugar value chain $ millions	Sugarcane growing $ millions	Whole sugar value chain $ millions
Direct	175.6	352.7	0.11%	0.22%
Indirect–supply chain	121.6	632.4	0.07%	0.39%
Indirect–consumption induced	82.0	375.5	0.05%	0.23%
Indirect–total	203.6	1,007.9	0.12%	0.62%
Total	**379.3**	**1,360.6**	**0.23%**	**0.83%**

Source: QEAS, 2019

Queensland sugar's contribution to Commonwealth and state taxes and local government rates, 2017-18

Level of government	Sugarcane growing $M	Whole sugar value chain $M
Commonwealth	226.0	823.5
State	42.2	153.7
Local	103.7	103.7*
Total	**371.9**	**1,080.9**

Source: QEAS, 2019

REVIEW

Everything you must know about *Azospirillum* and its impact on agriculture and beyond

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Abstract

Azospirillum is one of the most studied plant growth-promoting bacteria (PGPB); it represents a common model for plant-bacterial interactions. While *Azospirillum brasilense* is the species that is most widely known, at least 22 species, including 17 firmly validated species, have been identified, isolated from agricultural soils as well as habitats as diverse as contaminated soils, fermented products, sulfide springs, and microbial fuel cells. Over the last 40 years, studies on *Azospirillum*-plant interactions have introduced a wide array of mechanisms to demonstrate the beneficial impacts of this bacterium on plant growth. Multiple phytohormones, plant regulators, nitrogen fixation, phosphate solubilization, a variety of small-sized molecules and enzymes, enhanced membrane activity, proliferation of the root system, enhanced water and mineral uptake, mitigation of environmental stressors, and competition against pathogens have been studied, leading to the concept of the Multiple Mechanisms Hypothesis. This hypothesis is based on the assumption that no single mechanism is involved in the promotion of plant growth; it posits that each case of inoculation entails a combination of a few or many mechanisms. Looking specifically at the vast amount of information about the stimulatory effect of phytohormones on root development and biological nitrogen fixation, the Efficient Nutrients Acquisition Hypothesis model is proposed. Due to the existence of extensive agriculture that covers an area of more than 60 million hectares of crops, such as soybeans, corn, and wheat, for which the bacterium has proven to have some agronomic efficiency, the commercial use of *Azospirillum* is widespread in South America, with over 100 products already in the market in Argentina, Brazil, and Uruguay. Studies on *Azospirillum* inoculation in several crops have shown positive and variable results, due in part to crop management practices and environmental conditions. The combined inoculation of legumes with rhizobia and *Azospirillum* (co-inoculation) has become an emerging agriculture practice in the last several years, mainly for soybeans, showing high reproducibility and efficiency under field conditions. This review also addresses the use of *Azospirillum* for purposes other than agriculture, such as the recovery of eroded soils or the bioremediation of contaminated soils. Furthermore, the synthetic mutualistic interaction of *Azospirillum* with green microalgae has been developed as a new and promising biotechnological application, extending its use beyond agriculture.

Keywords *Azospirillum* · Phytohormones · Nitrogen fixation · Plant growth promotion bacteria

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Introduction

Azospirillum is a Gram-negative, microaerophilic, non-fermentative, and nitrogen-fixing bacterial genus. It has been one of the most studied plant growth-promoting bacteria (PGPB) since its discovery by Martinus Beijerinck in the Netherlands in 1925. However, as a result of the research conducted by Johanna Döbereiner in Brazil in the 1970s, two main characteristics are used to define this bacterial genus: its ability to fix atmospheric nitrogen (N) (Day and Döbereiner 1976) and produce several phytohormones, including auxins, cytokinins, and gibberellins (Reynders and Vlassak 1979; Tien et al. 1979). Consequently, in subsequent studies, these two characteristics have been considered the cornerstone of the effect of this genus on plant growth and crops. Because *Azospirillum* is one the most studied PGPB worldwide, and it has been commercialized in several South American countries, including Argentina, Brazil, Uruguay, and Paraguay (Okon and Labandera-Gonzalez 1994; Cassán and Diaz-Zorita 2016), a significant amount of knowledge has been accumulated, demonstrating different aspects of the plant-bacteria interaction under *in planta* and in vitro conditions. It is difficult to identify and quantify the agronomical use of *Azospirillum* in countries other than those in South America. We are aware of products in Mexico, India, China, the United States (US), South Africa, Australia, and France, but no official information is available about the number of hectares (ha) treated, type of crops, type of products, and strains used. Therefore, this review focuses on its use in the South American countries, and several of the available references presenting the data are either in Spanish or Portuguese.

Major changes in the plant root architecture is the main outcome of inoculation with *Azospirillum*. It is generally accepted that these developmental responses are triggered by the production of bacterial phytohormones, and more specifically by the biosynthesis of indole-3-acetic acid (IAA) (Cassán et al. 2014). Despite exhaustive efforts to define a single mode of action to explain the plant growth facilitated by inoculation with *Azospirillum*, the mode is still undefined. However, some hypotheses have been proposed to better understand the benefits of the *Azospirillum*-plant interaction (Bashan et al. 2004; Bashan and de-Bashan 2010). This review aimed to understand the evolution of the research on the agronomical use of *Azospirillum* conducted over the last several decades, and to identify its novel use for environmental purposes and biotechnological applications beyond the agricultural industry. Based on the gathered information and new evidence brought to light in the past several years, a novel hypothesis is proposed to explain the plant growth promotion capability of these bacteria.

The genus *Azospirillum*

The *Azospirillum* species (*Azospirillum* spp.) are alpha-proteobacteria that are members of the *Rhodospirillaceae* family (Baldani et al. 2005). While most of the representatives of this family are found in aquatic environments, *Azospirillum* spp. have mainly been isolated from soil. Genomic analysis suggests that, throughout the evolutionary process, this genus transicioned from aquatic to terrestrial environments significantly later than the major Precambrian divergence of hydrobacteria and terrabacteria (nearly 2.5 billion years ago), coinciding with the major radiation of vascular plants on land 400 million years ago (Wisniewski-Dyé et al. 2011). However, for scientists, the history of this genus begins in 1925 when Beijerinck first observed a spirillum-like bacterium isolated from garden soil that was able to increase the N content in nitrogen-deficient malate-based media. Beijerinck initially named the organism *Azotobacter largimobile*; 3 years later, he renamed it *Spirillum lipoferum* (Beijerinck 1925). For 50 years, the importance of this bacterial genus as a research subject decreased until 1974 when its capacity to form a strong association with plant roots was discovered (Von Bülow and Döbereiner 1975). Another fact that awakened interest in these bacteria was their isolation from several types of soil and the roots of grasses and grain crops (Döbereiner et al. 1976). The genus *Azospirillum* was first proposed by Tarrand et al. (1978). Initially, *A. lipoferum* and *A. brasilense* were the only two species described (Tarrand et al. 1978). Since then, as summarized in Fig. 1 and Supplementary Fig. 1, a total of 22 species belonging to this bacterial genus have been identified, including *A. halopraeferens* (Reinhold et al. 1987), *A. largimobile* (Ben Dekhil et al. 1997), *A. doebereinerae* (Eckert et al. 2001), *A. oryzae* (Xie and Yokota 2005), *A. melinis* (Peng et al. 2006), *A. canadense* (Mehnaz et al. 2007a), *A. zeae* (Mehnaz et al. 2007b), and *A. rugosum* (Young et al. 2008). Subsequently, new species have been reported: *A. picis* (Lin et al. 2009), *A. palatum* (Zhou et al. 2009), *A. thiophilum* (Lavrinenko et al. 2010), *A. formosense* (Lin et al. 2012), *A. humicireducens* (Zhou et al. 2013), *A. fermentarium* (Lin et al. 2013), *A. himalayense* (Tyagi and Singh 2014), *A. soli* (Lin et al. 2015), and *A. agricola* (Lin et al. 2016). Three new species were identified in 2019: *A. ramasamyi* (Anandham et al. 2019), *A. griseum* (Zhang et al. 2019), and *A. palustre* (Tikhonova et al. 2019). *A. amazonense* (Falk et al. 1985) and *A. irakense* (Khammas et al. 1989) were relocated to separate genera, *Nitrospirillum* and *Niveispirillum*, respectively (Lin et al. 2014).

The genus *Azospirillum* is distributed worldwide and different strains and species have been isolated from several countries, including Argentina, Brasil, China, Taiwan, Korea, Russia, Pakistan, and Irak, among others (Table 1). This genus is considered to be versatile because it has been isolated from different environments (Reis et al. 2015). Although less common, *Azospirillum* spp. have also been found under extreme conditions, such as saline soil, oil-contaminated soil, fermented products, fermentation tanks,

Fig. 1 *Azospirillum* phylogenetic analysis using rpoD sequences obtained from NCBI database. Reference strains of each specie were used. Other members of the *Rhodospirillaceae* family and *B. japonicum* E109 were used as outgroups. Analysis was made by Maximum Likelihood method, Tamura-Nei substitution model, and a Bootstrap testing of 1000 iterations (Jones et al. 1992). Bootstrap values ≥ 50 are shown in the corresponding nodes. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)
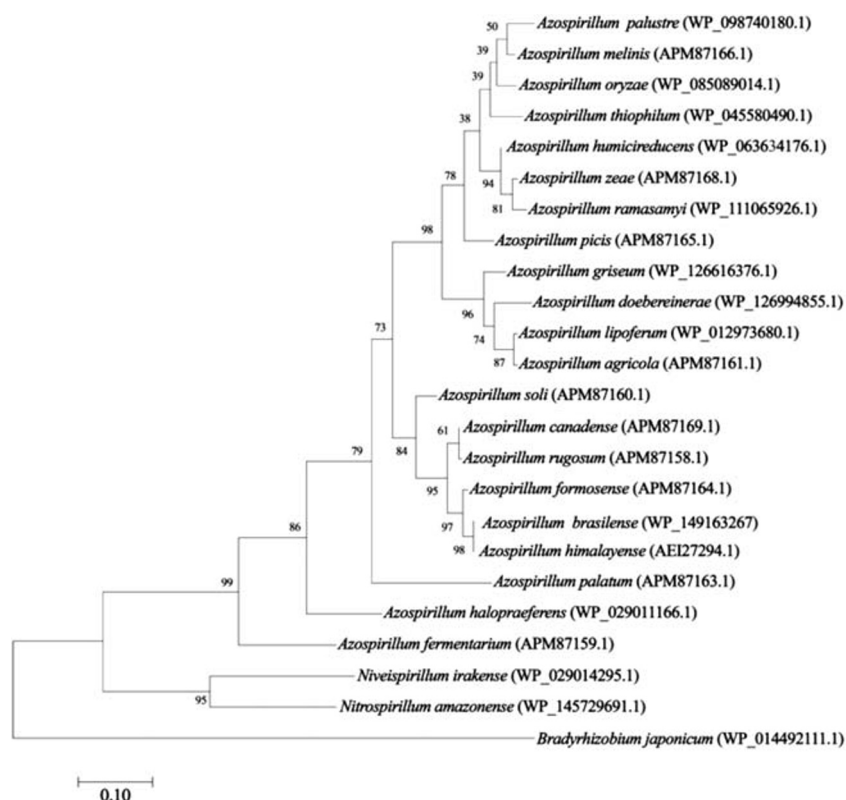
sulfide springs, and microbial fuel cells (Reis et al. 2015; Anandham et al. 2019; Tikhonova et al. 2019). Moreover, one member of the *Azospirillum* spp. was isolated from the Himalayan valley and others were found in Baiyang Lake (Reis et al. 2015; Zhang et al. 2019). Nearly 100 years have elapsed since the genus *Azospirillum* was first identified, and the taxonomy information about this type of bacteria continues to grow. Advances in molecular biology allow a better clasification of organisms, and the C. C. Young Group from National Chung Hsing University (Taichung, Taiwan) has made the greatest contribution to this research area (Young et al. 2008; Lin et al. 2009, 2012, 2013, 2014, 2015, 2016). Not only have they discovered a significant number of new species and redistributed other species, they have also made significant advances by developing methodologies for the identification of *Azospirillum* strains using polymerase chain reaction (PCR) (Lin et al. 2011).

Functional analysis of plant growth promotion

Azospirillum spp. have been associated to several mechanisms to promote plant growth and a wide range of studies have detailed the beneficial effects of inoculation with these rhizobacteria. The improvement of plant growth by *Azospirillum* spp. has been mostly attributed to their capacity to fix atmospheric N and to produce phytohormones; it is less attributed to the bio-disposition of nutrients, expression of enzymes, synthesis of compounds related to plant stress mitigation, and competition against phytopathogens, among other mechanisms. However, taken individually, none of these mechanisms has been found to be fully responsible for the changes observed in inoculated plants (Bashan and de-Bashan 2010). *Azospirillum* spp. modes of action were initially explained by the Additive Hypothesis where the effects of small mechanisms operating either at the same time or consecutively create a larger final effect on plants (Bashan and Levanony 1990). In 2010, this hypothesis was replaced by the Multiple Mechanisms Hypothesis, which posits that no single mechanism is involved in the promotion of plant growth; rather, in each case of inoculation a combination of a few or many mechanisms is responsible for the beneficial effect (Bashan and de-Bashan 2010). In the following sections, evidence related to the mechanisms most often studied is summarized to explain the plant growth resulting from inoculation by *Azospirillum* spp.

N fixation

N fixation was the first mechanism to be identified that demonstrated the way in which *Azospirillum* positively affects plant growth (Döbereiner et al. 1976; Okon et al. 1983); therefore, many studies have investigated it and a substantial about of information about it has been published (Kennedy et al. 2004; Baldani and Baldani 2005; Bashan and de-Bashan

Table 1 Country of origin and source of isolation of *Azospirillum* species

Azospirillum	Origin[a]	Isolated from[b]	Reference
A. lipoferum	Brazil	Wheat roots	Beijerinck (1925)
A. brasilense	Brazil	*Digitaria decumbens* roots	Tarrand et al. (1978)
A. halopraeferens	Pakistan	Roots of Kallar grass grown on salt-affected soils	Reinhold et al. (1987)
A. largimobile	Australia	Fresh lake water	Ben Dekhil et al. (1997)
A. doebereinerae	Germany	*Miscanthus sinensis* cv. "*giganteus*," washed roots	Eckert et al. (2001)
A. oryzae	Japan	Rhizosphere of *Oryza sativa*	Xie and Yokota (2005)
A. melinis	China	Tropical molasses grass (*Melinis minutiflora*)	Peng et al. (2006)
A. canadense	Canada	Corn rhizosphere	Mehnaz et al. (2007a)
A. zeae	Canada	Corn rhizosphere	Mehnaz et al. (2007b)
A. rugosum	Taiwan	Oil-contaminated soil near the oil refinery	Young et al. (2008)
A. picis	Taiwan	Discarded road tar	Lin et al. (2009)
A. palatum	China	Forest soil	Zhou et al. (2009)
A. thiophilum	Russia	Bacterial mat of a sulfide mineral spring	Lavrinenko et al. (2010)
A. formosense	Taiwan	Paddy soil	Lin et al. (2012)
A. humicireducens	China	Microbial fuel cell	Zhou et al. (2013)
A. fermentarium	Taiwan	Industrial fermentative tank	Lin et al. (2013)
A. himalayense	India	Himalayan Valley soil[a]	Tyagi and Singh (2014)
A. soli	Taiwan	Agriculture soil	Lin et al. (2015)
A. agricola	Taiwan	Cultivated soil	Lin et al. (2016)
A. ramasamyi	Korea	Fermented bovine products [a]	Anandham et al. (2019)
A. griseum	China	Water at Baiyang Lake[a]	Zhang et al. (2019)
A. palustre	Russia	Sphagnum-dominated raised peatland[a]	Tikhonova et al. (2019)

[a] Information obtained from original report

[b] Information obtained from Global Catalogue of Microorganisms [http://gcm.wfcc.info/]

2010). The emphasis on this mechanism is due to the significant increase in the total amount of N in shoots and grains observed after *Azospirillum* inoculation in wheat, sorghum, and panicum, among other cereal and grass species (Kapulnik et al. 1981). However, the evidence collected during subsequent decades is controversial. Numerous greenhouse and field experiments demonstrated the contribution of fixed N by bacteria on crops by a reduction in the doses of N fertilizers used under field conditions (for a review, see Bashan and de-Bashan et al. 2010). Incorporation of atmospheric N into the host plant by inoculating with *Azospirillum* was initially evaluated using the acetylene reduction assay (ARA) and later using isotopic $^{15}N_2$ and ^{15}N-dilution techniques. ARA has contributed to the understanding of *Azospirillum*-gramineae associations, but in its use for definitive quantification of biological nitrogen fixation (BNF), it has many disadvantages, mainly due to the fact that it is a short-term assay of enzyme activity and such activity is drastically reduced when plants are disturbed. While isotope techniques (^{15}N) have been more popular, they are not easily adaptable under field conditions due to the uniform labeling of soils and the selection of suitable non-N_2-fixing control plants (Boddey and Knowles 1987). Solid evidence that N fixation contributes to the N balance of plants has been mainly based on the observation of an increase in the nitrogenase activity within inoculated roots with sufficient magnitude to increase the total N yield of the inoculated plants (Bashan and Holguin 1997; Kennedy et al. 1997). However, many studies have shown that the contribution of N fixation by *Azospirillum* to plants is minimal (an increase of 5–18% in the total N of inoculated plants); consequently, plant growth promotion was induced by other mechanisms. These findings almost resulted in the abandonment of the N fixation aspects of *Azospirillum*, except in pure genetic and molecular studies.

In the last years, several studies have focused on N metabolism within bacterial cells, and many details of molecular mechanisms have been studied in *Azospirillum*, which is considered a bacterial model for investigating non-symbiotic N fixation. In this sense, during the genomic era, the Sp245 strain of *A. brasilense* has been used as a model to understand the N metabolism pathways since its genome had been completely sequenced and this strain has been physiologically

characterized. The *nif* gene cluster was identified in two specific positions of the genome; in one case, it was probably codified for an alternative iron or vanadium nitrogenase. The ammonia assimilation in *Azospirillum* occurs via two pathways, one involving glutamate dehydrogenase (*gdhA*) under a high NH_4^+ concentration and the other involving glutamine synthetase (*glnA*) and glutamate synthase (*gltBgltD*) under limiting NH_4^+. The genes involved in both pathways are present in all the *Azospirillum* species that have been analyzed to date (de Souza and Pedrosa 2015).

Two innovative approaches regarding N fixation research have been developed in the last decades: (a) obtaining the spontaneous ammonium excreting mutants of *A. brasilense* (see Bashan and de-Bashan 2010) and (b) induction of a specialized sites for N fixation on the roots of legume plants known as paranodules. Externally, paranodules resemble a legume nodule and they can be induced in grasses by exogenous application of auxins (Tchan et al. 1991). Under the premise that *Azospirillum* does not secrete significant amounts of ammonium obtained from BNF on plant tissues, *A. brasilense* cells were inoculated into rice and evaluated for their capacity to colonize root paranodules previously induced by treating the roots with auxins. The bacteria colonization of paranodules in the treated plants was correlated with significant increases in plant biomass in comparison to the non-inoculated plants (Christiansen-Weniger and van Veen 1991). Additionally, the nitrogenase activity was significantly higher in the *Azospirillum*-inoculated paranodules of the roots of the rice plants in comparison to the control plants (Christiansen-Weniger 1997). According to Christiansen-Weniger (1997), this was likely because nitrogenase was less sensitive to the oxygen tension in the paranodules than in the rest of the root. Similar increases in nitrogenase activity were reported by Tchan et al. (1991), Zeman et al. (1992), and Yu et al. (1993) in wheat roots containing paranodules colonized by *Azospirillum*. In addition to rice and wheat (Katupitiya et al. 1995), paranodules were also obtained on the roots of maize seedlings (Saikia et al. 2004, 2007).

Machado et al. (1991) characterized a spontaneous mutant, HM053, derived from *A. brasilense* FP2 (Sp7 ATCC 29145, SmR, NalR), which was resistant to ethylenediamine (EDAR). This mutant was able to excrete ammonium and fix N in the presence of high concentrations of NH_4^+; hence, it is an interesting candidate for use as a biofertilizer to supply N to gramineaceous plants. Machado et al. (1991) suggested that the mutant HM053's ability to excrete ammonium is related to low glutamine synthetase activity, resulting in a deficiency of NH_4^+ assimilation; this explains the excretion of excess ammonium produced during N fixation. Pankievicz et al. (2015) showed that *Setaria viridis* inoculated with the HM053 strain incorporates a significant N level via BNF, and this level may be enough to provide the plant's daily N demand. Moreover, HM053 was able to promote wheat and barley growth (Santos et al. 2017) and *nif* expression *in planta* during wheat root colonization, which was shown to be about 300-fold higher growth than with the wild type strain. The same strain outperformed the parental strain in field experiments, leading to a maize yield increase of up to 28% (Pedrosa et al. 2019). Similar ammonium excreting mutants of *A. brasilense* have been reported to enhance plant growth (Van Dommelen et al. 2009). Moreover, some of the mutants have been evaluated using the paranodules colonization system (Christiansen-Weniger and Van Veen 1991).

Phytohormone production

Due to evidence reported in studies published over the past 90 years, it is known that the *Azospirillum* genus is associated with the production of several phytohormones. Simultaneously, Reynders and Vlassak (1979) and Tien et al. (1979) reported the capacity of *Azospirillum* to produce indole-3-acetic acid (IAA) under in vitro and in vivo conditions, respectively. Additional investigations revealed the capacity to produce cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989), ethylene (Strzelczyk et al. 1994), and other plant growth regulators, such as abscisic acid (ABA) (Kolb and Martin 1985), nitric oxide (Creus et al. 2005), and polyamines, such as spermidine, spermine, and the diamine cadaverine (Thuler et al. 2003; Cassán et al. 2009). The plant growth regulators and phytohormones produced by *Azospirillum* have been summarized and ranked according their effects on plants in previous reports (see Table 1, Cassán and Diaz-Zorita 2016). In a culture medium, the concentrations of the most important groups of plant hormones produced by this bacterium, such as auxins, cytokinins, and gibberellins, increase with bacterial growth because these compounds are continuously accumulated in the medium according to a batch fermentation model (Ona et al. 2003; Cassán et al. 2009; Molina et al. 2018). Based on the active principles of inoculants, both the bacteria (cell number) and the metabolites (mainly phytohormones) are biosinthetized, released, and accumulated in the culture medium. Then, inoculants with a different metabolite profile should have a different capacity to promote the growth of inoculated plants, even if the number of cells is equal. In the case of seed inoculation, the use of inoculants containing *Azospirillum* and phytohormones in the culture medium will produce a "seed priming" effect. In this sense, Okon (1982) reported that, after seed inoculation, the number of viable *Azospirillum* cells decreases very rapidly. Then, the short-term benefits of seeds inoculation should not be strictly related to the presence of the bacterial cells in the inoculant; instead, they are, at least partly, related to the presence and concentration of several phytohormones and plant growth regulators. This has been defined as the hormonal effect of inoculation (Cassán et al. 2014).

Auxin metabolism

Auxins are a group of plant gowth regulators that are involved in numerous aspects of plant growth and development (Teale et al. 2006). IAA is the predominant plant gowth regulator found in plants. It is acknowledged that 80% of rhizobacteria, including *Azospirillum*, are able to produce IAA and the synthesis pathways are similar to those found in plants (Spaepen et al. 2007). At present, members of the genus *Azospirillum* have provided an excellent experimental model for investigating the physiological role of auxins in PGPB-plant interactions, and several naturally occurring auxin-like molecules have been described as products of bacterial metabolism. The genome sequence of *A. brasilense* Az39 revealed the existence of all the genes involved in the indole-3 pyruvate (IPyA) pathway: *hisC1* coding for an aromatic amino transferase, *ipdC* coding for an indole-3-pyruvate decarboxylase, which is considered to be the key enzyme of this pathway (Broek et al. 1999), and an aldehyde dehydrogenase gene (see Table 1, Cassán et al. 2014). For the Sp245 and CBG497 strains, only the *hisC1* and *ipdC* genes were identified; no evidence of aldehyde dehydrogenase was observed in these genomes. Considering that the genome sequences of *A. brasilense* Sp245 and Az39 are very similar, it is not surprising that all the genes encoding for the IPyA pathway are very similar in both strains. No evidence has been found for the existence of *ipdC* or aldehyde dehydrogenase in the genome sequence of *A. lipoferum* 4B. Only a putative aromatic amino transferase sequence with homology to AAT1 from *A. brasilense* Sp7 has been identified (Wisniewski-Dyé et al. 2011). *Azospirillum* sp. B510 genome sequence analysis revealed a putative aromatic amino transferase with homology to AAT1 from *A. brasilense* Sp7 (Wisniewski-Dyé et al. 2011). Kaneko et al. (2010) proposed that two candidate genes are involved in the indole acetamide (IAM) pathway, but we question their role in IAA biosynthesis due to the low similarity (especially for the putative *iaaM* gene) between them and the known *iaaM* and *iaaH* genes. Finally, gene encoding nitrilases have also been identified in the *Azospirillum* sp. B510 genome (Wisniewski-Dyé et al. 2012).

In addition to IAA, other molecules, such as indole-butyric acid (IBA) (Martínez-Morales et al. 2003), phenyl acetic acid (PAA) (Somers et al. 2005), indole-3-lactic acid (ILA), indole-3-ethanol and indole-3-methanol (Crozier et al. 1988), indole-3-acetamide (IAM) (Hartmann et al. 1983), indole-3-acetaldehyde (Costacurta et al. 1994), tryptamine (TAM), and anthranilate (Hartmann et al. 1983), have been identified in an *Azospirillum* spp. culture medium. At least four different IAA biosynthesis pathways have been proposed in *Azospirillum* spp.: the tryptophan-dependent pathways IPyA, IAM, and TAM, and a putative tryptophan-independent pathway (Prinsen et al. 1993). Despite this diversity, IPyA is considered to be the most important pathway for IAA

biosynthesis in this genus. The question about why some bacteria are able to produce phytohormones remains unanswered; however, in the case of auxins, a co-evolutionary mechanism could be hypothetized. Plants release several compounds, such as amino acids and organic acids, into the rhizosphere through root exudates. In the case of amino acids, and particularly for L-trp, this precursor could be used by auxin-producing bacteria to biosynthetize IAA. This molecule increases the amount of this hormone in the rhizosphere, which induces changes in the plant, increasing its root morphology and growth. Thus, a higher amount of root exudate in the rhizoshere will increase the availability of nutrients for the bacteria living in the rhizosphere, enhancing their population. Higher levels of IAA in the rhizosphere will induce a higher *ipdC* gene expression by *Azospirillum,* thereby enhancing the IAA concentration in the rhizosphere and stimulating root growth. In other words, some bacteria are able to increase their own population within the rhizosphere by producing IAA using the L-trp produced by plants as a co-evolutionary mechanism. How do plants regulate the IAA levels in the rhizosphere? This should be the most important question for this model; the answer is related to the ability of the plant to regulate the release of L-trp and other amino acids in the rhizosphere by the exudates. In this sense, the full IAA metabolism of *A. brasilense* has been recently revealed (Rivera et al. 2018). Rivera et al. (2018) found that some amino acids, such as L-met, L-val, L-cys, and L-ser, inhibit bacterial growth and reduce IAA biosynthesis, while the expression of *ipd*C and IAA biosynthesis, but not bacterial growth, are affected by L-leu, L-phe, L-ala, L-ile, and L-pro. Furthermore, L-arg, L-glu, L-his, L-lyis, L-asp, and L-thr do not affect bacterial growth, IAA biosynthesis, or *ipd*C gene expression; this fact should have some impact on the rhizosphere during plant-microbe interactions (see Fig. 2, Rivera et al. 2018). It was also confirmed that the *A. brasilense* strains Sp245, Az39, and Cd can only produce IAA in the presence of L-trp (biosynthesis); these strains are unable to degrade auxins (catabolism), conjugate IAA with sugars and/or L-amino acids (conjugation), or hydrolize conjugates to release free IAA (hydrolysis). IAA biosynthesis was also evaluated under abiotic and biotic stress conditions; it was found to increase with daylight or in the presence of PEG_{6000}, ABA, salicylic acid (SA), chitosan, and a filtered supernatant of *Fusarium oxysporum*. In contrast, exposure to 45 °C or treatment with H_2O_2, NaCl, Na_2SO_4, 1-aminocyclopropane 1-carboxylic acid, methyl jasmonate, and a filtered supernatant of *Pseudomonas savastanoi* decreases IAA biosynthesis (Molina et al. 2018).

Root growth phytostimulation

Roots are the plant organs that are preferentially modified by *Azospirillum* (see Bashan and de-Bashan 2010). In the 1990s, enhanced water and mineral uptake by roots was frequently
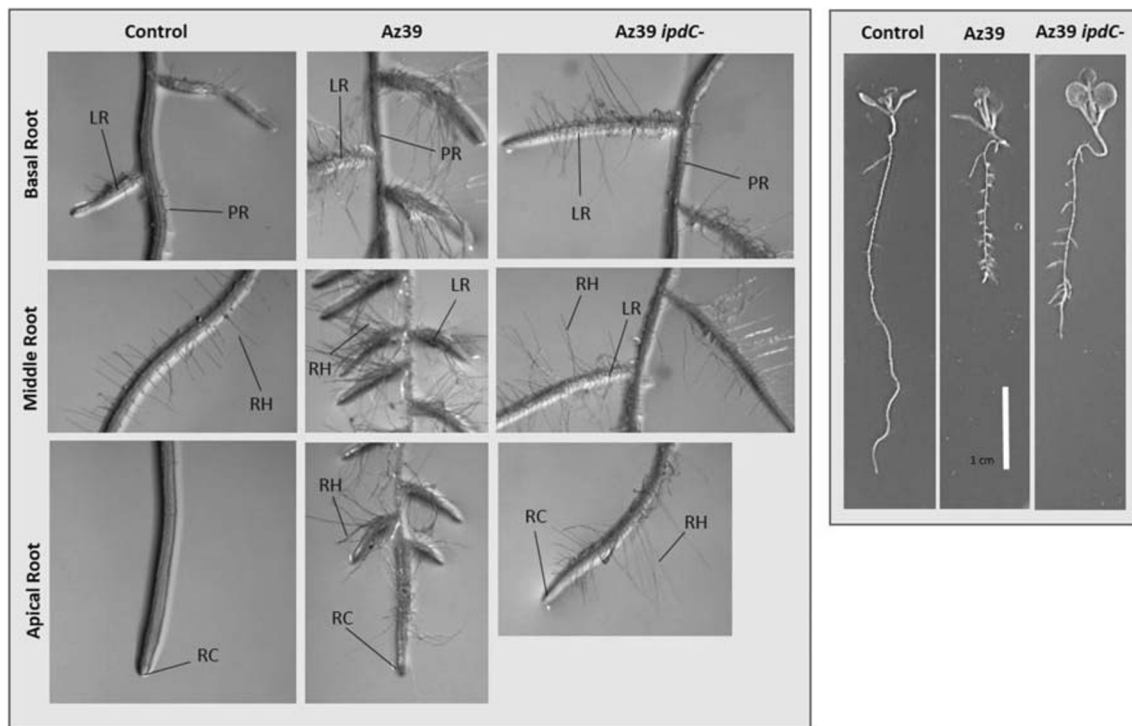
Fig. 2 Changes in the architecture of the 12-day-old seedling root system of *Arabidopsis*. Seedlings were grown for 7 days on MS medium, then were inoculated for 5 days with *A. brasilense* Az39 or Az39 *ipdC*-mutant deficient in IAA production. Images show different zones of the root: basal, middle, and apical root. Primary root (PR), lateral roots (LR), hair root (HR), root cap (RC). Photography credits: Mora V

used to explain the beneficial effects of *Azospirillum* inoculation (see Bashan and Levanony 1990; Bashan and Holguin 1997). Increased mineral uptake and water absorption have been related to changes in root growth, architecture, and volume instead of any specific metabolic enhancement process (Murty and Ladha 1988). This fact has been strictly related to the bacterial capacity to produce phytohormones. However, the descriptive data presented thus far have not shown whether these improvements are the cause or the result of other mechanisms (Bashan and de-Bashan 2010). The first evidence of phytostimulation by *A. brasilense* was observed in pearl millet and sorghum seedlings, and it was similar to that observed by exogenous application of IAA (Tien et al. 1979). Later, it was shown that inoculation of *Beta vulgaris* increased the number of lateral roots in the inoculated plants in comparison to the uninoculated plants. This effect was correlated with the high levels of IAA produced by bacteria in a pure liquid culture medium and it was mimicked by the exogenous addition of similar concentrations of the phyhormone (Kolb and Martin 1985). The current model of root growth phytostimulation by *Azospirillum* includes a number of morphological changes that can be summarized as follows: (1) decrease in the elongation of the main root (Dobbelaere et al. 1999; Spaepen et al. 2007); (2) increase in the lateral and adventitious roots (Fallik et al. 1994; Molina-Favero et al. 2008); (3) increase in the number of root hairs (Okon and Kapulnik 1986; Hadas and Okon 1987); (4) branching of the root hairs (Jain and Patriquin

1985); and (5) significant increase in the root surface and volume, probably related to the improvement in water and nutrient acquisition (Spaepen et al. 2014). Modifications in the root architecture mediated by *Azospirillum* have shown that there is an IAA-dependent response to inoculation. However, recent evidence suggests that other molecules or cell components would be able to induce an IAA-like response to inoculation (IAA independent response). In this sense, and as shown in Fig. 2, *A. brasilense* Az39 is able to induce the typical root phytostimulation effect in *Arabidopsis thaliana* under in vitro conditions due to IAA production. However, inoculation with *A. brasilense* Az39 *ipdC*- (a non-IAA producer mutant) still induced a stimulatory effect similar to the one induced by IAA on *Arabidopsis* roots (V. Mora, personal communication). This result increases the complexity of the current model and forces us to work with alternative hypotheses to establish the definitive model, which, in spite of many published papers and a significant amount of effort, has not yet been finalized.

The stimulation of plant root growth by *Azospirillum* induces an increase in the water absorption and nutrient adquisition rates (including N), which clearly improves the assimilation of N in the biomass and, more generally, plant growth. This capacity would be mediated by the bacterial colonization of the roots and/or their ability to produce different phytohormones, mostly during early stages of plant development. Consequently, the increase in the root biomass would

increase the supply of root exudates into the rhizosphere, which would increase the bacterial population associated with the roots and improve their ability to colonize this organ and the rest of the plant. Once the plant is colonized with a high number of bacteria, e.g., $> 10^5$ cfu g^{-1} according to Okon (1982), these cells would be able to provide the plant with significant amounts of NH_4^+ via BNF. During the advanced stages of plant development, this would have a greater impact on the N economy for the plant. In summary, the Eficient Nutrients Acquisition Hypothesis by inoculated plants would depend on both biological N fixation and phytohormone bio-synthesis by the effectively colonized bacteria.

The impact of *Azospirillum* inoculation on agriculture

Worldwide, the market of inoculants containing *Azospirillum* spp. is flourishing in South America. Here, the inoculation was initially focused on cereal production, but nowadays, and mostly in Brazil, inoculation is additionally focused on legumes, such as soybeans, combining it with rhizobia inoculants (co-inoculation). The changes in plant growth observed by *Azospirillum* inoculation and the bacterial capacity to improve the negative effects of abiotic stress on crops has attracted the attention of researchers interested in developing field applied studies (Okon and Labandera-Gonzalez 1994). Okon et al. (2015) suggested that because the diverse modes of action of *Azospirillum* mostly stimulate plant root growth, inoculation with this microbe could contribute to the increase and stabilization of crop production. However, evaluations of the efficacy of *Azospirillum* under current crop management practices and at regular environmental conditions are scarce and have been conducted on different crops and in different regions.

Based on 347 trials obtained from 12 countries, including Brazil, Argentina, and several countries in Southeast Asia, and 47 published articles, mainly focusing on maize and other cereals, the impact of *Azospirillum* inoculation has been analyzed (Díaz-Zorita et al. 2015). From this analysis, the greatest contribution of *Azospirillum* inoculation to grain yield was observed in winter cereals followed by summer cereals and other crops (Fig. 3). The reviewed studies on inoculation with *Azospirillum* showed variable results and a multiplicity of interactions related not only to crop management practices but also to environmental conditions. Most field assays have been performed in single geographical locations during one or two consecutive seasons. Thus, of the ability to analyze the performance of bacterial inoculation under random temporal and spatial conditions is limited.

Based on a total of 316 field experiments performed in the pampas region (Argentina), the relative yield increase in maize due to inoculation with *A. brasilense* showed positive results, ranging between 66 and 80% of positive responses in comparison with untreated control (Díaz-Zorita et al. 2015).
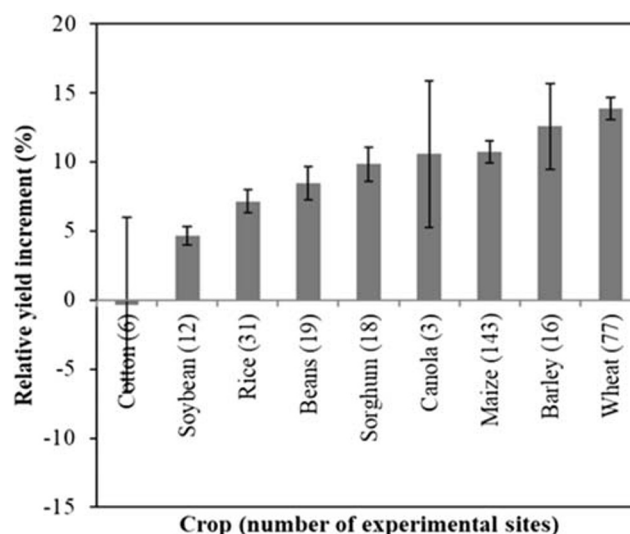

Fig. 3 Mean contribution of the inoculation with *Azospirillum* sp. on crop grain production reviewed from 47 worldwide published field trials under regular production practices. (Adapted from Díaz-Zorita et al. 2015)

Among the growing seasons, the relative contribution of *Azospirillum* to maize yield increases under conditions with less rainfall during the early growth stages (Supplementary Fig. 2). In wheat, the early season effects of inoculation with *Azospirillum* decrease if favorable growing conditions occur during the seed filling stage (Kazi et al. 2016). Okon and Labandera-Gonzalez (1994) and Díaz-Zorita and Fernández-Canigia (2009) found that the grain production responses to inoculation with *Azospirillum* spp. in wheat and other crops were successful in 70–80% of the cases, regardless of the production conditions. In part, this behavior is caused by the complexity of the impact of *Azospirillum* on plants interacting with the impact of several abiotic stressful conditions. Kazi et al. (2016) reported that azospirilla inoculation increased the bacteria population in the rhizosphere during the early stages of growth. Most of the benefits have been observed during the early growth stages of plants with greater and more consistent responses seen in the root and shoot dry matter production and a minimal contribution to the grain yield components during the seed filling period (Díaz-Zorita and Fernández-Canigia 2009; Veresoglou and Menexes 2010). Based on the analysis of 480 greenhouse and field experiments, Veresoglou and Menexes (2010) validated the benefits of wheat inoculation with *Azospirillum*, but they considered variable responses based on differences in the management practices, such as N fertilization, wheat genotype, or *Azospirillum* strain, that were used to inoculate the crops. Although N fertilization benefits wheat production (Saubidet et al. 2002), the relative contribution of *Azospirillum* decreased as the dose of the N fertilizer increased. Under high N availability, the bacterial response was not observed (Ozturk et al. 2003).

The combined inoculation of legumes with rhizobia and azospirilla, defined as co-inoculation, could improve plant

performance due to the complementary nature of the mechanisms of both bacteria. In soybean crops, co-inoculation resulted in both early initiation of nodule ontogenesis and an increase in the number of nodules, thereby increasing the concentration of N in the shoots and improving the plant growth, particularly under drought conditions (Chibeba et al. 2015; Cerezini et al. 2016). Although the contribution of co-inoculation to the productivity of diverse legume crops is promising, the available information about its use under large production conditions is limited. The results from 21 field trials with alfalfa performed in the pampas region (Argentina) showed that the seed treatment combining *Ensifer meliloti* and *A. brasilense* resulted in a response that was nearly two times better than the response obtained from a single inoculation with rhizobia (Díaz-Zorita 2012). Hungria et al. (2013) also reported an increase in grain yields in soybeans and common beans (*Phaseolus vulgaris*) when combining rhizobia seed inoculation with in-furrow application of *A. brasilense* at four sites in Brazil. The single inoculation of *Bradyrhizobium* in soybeans resulted in mean grain yield increases of 8.4% in comparison to the uninoculated control, whereas co-inoculation with *Bradyrhizobium* and *A. brasilense* resulted in an increase of 16.1%. For common beans, the single inoculation with *R. tropici* increased the yield by 8.3%, and co-inoculation of *R. tropici* and *A. brasilense* improved the yield by 19.6% (Hungria et al. 2013). The mean soybean yields from 37 field trials under regular management when *Bradyrhizobium* was co-inoculated with *A. brasilense* were 227 kg per ha greater than when the soybeans were inoculated with *Bradyrhizobium* alone and 335 kg per ha greater than the uninoculated control (Nogueira et al. 2018). The mean effects of co-inoculation on soybean nodulation were evaluated under 22 regular crop production conditions; the results showed differences in the effects between tropical-subtropical and temperate environments. On average, the percentage of soybean nodulation increased by around 5% at the Brazilian sites (Hungria et al. 2015; Fipke et al. 2016; Galindo et al. 2018) and around 12% at the Argentinian sites (Benintende et al. 2010; Ferraris and Couretot 2011, 2013; Morla et al. 2019). However, opposite results were found for grain yields. This limited dataset was insufficient to show a consistent and direct relationship between the use of co-inoculation and changes in nodulation and grain yield. Currently, the use of azospirilla inoculants in Brazil is increasing due to co-inoculation. In the state of Parana (Brazil), the use of co-inoculation between 2016 and 2018 increased by almost 30% (Prando et al. 2016, 2018).

Alternative methods of inoculation that are as effective as the standard seed inoculation technique may represent an important strategy to avoid the incompatibility that can occur between the inoculants and pesticides used during seed treatment. However, these technologies need to be thoroughly evaluated before promoting their extensive use.

Fukami et al. (2016) described the beneficial effects of spraying leaves with *Azospirillum* at the beginning of the vegetative phase. Morais et al. (2016) observed that seed furrow inoculation also increased the maize grain yield under current Brazilian production practices. The benefits of foliar inoculation with *A. brasilense* were evaluated and explained using an auxin signaling model (Puente et al. 2017). The results confirmed soybean growth promotion after seed treatment with *B. japonicum* and foliar co-inoculation with the IAA producer *A. brasilense* Az39. Both auxin production and *A. brasilense* colonization were responsible, via plant signaling, for the positive effects on plant growth and the symbiosis establishment (see Fig. 5 in Puente et al. 2017). An improvement in the nutritional quality of soybean grain due to foliar inoculation with *A. brasilense* Az39 under greenhouse and field conditions was reported 1 year later (Puente et al. 2018). These findings provide new insights into soybean agricultural technology.

Inoculants formulated with *Azospirillum* in South America

Currently, the use of azospirilla inoculants for crop production is a consolidated practice in South America (i.e., Brazil, Argentina, Uruguay, and Paraguay), where the extensive agriculture is frequent (Cassán and Diaz-Zorita 2016). In Argentina, Uruguay, and Brazil, there many biological products contain *Azospirillum* as an active principle. However, the first inoculant in the region was registered 23 years ago (1996) in Argentina with the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) using the name of Nodumax-L by Laboratorios Lopez SRL (Jesús Maria, Córdoba). It was formulated with *A. brasilense* Az39, one decade after the isolation and selection of this strain by Enrique Rodriguez Caceres from the Instituto Nacional de Tecnología Agropecuaria (INTA). The inoculant was initially recommended for the treatment of wheat and maize seeds, but it is now recommended for several crops. In Brazil, paradoxically, the first inoculant was registered by Stoller do Brasil SA (Campinas, São Paulo), 14 years after the first one was registered in Argentina. It was named Masterfix L gramineas, and it was formulated with a combination of the *A. brasilense* Abv5 and Abv6 strains. This product was initially recommended for the treatment of maize and rice seeds, but in the last several years, it has also been recommended in combination with *B. japonicum* for soybean co-inoculation. Finally, in Uruguay, the first inoculant product was registered in 2015 by Lage y CIA SA (Montevideo, Montevideo) under the name Graminosoil. It contains a combination of *A. brasilense* Az39 and CFN535. The product was initially recommended for the treatment of maize and sorghum. Currently in South America, there are 106 products (inoculants) produced by 74 companies representing 79 commercial brands. Most of them are

produced in Argentina (90 products); 14 products are produced in Brazil and two products are produced in Uruguay. All the available products for commercialization in the Argentinian market are produced in Argentina, but in Brazil and Uruguay, the inoculants are either locally produced or imported from Argentina. All of the products (100%) are formulated with *A. brasilense*, and the Az39 strain is the active principle in 75% of these inoculants (79 products). In 13 products, Az39 is combined with other *A. brasilense* strains (one product containing CFN535), *Pseudomonas fluorescens* (one product), or *B. japonicum* (11 products). In the last case, this is due to the increase in the number of products registered as a premium technology (co-inoculation) for soybeans. The combination of the *A. brasilense* Abv5 and Abv6 strains is used to formulate 18 products and the combination of the *A. brasilense* Az78 and Az70 strains is used to formulate three products. The rest of the azospirilla inoculants are formulated with single strains (Abv5, AzM3, AzT5, 1003, Tuc 27/85, Tuc 10/1, and 11005). Liquid carriers are most often used to formulate these biological products (94%); 6% of the products are formulated on solid carriers, such as peat or bentonite. In 2014, 82% of the formulations in the market were liquid carriers and 18% were solid carriers. This clearly shows the formulation preferences of the companies that are manufacturing these products. The most frequent shelf life of the registered products is approximately 6 months from production with a minimal concentration of 1×10^7 cfu ml^{-1} in Argentina or 1×10^8 cfu ml^{-1} in Brazil and Uruguay. Although the use of these biological products has been recommended for 16 types of crops, the registration is mainly for wheat (67), maize (65), sunflowers (16), and soybeans (12). The other plant species recommend for the treatment with *A. brasilense* are sorghum (*Sorghum bicolor*) (9), grasses, and winter cereals for grazing (4), rice (5), barley (3), cotton (*Gossypium hirsutum*) (3), oats (*Avena sativa*) (2), sugar cane (*Saccharum officinarum*) (1), tobacco (*Nicotiana tabacum*) (1), and lettuce (*Lactuca sativa*) (1). In Brazil, most of the commercialized products are allocated in the maize and soybean grain production market. Based on 2018 data, approximately 7.0 million doses of azospirilla inoculants were commercialized, covering almost 5.0 million ha in South America. In 2014, 3 million ha of plants were inoculated with *A. brasilense* corresponding to 3.5 million doses of these products. This shows a clear trend in the region of increased use of products formulated with these bacteria.

Extending the use of *Azospirillum* beyond the agricultural industry

In addition to its proven usefulness in agriculture, *Azospirillum* possesses the potential to solve environmental problems, such as preventing soil erosion by improving the growth of plants on barren and degraded lands that have lost their capacity to support regeneration, and participating in phytoremediation strategies to decontaminate soils, all leading to healthier environments (de-Bashan et al. 2012). Although these uses are not yet widespread, some examples are presented in this section.

Puente and Bashan (1993) demonstrated that *A. brasilense* inoculated on the cardon cactus, *Pachycereus pringlei*, the world's largest cactus that stabilizes topsoil in its usual scrub habitat in the Sonoran Desert (Mexico), improves the growth characteristics of the plant. In a field trial, three species of cacti inoculated with *A. brasilense* had a significantly higher survival rate in comparison to the non-inoculated controls. The most important outcome from this trial was the significant reduction in soil erosion and the reclamation of topsoil (Bashan et al. 1999). Growth chamber experiments have demonstrated that *A. brasilense* enhances enzymes in the phosphogluconate pathway and facilitates the growth of mesquite seedlings (*Prosopis articulata*) that are cultivated in poor soils (Leyva and Bashan 2008). In a greenhouse environment, the effect of *A. brasilense* combined with *Bacillus pumilus*, unidentified arbuscular mycorrhizal (AM) fungi (mainly *Glomus* spp.) and compost, were measured on the growth of leguminous trees, such as mesquite, yellow palo verde (*Parkinsonia microphylla*), and blue palo verde (*Parkinsonia florida*), used in desert reforestation and urban gardening in arid northwestern Mexico and the southwestern region of the US (de-Bashan et al. 2012). The mesquite and yellow palo verde had different, positive responses to several parameters, while blue palo verde did not respond (de-Bashan et al. 2012). Later, seven field trials were undertaken with cardon cacti and the same species of leguminous trees (Bashan et al. 2012). The trial showed that, a decade later, a combination of a legume tree with a cardon cactus, while detrimental to the legume, significantly increased the chances of the cactus surviving and growing in degraded soil. (Moreno et al. 2017). Recently, inoculation of *Brachiaria* spp. with *A. brasilense* demonstrated the potential for successful reclamation of degraded pastures in Brazil (Hungria et al. 2016).

In terms of phytoremediation, *A. brasilense* improved the growth of the shrub quailbush, *Atriplex lentiformis*, and affected the rhizosphere microbial community in acidic, metalliferous tailings in Arizona (de-Bashan et al. 2010). Tugarova et al. (2013) proved the capacity of *A. brasilense* strains to reduce selenium (IV) to selenium (0), indicating the possibility of applying *Azospirillum* as a microsymbiont for the phytoremediation of selenium-contaminated soils; moreover, the bioremediation potential of *Panicum virgatum* (switchgrass), along with AM fungi and *Azospirillum*, was tested against lead and cadmium in pot trials (Arora et al. 2016).

In 2000, the Yoav Bashan research group began an interesting study to investigate extending the use of *Azospirillum* from agricultural plants to aquatic green microalgae (Gonzalez and Bashan 2000). Specifically, they created a synthetic mutualism between the microalga *Chlorella* spp. and

A. brasilense, and they proposed it as a simple, quantitative experimental model to study the beneficial interactions between the plant and the bacteria (Fig. 4). To facilitate the interaction and maintain the mutualistic associations, the two microorganisms were initially immobilized in small alginate beads (de-Bashan and Bashan 2008). The hypothesis behind proposing such an interaction was that, as an unspecified PGPB, *A. brasilense* would affect green microalgae in ways that were similar to how it impacted higher plants. They found that the effects occurred at all levels, presenting a new avenue for the application of *A. brasilense*.

Thus far, physiological studies have shown the effects of *A. brasilense* on microalgae pigments (de-Bashan et al. 2002), carbohydrates (Choix et al. 2012a, b, 2018), total lipids (Leyva et al. 2015), and vitamins (Palacios et al. 2016). Similar to higher plants, the production of IAA is a key mechanism affecting microalgae (de-Bashan et al. 2008a). *Azospirillum* enhances the growth of *Chlorella* spp., *Scenedesmus obliquus*, and *Chlamydomonas reinhardtii* (de-Bashan and Bashan 2008; Choix et al. 2018), but it also affects the activities of enzymes, including glutamine synthetase and glutamate dehydrogenase in *C. vulgaris*. A higher uptake of N from the culture medium and a higher accumulation of intracellular N were observed in the plants inoculated with *Azospirillum* than those that were not inoculated (de-Bashan et al. 2008b; Meza et al. 2015). Similarly, it was found that *Azospirillum* had an effect on ADP-glucose pyrophosphorylase, leading to increased accumulation of starch (Choix et al. 2014) and on acetyl-CoA carboxylase, resulting in higher synthesis of fatty acids (Leyva et al. 2014) in microalgae. A direct exchange of N and C between *A. brasilense* Cd and *C. sorokiniana* was demonstrated by nanoSIMS (de-Bashan et al. 2016), and the positive effect of the volatile compounds produced by *A. brasilense* in

C. sorokiniana was also reported (Amavizca et al. 2017). Lopez et al. (2019) showed that riboflavin and lumichrome produced by *A. brasilense* had a significant effect on photosynthetic and auxiliary pigments in *C. sorokiniana*. The combination has been successfully used for wastewater treatment (de-Bashan et al. 2002; Bashan et al. 2004; Perez-Garcia et al. 2010) and recovery of desert degraded soil after amendment of wastewater debris (Trejo et al. 2012; Lopez et al. 2013).

Overall, these results have extended the use of *Azospirillum* beyond agriculture to tackling environmental issues, such as revegetation, reforestation, phytoremediation, and wastewater treatment programs.

An overview of the research timeline

Over the last 90 years, studies on *Azospirillum*-plant interaction have suggested a wide range of mechanisms through which the bacterium enhances plant growth, as summarized in Supplementary Fig. 1.

Despite this body of evidence, two main mechanisms have defined this genus as a model of PGPB: BNF and phytohormone production. The history of the effects of *Azospirillum* as a bacterium capable of fixing atmospheric N dates to 1976 in Brazil. It was revealed for the first time that *A. lipoferum* was able to efficiently fix N in the roots of *Digitaria decumbens* (Day and Döbereiner 1976). This mechanism lost its research importance because the results obtained in greenhouse and field experiments were controversial; however, new mechanisms were proposed to explain the positive effects of inoculation. That was how, at the Katholieke Universiteit Leuven (Belgium), it was demonstrated for the first time that tryptophan was involved in IAA production since *A. brasilense* was able to convert tryptophan into IAA (Reynders and Vlassak 1979). Meanwhile, a study conducted in the US reported that
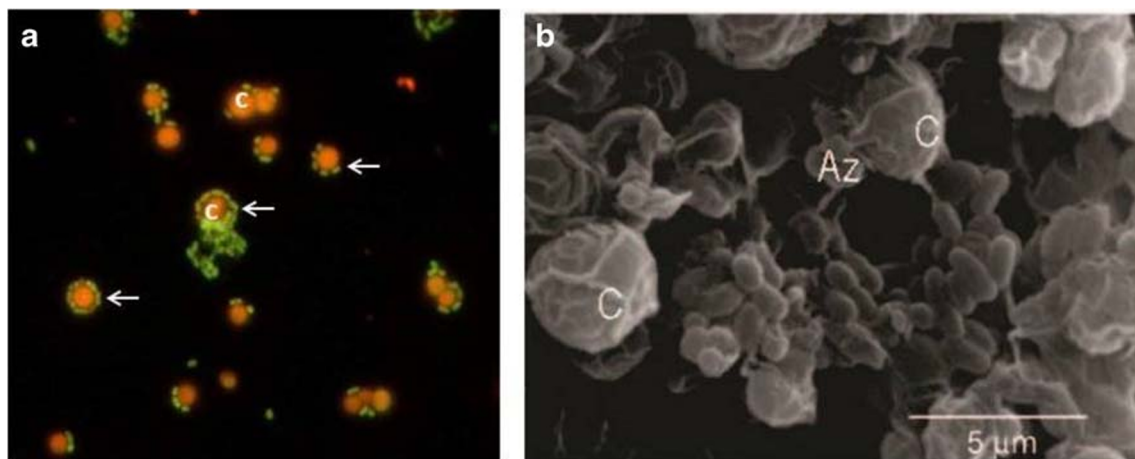

Fig. 4 *Chlorella sorokiniana* and *Azospirillum brasilense* in co-culture. **a** Auto-fluorescence of microalgae appears in orange while bacteria appears in green, as result of fluorescent in situ hybridization (FISH) using three specific probes targeting Eubacteria (FAM dye) and one specific probe for *A. brasilense* (CY3 dye). **b** Scanning electron microscopy (SEM). **c** C: *C. sorokiniana*. Az: *A. brasilense*. Arrows show cells of *A. brasilense* attached to the microalgae

A. brasilense was able to produce plant growth substances, such as auxins, cytokinins, and gibberellins (Tien et al. 1979). These two reports were the first to show that *Azospirillum* had the ability to improve plant growth due to the production of phytohormones. Two years later, Oliveira and Drozdowicz (1981) demonstrated for the first time the ability of the genus *Azospirillum* to produce bacteriocins (molecules able to inhibit bacterial growth) in a pure culture medium. One year later, Reynders and Vlassak (1982) investigated the use of *A. brasilense* as a biofertilizer in intensive wheat cropping. Simultaneously, a selective culture medium, Congo Red medium (CR), was developed to isolate *Azospirillum* spp. from soil or seeds (Rodriguez Caceres 1982).

As the interest in the phytohormonal effects of *Azospirillum* in plants intensified, numerous field studies were conducted in the 1980s to analyze the growth and yield of inoculated crops. Thus, *Azospirillum* began to emerge as a powerful crop inoculant in co-inoculation systems. The first co-inoculation studies were conducted using *A. brasilense* and the mycorrhizal fungus, *Glomus mosseae*, to study their effects on the growth and nutritional quality of maize and ryegrass (Barea et al. 1983). Furthermore, co-inoculation of *Azospirillum* and *Rhizobium* was found to have a positive effect on winged beans and soybeans (Iruthayathas et al. 1983). Sarig et al. (1984) reported that the best effects on plants inoculated with *Azospirillum* were obtained when the culture conditions were sub-optimal. It was first found that the grain yield of non-irrigated *Sorghum bicolor* increased under abiotic stress when inoculated with *Azospirillum* (Sarig et al. 1984). As interest in the phytohormonal effects of these bacteria continued, it was observed that *A. brasilense* was able to produce ABA in a chemically defined culture medium (Kolb and Martin 1985). The production of plant growth substances (phytohormones), classified as cytokinins, by *Azospirillum* and other related bacteria continued to be analyzed (Horemans et al. 1986). Four years later, gibberellins A$_1$, A$_3$, and iso-A$_3$ were identified in cultures of *A. lipoferum* (Bottini et al. 1989). Similar results were obtained using *A. brasilense* (Janzen et al. 1992). Later, ethylene production by *Azospirillum* was evaluated in chemically defined media modified with the amino acid L-methionine (Strzelczyk et al. 1994).

The arrival of the molecular biology and genomics era shifted the focus to investigating the functional effects of *Azospirillum* on plants at the molecular level. The first studies to emerge focused on *Arabidopsis* plants as a model to investigate the *A. brasilense*-*Arabidopsis* root interaction system; they demonstrated that this bacterium more than doubled the root hair growth in comparison to the non-inoculated control in a consistent and reproducible way (Dubrovsky et al. 1994). Subsequently, it was established that this effect had a strong phytohormonal component mediated by IAA (Spaepen et al. 2014).

The study of the *Azospirillum* genome began in 2000 with the analysis of five *Azospirillum* spp. genomes using pulsed-field gel electrophoresis (Martin-Didonet et al. 2000). This biochemical characterization continued, and new plant growth-promoting mechanisms were proposed. It was found that *Azospirillum* was able to solubilize insoluble phosphates through the production of gluconic acid (Rodriguez et al. 2004). The same year, the sequence of the pRhico plasmid in *A. brasilense* Sp7 was analyzed and it was found to have an important role in plant-root interactions and bacterial viability (Vanbleu et al. 2004). In 2005, it was demonstrated that the nitric oxide produced in vitro by *A. brasilense* Sp245 was a promoter of lateral root initiation in tomato seedlings (Creus et al. 2005). Another interesting mechanism emerged in 2006. Four strains belonging to *A. lipoferum* isolated from the rice rhizosphere were able to synthesize N-acyl-homoserine lactones (AHLs), which regulate crucial functions for plant-bacteria interactions (Vial et al. 2006). A similar paper reported the production of cadaverine by *A. brasilense* Sp245 and Az39 (Perrig et al. 2007). Another study confirmed that *A. brasilense* had the capacity to produce several polyamines, such as putrescine, spermine, and spermidine, under similar culture medium conditions (Thuler et al. 2003). Supporting evidence was reported in later studies. It was reported that *A. brasilense* Az39 promoted root growth and helped mitigate osmotic stress in rice seedlings, in part due to cadaverine production (Cassán et al. 2009).

In 2010, the complete genomic structure of *Azospirillum* sp. B510 isolated from stems of rice plants was obtained (Kaneko et al. 2010). That study was the first to report on the genome structure of a member of the genus *Azospirillum*. In the same year, a new hypothesis about the action of *Azospirillum* on plants was proposed (Bashan and de-Bashan 2010). A year later, Wisniewski-Dyé et al. (2011) obtained the genome sequences of the model strains *A. brasilense* Sp245 and *A. lipoferum* 4B and analyzed the taxonomic origin of this bacterial genus. Through genome sequencing and analysis, they showed that *Azospirillum* spp. transitioned from aquatic to terrestrial environments. Most of the *Azospirillum* genes were acquired horizontally, and they encode functions that are critical for rhizosphere-plants adaptation and interaction. In 2014, the complete genome sequence of *A. brasilense* Az39 was presented (Rivera Botia et al. 2014); it is one of the strains that is most often used for agriculture in South America. One year later, the complete genome sequences of *A. brasilense* Sp7 (Kwak and Shin 2015) and *A. thiophilum* isolated from a sulfide spring (Fomenkov et al. 2016) were analyzed and annotated. More recent studies have identified the draft genome sequences of *Azospirillum* sp. B2, isolated from a raised *Sphagnum* bog (Grouzdev et al. 2018), *A. brasilense* strains Ab-V5 and Ab-V6 (Hungria et al. 2018), extensively used as biofertilizers in Brazil, and *A. brasilense* REC3 (Fontana et al. 2018), isolated from

strawberry plants in Argentina. Recently, the quorum-sensing and quorum-quenching mechanisms based on N-acyl-L-homoserine lactones in *A. brasilense* Az39 were analyzed in silico and in vitro (Gualpa et al. 2019). That study reported that although *A. brasilense* Az39 this strain is a silent bacterium unable to produce AHL signals, it can interrupt the communication between other bacteria and/or plants via its quorum-quenching activity.

Concluding remarks and perspectives

Since its re-discovery in the 1970s, *Azospirillum* has become a cornerstone in the study of PGPB. Its potential as an effective inoculant for a wide variety of crops has been recognized. Yet, the exact mode of action is still not completely understood. *Azospirillum* modes of action were initially explained by the Additive Hypothesis; 20 years later, that was replaced by the multiple mechanisms hypothesis. In this review, we proposed the Eficient Nutrients Acquisition Hypothesis, which posits that plant growth promotion occurs via two major mechanisms, biological N fixation and phytohormone production, which are effectively induced by the colonized bacteria. Thus, some of these molecules have the capacity to alter the root morphology, thereby improving mineral uptake and inducing higher yields, even if using lower doses of chemical fertilizers. The contribution of N fixation is more controversial, and its effect may be less potent than previously believed. Although mixed results have been reported for inoculation, this has not prevented numerous companies around the world from offering inoculants containing *Azospirillum*. More specifically, in South America, 10 million doses of inoculants containing *Azospirillum* were used in 2018.

The use of *Azospirillum* under field conditions has been widely shown to improve plant growth and crop productivity. Thus, the use of azospirilla inoculants for crop production should be understood as a consolidated practice, in terms of grain yield production in summer and winter cereals, as well as legume production (co-inoculation). As an improvement in the use of *Azospirillum*, co-inoculation with rhizobia has proven to be a novel technology to enhance legume performance. Part of the current challenges of azospirilla inoculants has been the need for inoculant companies to develop effective formulations that can be used for diverse applications and under different storage handling and environmental conditions. In summary, the development of alternative application systems, such as the delivery of azospirilla by foliar inoculation, is seen as a solution to overcoming the limitations of on-seed treatment. There is an urgent need to promote a regional coordinated communication program about the already measured benefits of inoculation with *Azospirillum* as a complement to current extensive and intensive crop practices. These networks should include direct users of these products as well as other actors from rural and urban environments and local regulatory agencies.

Additionally, *Azospirillum* inoculation may serve as a valuable method for the remediation of contaminated soil and water and the revegetation and reforestation of degraded lands. Furthermore, the interaction of *Azospirillum* with green microalgae was proven to be an independent sub-field of *Azospirillum* research, presenting a new and interesting avenue to produce metabolites, such as lipids and pigments. However, this biotechnological application is yet to be tested under scale-up conditions to evaluate its real-life potential.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

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Agroecosystems, Nitrogen-use Efficiency, and Nitrogen Management

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Agroecosystems, Nitrogen-use Efficiency, and Nitrogen Management

The global challenge of meeting increased food demand and protecting environmental quality will be won or lost in cropping systems that produce maize, rice, and wheat. Achieving synchrony between N supply and crop demand without excess or deficiency is the key to optimizing trade-offs amongst yield, profit, and environmental protection in both large-scale systems in developed countries and small-scale systems in developing countries. Setting the research agenda and developing effective policies to meet this challenge requires quantitative understanding of current levels of N-use efficiency and losses in these systems, the biophysical controls on these factors, and the economic returns from adoption of improved management practices. Although advances in basic biology, ecology, and biogeochemistry can provide answers, the magnitude of the scientific challenge should not be underestimated because it becomes increasingly difficult to control the fate of N in cropping systems that must sustain yield increases on the world's limited supply of productive farm land.

INTRODUCTION

The focus of this paper is on nitrogen-use efficiency (NUE) in cereal production systems because maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) provide more than 60% of human dietary calories either as cereals for direct human consumption or embodied in livestock products produced from animals fed with feed grains and their by-products (http:/apps.fao.org/, agricultural production). It is likely that these same cereal crops will continue to account for the bulk of the future human food supply because they produce greater yields of human-edible food, are easily grown, stored, and transported, and require less fuel and labor for processing and cooking than other food crops. Our analysis will examine the NUE of these primary cereals in the world's major cropping systems, which also account for the majority of global N fertilizer use. We define the NUE of a cropping system as the proportion of all N inputs that are removed in harvested crop biomass, contained in recycled crop residues, and incorporated into soil organic matter and inorganic N pools. Nitrogen not recovered in these N sinks is lost from the cropping system and thus contributes to the reactive N (Nr) (1) load that cascades through environments external to the agroecosystem.

Our evaluation will focus on NUE in on-farm settings because estimates of NUE from experimental plots do not accurately represent the efficiencies achieved in farmers' fields. This lack of agreement results from differences in the scale of farming operations and differences in N-management practices—some of which are only feasible in small research plots. The effect of scale not only influences N fertilizer application, but all other management operations such as tillage, seeding, weed and pest management, irrigation, and harvest, which also affect efficiency. As a result, N-fertilizer efficiency in well-managed research experiments is generally greater than the efficiency of the same practices applied by farmers in production fields. For example, the average N-fertilizer uptake efficiency (defined as the percent-age of fertilizer-N recovered in aboveground plant biomass during the growing season—hereafter called the N-fertilizer recovery efficiency – RE_N), achieved by rice farmers is 31% of applied N based upon on-farm measurements in the major rice-production regions of four Asian countries (Table 1). In contrast, RE_N for rice in well-managed field experiments typically range from 50–80% (3–5). In the authors' experience, similar overestimation of RE_N in small plot experiments occurs for irrigated and rain-fed maize in the North-Central USA and for irrigated wheat in California.

The need to improve RE_N will be emphasized because N fertilizer is the largest source of N input to and losses from cereal cropping systems. A recent study estimates total N input to the world's cropland at 169 Tg N yr^{-1} (6). Inorganic N fertilizer, biological N fixation from legumes and other N-fixing organisms, atmospheric deposition, animal manures, and crop residues account for 46%, 20%, 12%, 11%, and 7%, respectively, of this total. Hence, crop-management practices that increase RE_N have a substantial impact on the amount of Nr that escapes from cereal production systems. While we recognize that solutions to global concerns about effects of Nr on the environment must involve integrated management of both inorganic and organic N sources to maximize NUE, other papers in this issue of Ambio and elsewhere address issues of N efficiency in livestock production systems and the contributions of organic N sources such as legume crops and green manures (6, 7).

NITROGEN-USE EFFICIENCY TODAY

Applied N not taken up by the crop or immobilized in soil organic N pools-which include both microbial biomass and soil organic matter—is vulnerable to losses from volatilization, denitrification, and leaching. The overall NUE of a cropping system can therefore be increased by achieving greater uptake efficiency from applied N inputs, by reducing the amount of N lost from soil organic and inorganic N pools, or both. In many cropping systems, the size of the organic and inorganic N pools has reached steady-state or is changing very slowly, and the N inputs from biological N_2 fixation and atmospheric deposition are relatively constant. For example, analysis of the N balance in long-term experiments on irrigated rice in Asia suggests that many of these systems have reached steady-state (8), and similar evidence suggests that some maize-based cropping systems in the USA corn belt are also near steady-state (9). In these cases, the overall NUE of a cereal cropping system is equal to the RE_N.

In contrast to systems at steady-state, adoption of new management practices or crop rotations that affect the soil carbon (C) balance will also affect the N balance because the C/N ratio of soil organic matter is relatively constant. In such cropping systems, the overall NUE of the cropping system must include changes in the size of soil organic and inorganic N pools in addition to the RE_N. When soil-N content is increasing, the amount of sequestered N contributes to a higher NUE of the cropping system, and the amount of sequestered N derived from applied N contributes to a higher RE_N. Conversely, any decrease in soil-N stocks will reduce NUE and RE_N.

Unfortunately, there is a paucity of reliable data on RE_N based

on measurements from on-farm studies in the major cereal production systems. Likewise, we are not aware of measurements of on-farm NUE that include the contributions from both RE_N and changes in soil-N reserves. This shortage of information reflects the logistical difficulty and high cost of obtaining direct on-farm measurements and the lack of funding for what appear to be routine on-farm evaluations. Available data indicate a very low mean RE_N of 31% in continuous irrigated rice systems in Asia (2, 10), and somewhat higher efficiency of 37% for maize in the major maize-producing states of the USA (Table 1). In contrast, mean RE_N for wheat in rice-wheat systems of India was 18% in one year and 49% the next. This difference was associated with low grain yields in the first year caused by unfavorable weather, and highlights the importance of robust crop growth and yield to greater RE_N. Good crop management and high yields of rain-fed wheat in northwestern Europe also contribute to relatively high RE_N in those systems (11). Most other estimates of RE_N in the literature are obtained from experimental plots at research stations, which tend to overestimate RE_N for the reasons previously described.

Two methods are commonly used for direct measurement of RE_N, and both have inherent weaknesses (12). The 'N-difference' method is based on the difference in N uptake between a crop that receives a given amount of applied N and N uptake in a reference plot without applied N. Another technique uses ^{15}N-labeled fertilizer to estimate crop recovery of applied N. Each of these methods can be confounded by 'added-N effects' when the applied N alters the ability of the plant root system to acquire N from soil, the rate of net N mineralization from organic N pools, or both. In addition, the ^{15}N-fertilizer technique can also be confounded by 'pool substitution' whereby N from applied ^{15}N-fertilizer replaces N in the various soil N pools during the processes of N immobilization-mineralization turnover from or-

ganic matter and microbial biomass. Because estimates of RE_N by the N-difference method are influenced by fewer confounding factors, we believe it is preferable to the ^{15}N-fertilizer technique. The data in Table 1 and cited throughout this paper are based on this method.

The NUE of agricultural systems also have been calculated using aggregate databases on crop production statistics and literature-based assumptions about N cycling to estimate N inputs and outputs on a regional or global basis. For example, Smil's (6) elegant global N balance for crop production estimates an average N recovery efficiency in crop biomass of 50% from all sources of N input—including fertilizers, atmospheric deposition, biological N_2 fixation, recycled crop residues, and manures. However, N recovery efficiencies can differ substantially from each of these N sources, and therefore it is not possible to estimate RE_N by this approach. The much lower estimates of RE_N based upon direct on-farm measurements for rice in Asia and maize in the North-Central USA (Table 1) may reflect higher N uptake efficiency from indigenous N sources than from applied fertilizer. Moreover, the overall NUE of these systems would be higher or lower depending on whether soil N reserves are increasing or decreasing over time.

In recent years, significant strides towards increasing RE_N are suggested from aggregate data of fertilizer use and crop yields. Since the early 1980s, the ratio of crop yield per unit of applied N fertilizer (called the partial factor productivity for N fertilizer—PFP_N) has increased in Japan (13), and the USA (14). For USA maize, PFP_N increased by 36% in the last 21 years, from 42 kg kg^{-1} in 1980 to 57 kg kg^{-1} 1 in 2000 (Fig. 1). Because crop dry matter accumulation and grain yield are closely correlated with N uptake, the increase in PFP_N since 1980 suggests an associated increase in RE_N—assuming the indigenous N supply from net mineralization of soil organic matter, atmospheric N inputs, and biological N fixation have remained relatively constant during this period. In contrast, there appears to have been little improvement in RE_N of irrigated rice in tropical Asia; on-farm efficiencies measured in the late 1960s and early 1970s (15) are comparable to estimates made in the late 1990s as given in Table 1. Understanding the reasons for these trends in PFP_N and RE_N and the prognosis for improving them depends on knowledge of the factors that govern N demand and supply in cereal cropping systems.

BIOPHYSICAL DETERMINANTS OF CROP NITROGEN REQUIREMENTS

Crop-N demand is determined by biomass yield and the physiological requirements for tissue N. Crop-management practices and climate have the greatest influence on yield. Climate varies considerably from year to year, which causes large differences in yield potential. In irrigated systems, the yield potential of a given crop cultivar is largely governed by solar radiation and temperature. In dryland systems, rainfall amount and temporal distribution also have a large influence on yield potential. While solar radiation, temperature, and moisture regimes determine the genetic yield ceiling, actual crop yields achieved by farmers are generally far below this threshold because it is neither possible, nor economic, to remove all limitations to growth from sub-

Table 1. Nitrogen fertilizer-uptake efficiency[+] (or recovery efficiency, RE_N) by maize, rice, and wheat crops based on data obtained from on-farm measurements in their major cropping systems.

Crop	Region/Countries (cropping system)	Number of farms	N fertilizer (kg ha^{-1}) mean (+/- SD)	RE_N (% of applied) mean (+/- SD)
Maize[++]	North-central USA (various rotations)	55	103 (85)	37 (30)
Rice[+++]	China, India, Indonesia, Phillipines, Thailand, Vietnam (rice-rice)	179 179	117 (39) 112 (28)	31 (18) 40 (18)
Wheat[++++]	India (rice-wheat)	23 21	145 (31) 123 (30)	18 (11) 49 (10)

+   Recovery efficiency is the proportion of applied N fertilizer that is taken up by the crop. It is determined by the difference in the total amount of N measured in aboveground biomass at physiological maturity between replicated plots that receive N fertilizer and control plots without applied N. Except for the omission of N in control plots, all crop-management practices are determined and applied by the farmer of each field.

++   Data obtained from on-farm experiments located in Illinois, Michigan, Minnesota, Missouri, Nebraska, and Wisconsin. Experiments were conducted from 1995–1999 by researchers in the NC 218 Regional Research Project. At each site, replicated plots received N-fertilizer across a wide range of N-application rates, including a control without applied N. Management practices other than N-fertilizer rate were imposed by the farmer. RE_N was estimated as described above.

+++ Data from on-farm experiments conducted at 179 sites in major irrigated rice domains of Asia from 1997 to 2000 with measurements taken in 4 consecutive rice crops at each site (2). The first row of data were taken from the field-at-large where nutrient management practices were applied by the farmer without guidance from researchers, whereas the second row represents field-specific nutrient management whereby the amount of applied fertilizer was adjusted to account for the balance between soil nutrient supply capacity and crop demand.

++++ Data from on farm studies of rice-wheat systems in North India (A. Dobermann, C. Witt, and B. Mishra, unpubl. data) following similar methods as for rice (2). Data in the first row were from a year in which mean yields were relatively low because of unfavorable weather (1998: average grain yield 2.3 Mg ha^{-1}), whereas the second row of data is for a favorable year with considerably larger mean yields (1999: average grain yield 4.8 Mg ha^{-1}).

optimal nutrient supply, weed competition, and damage from insects and diseases. Hence, the interaction of climate and management causes tremendous year-to-year variation in on-farm yields and crop N requirements.

Crop physiological N requirements are controlled by the efficiency with which N in the plant is converted to biomass and grain yield. Because cereal crops are harvested for grain, the most relevant measure of physiological N efficiency (PE_N) is the change in grain yield per unit change in N accumulation in aboveground biomass. Crop-PE_N is largely governed by 2 factors: *i*) the genetically determined mode of photosynthesis—either the C_3 or C_4 photosynthetic pathway; and *ii*) the grain N concentration—also under genetic control but affected by N supply as well. Both rice and wheat are C_3 plants while maize is a C_4 plant. The C_4 plants tend to have greater PE_N than C_3 plants because the C_4 pathway has a higher photosynthetic rate per unit leaf-N content (16), which results in greater biomass production per unit of plant-N accumulation (Fig. 2, ref. 17).

Large genetic variation in grain-N concentration within each of the major cereal species has allowed plant breeders to develop cultivars with the desired grain-N concentration for specific end-use properties. Relatively low grain-N content of 10–12 g kg^{-1} here and elsewhere, grain-N concentration is given on a dry weight basis desired in rice for optimal cooking and eating quality. Maize-N content also is relatively low (13–14 g kg^{-1}) because maize products for human consumption or animal feed do not require high protein. In contrast, the N concentration of wheat must exceed 18 g kg^{-1} to have acceptable quality for bread or noodles. The relationship between grain yield and the N contained in aboveground biomass at physiological maturity provides a measure of PE_N across a wide range of production environments (Fig. 3). The line at the upper boundary of data points in this Figure provides an estimate of maximum N dilution in plant biomass, which occurs when N is the most limiting factor to plant growth. When N is no longer the most limiting resource and other factors such as water supply, pest damage, or deficiencies of other nutrients reduce crop growth, the amount of grain produced per unit N uptake decreases and moves off the line of maximum N dilution.

Across a wide range of production environments and management practices, maize tends to have a larger increase in grain yield per unit N uptake than rice because it is a C_4 plant. This advantage in PE_N is evident in the slopes of regression lines in Figure 3. Rice has a lower efficiency than maize because it is a C_3 plant although its lower grain N concentration partially offsets this disadvantage. Wheat has the smallest PE_N of the 3-major cereals because it is a C_3 plant with high grain protein (data not shown). Two additional points are noteworthy. First, the lines defining both maximum N dilution and the overall regression are curvilinear, which means there is a diminishing return to the conversion of plant N to grain as yields approach the yield

Figure 1. Trends in maize grain yield, use of N fertilizer, and Partial Factor Productivity from applied N fertilizer (PFP_N, kg grain yield kg^{-1} N applied) in the USA.
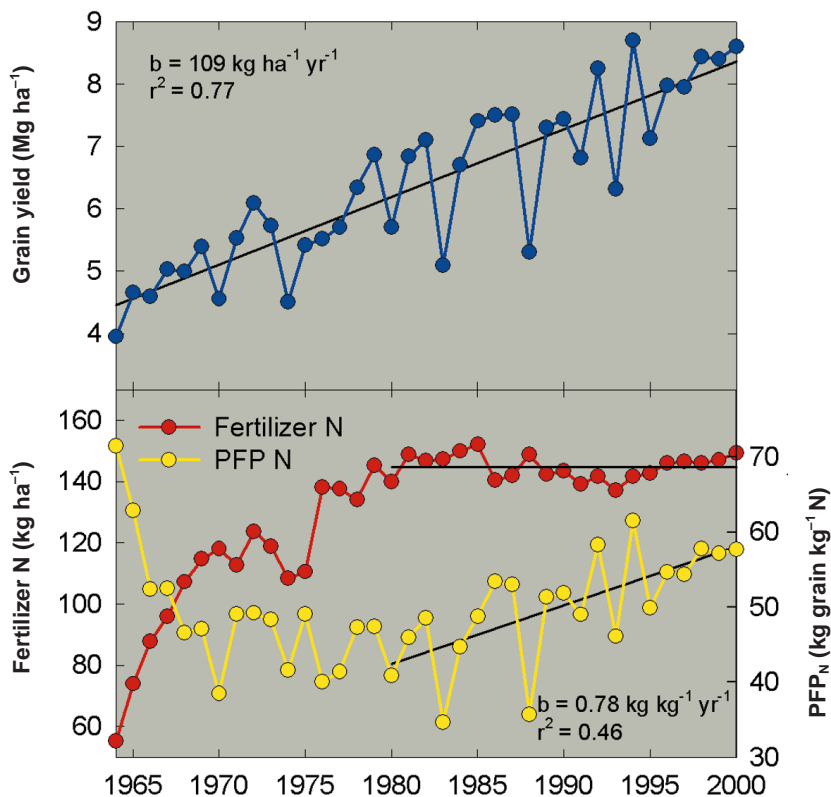
Sources of data: Mean annual maize yields, National Agricultural Statistics Service, USDA http://www.usda.gov/nass/; mean annual N fertilizer N use, USDA Annual Cropping Practices Surveys (> 2000 farms representing 80 to 90% of the USA maize area), Economic Research Service, USDA, http://www.ers.usda.gov/

Figure 2. Relationships between dry matter yield and nitrogen content of plant tissue for C_3 and C_4 crops. (Source: 17).
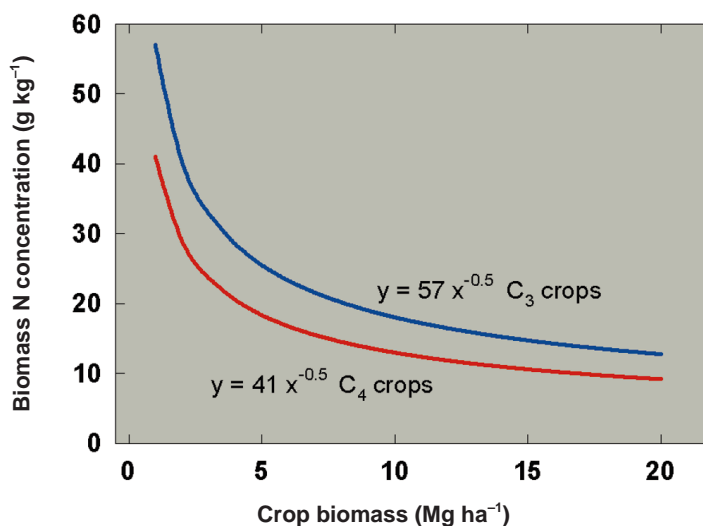
C_3 crops [site-years] include lucerne [12], fescue [7], French bean [1], potato [7], cabbage [1], wheat [2] and rape [4], n = 181. C_4 crops [site-years] include sorghum [10], maize [2] and setaria [2], n = 75.

Figure 3. Relationship between grain yield and plant-N accumulation in aboveground biomass at physiological maturity in maize and rice.
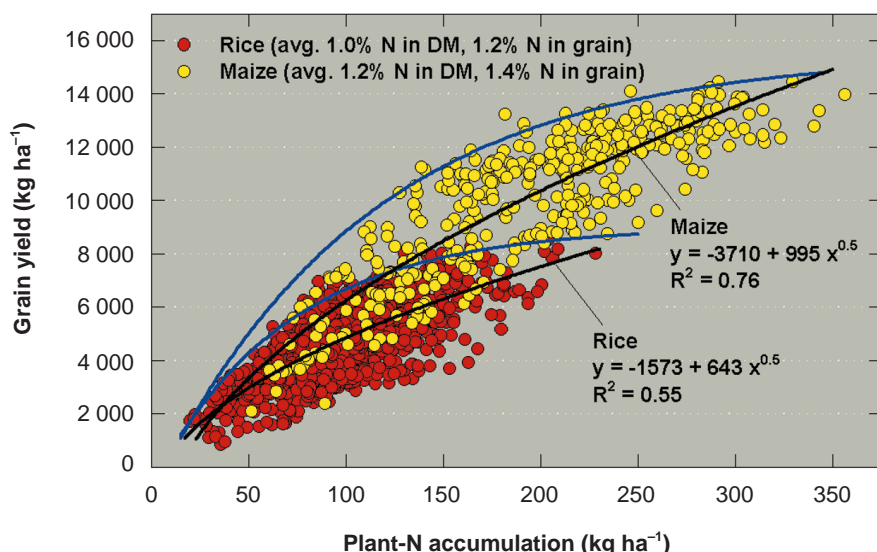
Data sources: *i*) for rice, data obtained from on-farm and research station experiments conducted across a wide range of agroecological environments in Asia from 1995 to 2000 (n = 1658); *ii*) for maize, data obtained from on-farm and research station experiments conducted across a wide range of agroecological environments in the North-Central USA from 1995 to 2000 (n = 470). Blue lines indicate the boundary of maximum dilution of N in the plant (maximum physiological efficiency), whereas the black lines depict the average physiological efficiency as obtained from nonlinear regression for the entire data set for maize and rice.

potential ceiling. Second, the N concentration of cereal straw and stover is much smaller than in grain, and differences among cereal crops or among cultivars of the same crop species are relatively small. Therefore, the amount of N remaining in straw or stover has a relatively small effect on PE_N unless factors other than N are limiting crop growth and grain yield.

DYNAMICS OF NITROGEN SUPPLY

Inorganic nitrate and ammonium ions are the primary source of N taken up by plant roots. Both indigenous soil resources and applied N inputs contribute to this plant-available N pool, which represents a very small fraction of total soil-N. For example, a typical irrigated rice soil in Asia contains about 2800 kg N ha^{-1} in the top 20 cm of soil where roots derive the majority of crop-N supply. Of this total, the amount of N derived from indigenous resources during a single cropping cycle typically ranges from 30–100 kg N ha^{-1} (Fig. 4a), which represents only 1–4% of total soil N. For cereal crops, we define the indigenous soil-N supply as the amount of N the crop obtains from the inorganic N pool, net N mineralization from soil organic matter and incorporated crop residues, biological N$_2$ fixation by soil microflora in the rhizosphere and floodwater (in the case of irrigated rice), and inputs of N from atmospheric deposition and irrigation water. Similarly, total-N in the top 20 cm of a fertile prairie soil in the USA corn belt is about 4000 kg N ha^{-1}, and the indigenous N supply typically ranges from 80–240 kg N ha^{-1} (Fig. 4b), which is 2–6% of total soil-N. Although small in size, the indigenous N supply has a very high N-fertilizer substitution value because of the relatively low RE_N from applied N fertilizer.

A maize crop that produces a grain yield of 10 000 kg ha^{-1} requires total uptake of about 190 kg N ha^{-1} (Fig. 3). The indigenous N supply typically provides about 130 kg N ha^{-1} (median value in Fig. 4b), which leaves 60 kg N ha^{-1} that must be provided by applied N. If RE_N is 37%, which is typical of on-farm conditions (Table 1), then an N-fertilizer rate of 162 kg N ha^{-1} must be applied to meet crop-N demand. If the indigenous N supply decreases from 130 to 100 kg N ha^{-1} (a 23% reduction), then the N-fertilizer requirement increases by 50% to 243 kg N ha^{-1}, assuming RE_N remains constant at this higher N fertilizer rate. However, RE_N typically

Figure 4. Variation in the indigenous N supply (I_N, plant N accumulation in on-farm plots that did not receive N fertilizer). of a. rice fields in Asia; and b. maize fields in the North-Central USA.
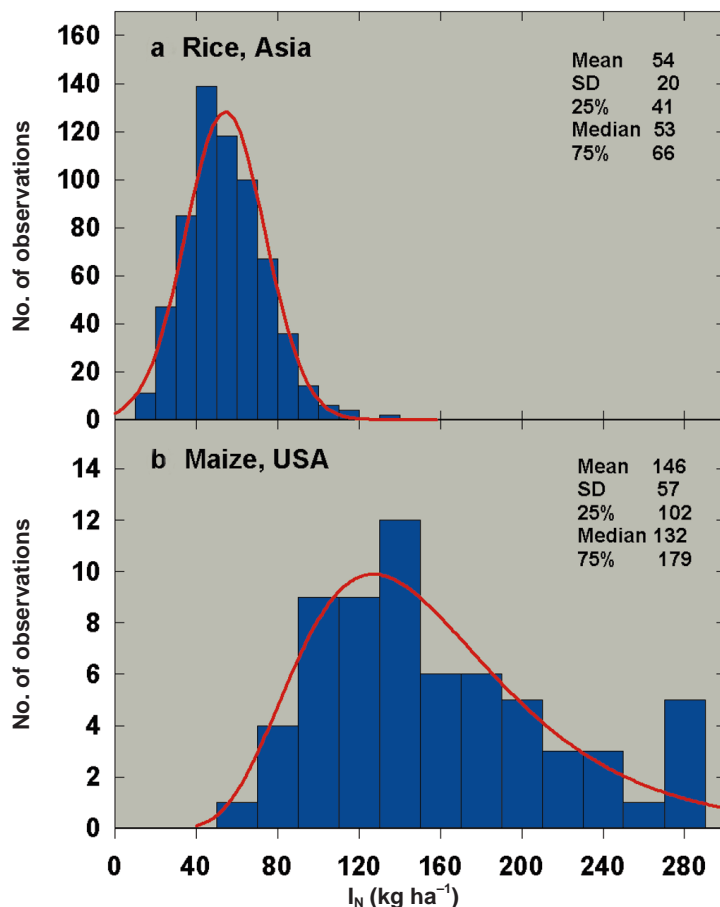
I_N for rice was measured at on-farm sites at 179 locations in South and Southeast Asia (Source: C. Witt and A. Dobermann, Reversing Trends of Declining Productivity in Intensive Irrigated Rice Systems, On-farm Monitoring Database, June 2000 release; IRRI, Los Banos, Philippines). I_N for maize was measured at 64 locations in 6 major maize-producing states in the North-Central USA in replicated field experiments and on-farm trials (Source: D. Walters, Univ. of Nebraska; North Central regional Research Project NC-218). For both rice and maize, the I_N at each site was measured as described in the footnotes to Table 1. For comparison, mean total soil N content in the 0-20 cm topsoil layer was 1.4 ± 0.7 g kg^{-1} at the rice sites in Asia and 1.6 ± 0.1 g kg^{-1} at the maize sites in North America.

decreases as the amount of N-fertilizer application increases (18), especially at high rates of fertilizer input, which further increases the fertilizer substitution value of indigenous N.

Given the large N-fertilizer substitution value of indigenous N, predicting the amount and temporal variation of the indigenous N supply during crop growth is essential for determining the optimal timing and amount of N-fertilizer applications. Accurate prediction is difficult, however, because the indigenous N supply is highly variable in the same field over time as well as in different fields within the same agroecological zone, even when the fields have similar soil type, management, and climatic conditions (3, 19). This high degree of variability is illustrated by the on-farm measurements of the indigenous N supply in rice- and maize systems (Fig. 4 a, b). Because of the high degree of variation and small size relative to the much larger background of total soil-N, prediction of the indigenous soil N supply is one of the key challenges for agronomic research.

The primary determinants of total plant-available N supply are the net rate of N release from soil organic matter and incorporated crop residues, which is controlled by the balance between N immobilization and mineralization as mediated by soil microbes, the contributions from applied organic and inorganic N sources, and losses from the plant-available N pool. Other contributions include wet/dry deposition from rainfall and dust, free-living biological N fixation (BNF), and, in irrigated systems, N contained in irrigation water. The contribution of BNF is of greatest importance in rice systems with an active floodwater flora and contributions typically range from about 30 to 50 kg N ha^{-1} crop^{-1} (20). Although there is often a flush of N mineralization after soil tillage, soil rewetting after a tropical dry season, or after thawing and warming in a temperate spring planting season, the rate of N mineralization is relatively constant during the period of active crop uptake. In contrast, most N fertilizers rapidly enter the plant-available N pool because they are composed of inorganic N in the form of nitrate, ammonium, or both. The amount and time course of available N-released from organic manures and other organic N sources depends on the amount of inorganic N they contain at the time of application and on subsequent rates of N mineralization. Regardless of N source, the potential for N losses is greatest whenever the size of the plant-available N pool exceeds crop uptake requirements.

Environmental conditions and crop management heavily influence the rate of net N mineralization from indigenous and applied organic N as well as the rate of N losses from the plant-available N pool. Most influential during the relatively short period of a single crop production cycle are temperature and moisture regime, soil tillage method, and the amount, chemical composition, and timing of carbon and N inputs from crop residues and roots, inorganic fertilizers, cover crops, and manures. Over longer periods, soil erosion, atmospheric N deposition, and soil acidification can have large cumulative effects on the overall N balance and amount of soil-N reserves. A detailed review of these N-supply components and the environmental and management factors that affect ecosystem N dynamics and N balance are beyond the scope of this paper. Fortunately, a wealth of information is available in comprehensive reviews on components of the soil-N cycle and the effects of environment and management on N transformations and fluxes (12, 20–23).

At issue here is how well current knowledge of controls on soil-N dynamics is distilled into practical management tools for identifying an N-fertilizer management regime that optimizes RE$_N$ and profit. While present understanding allows reasonably accurate predictions of the total soil N balance over long-term periods using mechanistic simulation models (24), such models have not proven sufficiently robust for predicting the size of the available N pool and crop uptake from it across a wide range of production environments (25, 26). The small size of this dynamic N pool and the complexity of interactive processes that govern

its availability over short periods, are difficult issues for realistic models. We would argue that the development of simulation models that can make reasonable predictions of the amount and time course of the indigenous N supply is a very high priority. However, we also recognize that it will be very challenging to make such models user-friendly for routine N-management decisions.

IMPROVING NITROGEN-USE EFFICIENCY AND PROFIT

The goal of reducing Nr while sustaining adequate rates of gain in cereal production to meet expected food demand will require increases in both NUE and RE$_N$, which in turn will require innovative crop- and soil-management practices. The economic 'benefit to cost ratio' has a large influence on farmer adoption of new technologies. While some management practices might increase NUE by reducing N losses or increasing the proportion of N inputs that are retained in soil organic and inorganic N pools, adoption by farmers is not likely without the promise of adequate economic return. Hence, management options for improving NUE of cereal production systems must also consider RE$_N$ and PE$_N$ because these parameters determine the economic impact on grain yield in relation to applied N inputs and crop-N accumulation.

Recent literature on improving NUE in crop-production systems has emphasized the need for greater synchrony between crop N demand and the N supply from all sources throughout the growing season (3, 27–30). This approach explicitly recognizes the need to efficiently utilize both indigenous and applied N and is justified by the fact that losses from all N-loss mechanisms increase in proportion to the amount of available N present in the soil profile at any given time. Hence, uptake efficiency from a single N-fertilizer application typically decreases in proportion to the amount of N-fertilizer applied (18). The same principle applies to available N derived from organic N sources such as legume green manures, cover crops and animal manures. Indeed, potential nitrate leaching from manures can be equal or greater than potential losses from inorganic N fertilizer when the available N supply from either source exceeds crop demand by similar amounts for comparable time periods (31, 32).

Increased yields also can contribute to greater NUE from both indigenous and applied N sources because fast growing plants have root systems that more effectively exploit available soil resources (33). Crop health, insect and weed management, moisture and temperature regimes, supplies of nutrients other than N, and use of the best adapted cultivar or hybrid all contribute to more efficient uptake of available N and greater conversion of plant N to grain yield. Assuming a well-managed crop, RE$_N$ and profit from applied N are therefore optimized with the least possible N losses when the plant-available N pool is maintained at the minimum size required to meet crop-N requirements at each stage of growth. Too little N reduces yields and profit while too much N is vulnerable to losses from leaching, volatilization, and denitrification.

The degree of synchrony between N supply and demand and its influence on RE$_N$ can be evaluated quantitatively when N demand and supply can be measured or estimated. For example, yield level provides an estimate of crop N demand and the indigenous N supply can be estimated by N uptake in plots that do not receive applied N. On-farm experiments with irrigated rice in Asia and maize in the North-Central USA illustrate the interactive effects of these factors across a wide range of environments based on the relationship between RE$_N$ and an expression that represents the degree of synchrony between N supply and demand:

$$F_N/(1–I_N/U_N) \qquad \text{Eq. 1}$$

Figure 5. Variation in nitrogen fertilizer uptake efficiency RE_N.
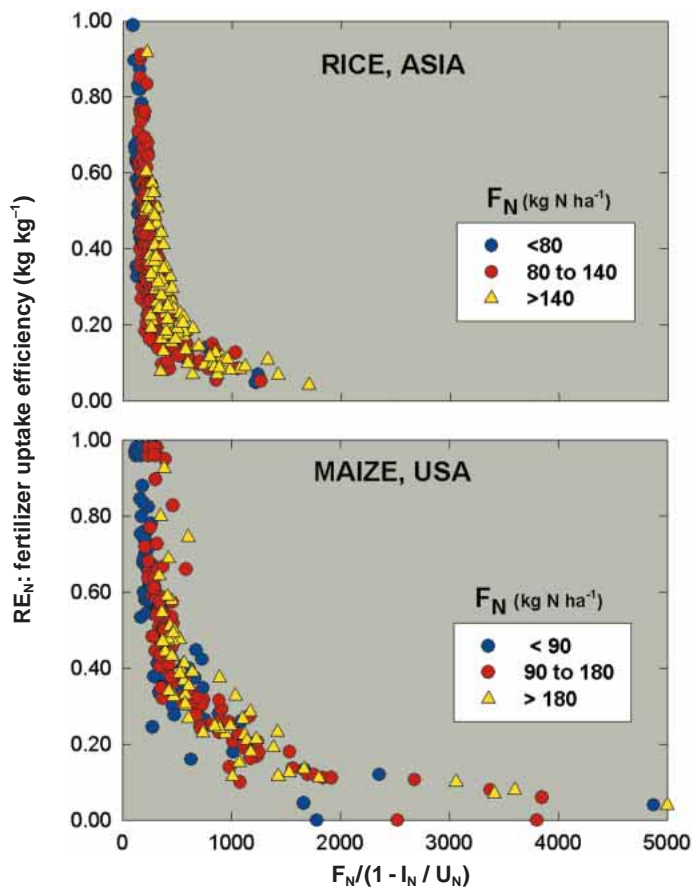
(RE_N: kg N fertilizer uptake kg^{-1} N applied) in relation to the degree of synchrony between crop N demand (U_N: in kg N ha^{-1} as measured by crop N accumulation in aboveground biomass at physiological maturity in N-fertilized plots) and the N supply from indigenous resources (I_N: in kg N ha^{-1} as measured by crop N uptake in control plots without applied N) and the amount of applied N fertilizer (F_N in kg N ha^{-1}). Smaller values on the abscissa indicate greater synchrony between N supply and demand. Data source for rice: C. Witt and A. Dobermann, On-Farm Monitoring Database, June 2000 release; IRRI, Los Banos, Philippines. Data source for maize: D. Walters, University of Nebraska; North Central Regional Research Project NC-218.

where F_N is the amount of applied N fertilizer (kg N ha^{-1}), I_N is the indigenous N supply as measured by crop-N uptake in plots without applied N (kg N ha^{-1}), and U_N is the measured crop-N uptake (kg N ha^{-1}) where farmers applied N fertilizer outside the N-omission plots (Fig. 5). Greater synchrony between supply and demand is indicated by smaller values for this expression. The RE_N from a given amount of applied N fertilizer for both crops increases when demand for N cannot be met by the indigenous N supply—a situation that occurs when I_N is small relative to U_N. Conversely, when the indigenous N supply can meet crop N requirements (I_N approaches U_N), RE_N is small because N does not limit crop growth. The data also demonstrate that it is possible to achieve high RE_N with relatively large N-fertilizer rates (F_N), but only when crop N demand is much larger than the indigenous supply. The scatter in these relationships reflects the effects of other management factors on crop growth and N uptake even though N was generally the most limiting production factor in these on-farm studies.

Improving N Efficiency in USA Maize Systems

The 'N synchrony framework' is useful for evaluating management options to improve NUE regardless of scale or technological sophistication of the crop production system. For example, 3 factors have contributed to improvement since 1980 in RE_N of USA maize (Fig. 1) where production systems are large scale and highly mechanized: i) increased yields and more vigorous crop growth associated with increased stress tolerance of modern hybrids (34); ii) improved management of production factors other than N such as conservation tillage and higher plant densities; and iii) improved N-fertilizer management. Improvements in N-management include significant reductions in fall-applied N-fertilizer with a shift to applications in spring or at planting, greater use of split N-fertilizer applications during the growing season rather than a single large N application, and development and extension of N-fertilizer recommendations that give N 'credits' for manure, legume rotations, and residual soil nitrate as determined by soil testing (35).

Each of these practices helps to better match the amount and timing of applied N to crop-N demand and the N supply from indigenous resources. They were developed through large investments in research at land-grant universities during a 30-year period from 1960–1990. Adoption by farmers required additional investments in extension education. Even with this tremendous effort and investment, not all farmers have adopted these practices. Of the total N fertilizer applied to maize in 1999, 28% was applied in the fall, 45% in the spring (preplanting or at planting), and 25% after planting. Soil testing was practiced on 37% of the total maize area, and the average number of N-fertilizer applications was 1.8, which means that some farmers still do not use split applications (36). Fall applications continue because N suppliers offer price discounts for N applied in the fall. These discounts reflect labor shortage and additional costs for storage, distribution, and application of N in the spring when many other field operations associated with tillage, planting, and weed control are in progress.

Despite the improvement in efficiency since 1980, our best estimate of average RE_N in farmer's fields is less than 40% of the applied N. This estimate is based on recent on-farm measurements in six of the major maize-producing states (Table 1). Eliminating fall applications, increased use of soil testing, and greater use of split applications rather than a single large application would contribute to further gains in efficiency. Continued expansion of no-till and other conservation tillage practices that reduce erosion will also help reduce N load in surface waters, but they can also increase N-fertilizer losses from denitrification and leaching (37).

Improving N Efficiency in Asian Rice Systems

In contrast to USA maize production, there is little evidence of improvement in RE_N of irrigated rice in Asia. Moreover, the rate of increase in yield of irrigated rice has slowed markedly in the past 20 years in part because average yields are approaching the yield potential ceiling in some of the major rice-growing domains (38). Recent studies also document that fertilizer practices used by rice farmers fail to match application amounts with crop demand and soil supply (2, 10, 19, 39). Despite tremendous variation in the indigenous N supply (Fig. 4), most extension services in developing countries provide a single, standard fertilizer recommendation for an entire district or region. Farmers apparently have few guidelines for adjusting N-fertilizer amount to account for the large differences in the indigenous N supply, indicating the need for a 'field-specific' approach to N management.

To test this hypothesis, a field-specific management approach was evaluated in on-farm experiments at 179 sites in 8 rice-producing domains of 6 Asian countries where continuous annual double-crop rice systems were the dominant agricultural land use. Fertilizer application rates for N, phosphorus (P), and potassium (K) were estimated for individual fields by accounting for the indigenous nutrient supply, yield goal, and nutrient de-

mand as a function of the interactions between uptake requirements for N, P, and K (2). Nitrogen was applied in as many as 4 split applications to better synchronize N supply with crop demand. A relatively small amount of N was applied at planting and several topdressings were made during the rapid crop-growth period. The timing of topdressings was determined by monitoring crop-N status with a chlorophyll meter, and the amounts applied were adjusted to meet crop-N demand as determined by the expected yield. The performance of this approach was compared in 4 successive rice crops with the existing practices used by farmers. Average grain yield increased by 0.5 Mg ha^{-1} (11%) and N-fertilizer rate decreased by 5 kg N ha^{-1} with field-specific management compared to the baseline farmers' fertilizer practice (2).

The increased grain yields and reduced N-fertilizer rates resulted in significant gain in RE$_N$ and profit. Several factors contributed to the increased efficiency with field-specific management. Farmers' practices typically relied on a large N-fertilizer application early in the season, when the capacity for crop uptake was small, and 1 additional N topdressing. In contrast, field-specific management utilized 2 or 3 topdressings that were applied to achieve greater synchrony with crop demand, and individual doses of preplant or topdressed N were smaller than those applied by farmers. As a result, mean RE$_N$ increased from 30% with farmers' practices to 40% with field-specific management. On average, across all sites and cropping seasons, profit increased by USD 46 ha^{-1} crop^{-1} through the use of field-specific management. This gain in efficiency was achieved using prilled urea, which is the most widely used N fertilizer in Asia, and without major changes in other cropping practices. Spreading N applications more evenly during the growing season probably made the largest contribution to improved RE$_N$. It would also reduce the risk for environmental pollution associated with gaseous N losses or losses from runoff and leaching.

These results highlight the potential for improving NUE at the farm level in small-scale farming systems in developing countries. They also demonstrate that such improvements occur in small increments and will require significant long-term investments in research and extension education. Several years of on-farm experimentation are required to develop an "optimal" N-management scheme for a particular location that is characterized by a set of common environmental, socioeconomic, and cropping characteristics. Seasonal variation is large and fine-tuning of N management must be accomplished in accordance with other management factors that influence NUE such as balanced supplies of macro- and micronutrients, water management, optimal plant density, and pest control (40).

RESEARCH AND POLICY PRIORITIES TO IMPROVE NITROGEN-USE EFFICIENCY

Although there have been improvements in NUE for some crops (Fig. 1) and in several countries (13–14), concerns about the negative effects of reactive N load on ecosystem function and environmental quality persist (41). Reliable estimates of N losses from the major agroecosystems are required to understand the contribution of agriculture to these problems. Here again there are few studies in which N losses have been measured in on-farm settings across a reasonable range of representative environments; most estimates are based on field experiments conducted at research stations. Although such studies provide useful information about the relative importance of different loss pathways and the biophysical factors controlling them, they do not give accurate estimates of actual N losses under on-farm conditions. Despite the lack of hard data on N losses from on-farm environments, nitrate concentration in ground water often exceeds acceptable thresholds and nitrate losses contribute to eutrophication of surface water bodies in many agricultural ar-

eas where intensive cropping systems are the dominant form of land use (42). In addition, atmospheric N$_2$O concentration has increased rapidly since the 1950s in concert with the increase in N fertilizer applied to cropland.

While specific tolerance thresholds for N losses from cropping systems cannot be determined without more reliable data on hydrology and current levels of N losses, most agricultural scientists and ecologists agree on a number of issues regarding productivity and environmental requirements of future agroecosystems: i) food production must increase substantially to meet the needs of a much larger and wealthier human population; ii) nearly all of this increase must come from achieving greater yields on existing agricultural land rather than expanding production to marginal land or by further encroachment into natural ecosystems such as rainforests, wetlands, or estuaries; iii) farmers must achieve significant improvements in NUE to maintain acceptable standards of environmental quality; and iv) farmers must make a profit to stay in business. Agreement on these issues provides common ground for examining research priorities and policies that foster the tripartite goals of food security, agricultural profitability, and environmental quality.

Research Priorities

Given continued population growth and limited land resources, a strong emphasis should be given to understanding and improving NUE in the major cereal cropping systems that are endowed with good soils and climate and can support both high yields and high NUE based on the biophysical principles governing N supply and crop demand. Indeed, the challenge of sustaining adequate rates of gain in cereal yields while significantly improving NUE must receive explicit emphasis in the global research agenda. The magnitude of this challenge should not be underestimated for 4 reasons: i) crop physiological N requirements are tightly conserved as determined by photosynthetic pathway and grain N concentration (Figs. 1 and 2); ii) the yield response to crop-N accumulation is curvilinear (Fig. 3); iii) increased yields require greater N accumulation (Fig. 3), which in turn requires a larger pool of plant-available soil-N to support additional crop growth, but which is also more vulnerable to N losses from all pathways; and iv) the plant-available soil-N pool is highly variable (Fig. 4) and difficult to predict.

While it has been argued that application of existing technologies can meet much of the needed improvement in on-farm NUE, we believe such assessments are too optimistic because they are based on overestimation of current levels of on-farm RE$_N$ and they assume increased inputs from nitrogen-fixing legumes (43). Increased N input from legumes to reduce dependence on N fertilizer is not likely in the developing countries of Asia, where the majority of increased food demand and production is projected to occur, because inclusion of legume crops in cereal production systems has decreased markedly during the past 30 years (44). Diverting land for green manure crops in this region has become uneconomical because land scarcity and wage rates are increasing rapidly. Moreover, green manures used in irrigated rice systems have similar or lower RE$_N$ than inorganic N fertilizer (45, 46). Although inclusion of grain-legumes in rotation with cereals can reduce N-fertilizer requirements compared to continuous cereal cropping, they generally do not increase soil-N stocks because more N is removed in harvested seed than is replenished by biological N fixation. And, despite greater N-fertilizer requirements of continuous maize systems, recent evidence suggests that nitrate leaching is greater in a maize-soybean rotation than from continuous maize (47).

Another scenario for meeting both food needs and alleviating environmental damage from N used for crop production relies on a projection for a doubling in the rate of cereal yield increase compared to current rates of gain (14). Such a scenario is questionable because the rate of yield gain for the major cereals has

been declining steadily during the past 30 years (48). In contrast to these rather optimistic scenarios, we view the dual goals of meeting food demand while protecting the environment from excess Nr as one of the greatest ecological challenges facing humankind.

What, then, are the highest priorities for research investment and policies to improve NUE? A short-list of research targets that *are not* likely to have a large impact will be considered first. We see little scope for genetic improvement in PE_N because the relationship between economic yield and crop-N uptake is tightly conserved. This in turn suggests only marginal gains in N efficiency from molecular engineering of N assimilation and biochemical transformation pathways within the plant. Likewise, N-uptake capacity of crop root systems does not appear to be a sensitive factor limiting the efficiency with which most crops acquire soil or fertilizer N (4, 49), especially when compared to potential improvements in NUE from better crop- and soil-management practices. Similarly, we see little biological or economic advantage from organic N sources over inorganic N fertilizer when both are used with 'best management' practices because the same biophysical factors govern N cycling processes regardless of N source. Moreover, nearly all available animal manure is already used as inputs to cropping systems and the scope for increased inputs from legumes, as described above, is small.

Instead of these less promising targets, we see the greatest gains in NUE and environmental protection accruing from "precision management" in time and space of all production factors to maximize the synchrony between crop-N demand and the supply of mineral N from soil reserves and N inputs in high-yield systems (27, 50). Such precision-management approaches will be required for both large-scale agriculture in developed countries and small-scale farming in developing countries. Balancing N demand and supply will require breakthroughs in fundamental understanding of crop and soil ecology and organic geochemistry to allow development of dynamic and cost-effective N-management approaches. For example, although theoretical predictions indicate significant environmental and economic returns from site-specific N-management in USA maize systems, it has been very difficult to document actual improvements in yields or RE_N under on-farm conditions (51). This discrepancy between theory and practice results from large gaps in our knowledge of plant response to spatial and temporal variations in soil conditions and in effects on crop response to indigenous and applied N. Similar knowledge gaps limit our ability to utilize remote sensing of plant N status and simulation models as cost-effective and practical tools for improved N management.

The long-term cumulative "feedback effects" of N and crop-management tactics on soil quality also must be considered with explicit emphasis on productivity and NUE of the entire agro-ecosystem. Soil organic-matter content is a key measure of soil quality in upland cropping systems. Upland soils that sequester carbon also sequester N, resulting in greater indigenous N supply and a reduction in N-fertilizer requirements. Management practices that lead to increased soil organic matter or alter organic matter composition to achieve better synchrony between soil net-N mineralization and crop demand provide efficiency benefits over the long term (30, 52).

Quantitative, on-farm evaluations of improved technologies and measurements of N losses are needed to provide reliable estimates of potential improvements in NUE in the major agro-ecosystems. While present knowledge of individual components of the N cycle and estimates of N inputs are generally adequate, large uncertainties exist in the magnitude of N losses from both crop and livestock production systems. Better estimates of losses of specific N compounds (NO_3, N_2O and N_2) also are needed for major agroecosystems throughout the world. Scientific uncertainties are especially large for net-N immobilization/mineralization rates in systems where soil organic-matter levels are

changing over time as a result of increased cereal cropping intensity, higher yield levels, and conservation tillage or residue-management practices. Without such data, research investments and policies may not accurately target crucial components of the N cycle or promote the most cost-effective technologies.

Effective Policies

While there is a large body of published research on technologies for increasing NUE, relatively few have been adopted by farmers because they are not cost-effective or practical. Adoption of improved technologies typically requires additional skills and labor or investments in new equipment. Information on expected costs and economic returns from such investments is required to convince farmers of the benefits from adoption. The only data directly available to farmers regarding NUE are the grain yield they obtain from their fields and the amount of N fertilizer they apply. Unfortunately, these data provide little information about the size of the indigenous N supply, RE_N, or PE_N, all of which are essential for identifying management practices that increase both NUE of the cropping system and economic return from applied N. Farmers also need estimates of the portion of yield obtained from indigenous soil-N and the yield increase from applied N. A more thorough understanding of these NUE components are essential for management decisions that maximize returns from both indigenous and applied N, and which in turn minimizes the potential for N losses.

Because of the cryptic nature of these NUE components, both the public and private sector must play a greater role in providing information to crop producers about how various management and technology options influence these components. Policies must support strong research and extension programs that develop this capacity, especially for cereal-cropping systems that are rapidly intensifying. Policies must also recognize the potential for interactions between different environmental goals. For example, some technologies proposed for decreasing P runoff from fields that receive applications of livestock manure may increase the potential for N-leaching losses (53).

Low profit margins of most cereal production systems make it difficult for farmers to absorb the costs of environmental regulations. Incentive programs to promote adoption of N-efficient management practices are preferred because regulations imposed on farmers in one country can have the unintended effect of exporting crop and animal production systems with high Nr leakage to countries with the least stringent environmental guidelines. If at some point in the future scientific evidence clearly supports more drastic action to reduce N load in the environment, a global plan may be needed to concentrate food-crop production in agroecosystems with the highest biophysical potential to maximize grain output in relation to N losses and the potential for environmental damage.

References and Notes

1. The term reactive N (Nr) includes all biologically active, photochemically reactive, and radiatively active N compounds in the atmosphere and biosphere of the Earth. Thus, Nr includes inorganic reduced forms of N (e.g. NH_3, NH_4^-), inorganic oxidized forms (e.g. NO_x, HNO_3, N_2O, NO_3^-), and organic compounds such (e.g. urea, amines, proteins).
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RESPONSE OF MICROPROPAGATED SUGARCANE VARIETIES TO INOCULATION WITH ENDOPHYTIC DIAZOTROPHIC BACTERIA

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ABSTRACT

Previous studies estimated that sugarcane could obtain up to 60% of total nitrogen accumulated from BNF. Here a mixture of five endophytic diazotrophic strains was tested in a field trial, inoculated in two micropropagated sugarcane varieties and three locals, to determine the effects on commercial crop conditions. The sugarcane plantlets were inoculated *in vitro*, and after 17 months of growing in the field, the productivity and BNF contribution showed to be influenced by the plant genotype and soil type. The highest BNF contributions was observed in the poorest soil for both varieties. Smaller increases in productivity were observed for SP 701143 variety grown in soil with low or medium fertility. In contrast, a decrease in the stem productivity was observed in the SP 813250 variety grown in the three localities.

Key words: endophytic bacteria inoculation, biological nitrogen fixation, plant growth promotion bacteria, sugarcane.

INTRODUCTION

Research studies using ^{15}N-isotopic dilution technique estimated than more than 60% of total nitrogen accumulated in some sugarcane varieties were derived from BNF (2). Recent inoculation studies using micropropagated sugarcane plants showed a maximum BNF contribution up to 30% of total Nitrogen, obtained with a mixture of five diazotrophic bacteria species (1). In this work, the BNF contribution and cane productivity of the inoculated micropropagated sugarcane plants with the same mixture of endophytic bacteria was evaluated in a field trial. The plants were grown in three different locations, comprising different soil fertility levels.

MATERIALS AND METHODS

Endophytic bacteria used in the mixture to inoculate the sugarcane plants are listed in Table 1.

Table 1. Mixture of inoculant used in this study, and isolation sources.*

Bacteria species	Sign	Strain	Plant tissue	Sugarcane variety
G. diazotrophicus	Gd	BR 11281	Roots	*Saccharum sp.*
H. rubrisubalbicans	Hs	BR 11335	Roots	SP 701143
H. rubrisubalbicans	Hr	BR 11504	Stems	SP 701284
A. amazonense	Aa	BR 11115	Roots	SP 775181
Burkholderia sp.	Bk	BR 11366	Buds	SP 711406

*Culture collection, Embrapa/Agrobiologia

Plant inoculation was performed as described by Oliveira *et al.* (1). The strains were grown overnight in Dyg's liquid media, and an initial inoculum of 2.0×10^7 cells/ml of each species was inoculated in the MS medium. The plants were incubated for 120 hours at 12-hour fotoperiod at 30ºC. The micropropagated

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sugarcane varieties *SP 701143* and *SP 813250* were provided by Copersucar (SP), and grown in three different locations in soil with different fertility level. The stem productivity was evaluated in 16 months old plants grown in the field. The BNF contribution was measured 9 months after field growth, using a mass spectrometer Delta Plus (Finnigam, UK). The following formula was applied to estimate the BNF:

$$\%BNF = 100 \times \frac{(\delta^{15}N \text{ control plant} - \delta^{15}N \text{ test plant})}{(\delta^{15}N \text{ control plant})}$$

RESULTS

The cane yield obtained in Ultisol (low fertility soil) was significantly lower than the cane productivity of the Oxisol or the Alfisol (medium and high fertility soil, respectively) for both varieties (Fig. 1).

A significant BNF contribution for the nitrogen nutrition of sugarcane plants for both varieties was detected by the isotopic analysis. However, the positive income was observed only for the experiments performed at the Ultisol and Alfisol soils. It was impossible to run the isotopic analysis of the plants grown in the Oxisol soil probably because the massive use of nitrogen fertilisation in previous experiments (Table 2).

DISCUSSION

A different response to inoculation was observed for each tested sugarcane variety. The inoculation showed a small
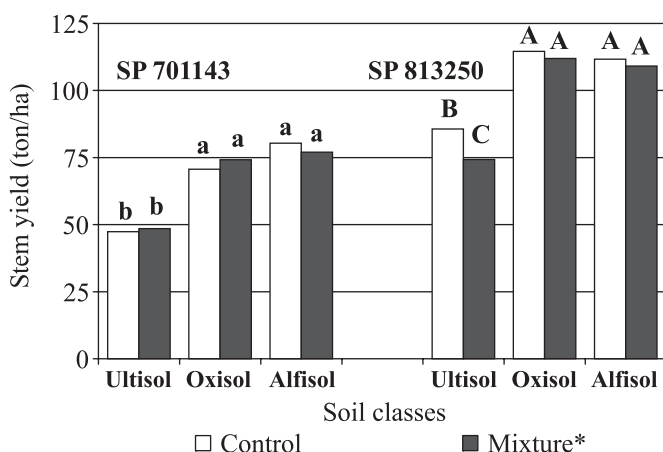

Figure 1. Stem productivity of two sugarcane varieties (16 months old plants) grown in three soil classes. Same letters do not differ statistically by LSD test at 5% of confidence. Means of 12 plots.

Table 2. Biological nitrogen fixation contributions to nitrogen nutrition of two endophytic diazotrophic bacteria inoculated sugarcane varieties grown in three soil classes.

Soil Class		% of total N derived from BNF	
	Locality	Var SP 701143	Var SP 813250
Ultisol	Seropédica - RJ	18.2 %*	31.4 %*
Oxisol	Piracicaba - SP	-4.5 %	-1.1 %
Alfisol	Jaú - SP	13.4 %*	5.9 %

* Significant at LSD test with 95% of confidence, obtained by comparison of $d^{15}N$ values of inoculated plants with $d^{15}N$ values of control plants. * See in Table 1.

increase (not significant) on the productivity of the SP 701143 variety when cultivated on Oxisol and Ultisol soil types, and decreased the stem yield when grown in the Alfisol. The SP 813250 variety showed a decrease of the stem yield with the inoculation, mainly for the plants grown in the Ultisol. This could be due to the breeding characteristics of the cane varieties used, where the SP 701143 was breeded to grow in low fertility soil classes, while the SP 813250 was much adapted to medium to high fertility soil classes. Those characteristics could influence the capacity of association with the inoculated bacteria.

In Alfisol, the BNF contribution as evaluated for the SP 701143 variety was up to 13.4% of total nitrogen accumulated in the plant, while in the SP 813250 variety it corresponded only to 5.9% of total N. Evaluations of plants grown in the Ultisol showed that the inoculation contributed with up to 18.2% of total nitrogen in the SP 701143 variety, while 31.4% of total N was derived from BNF in the SP 813250 variety (Table 2). Although, it was shown that substantial part of nitrogen was derived from the BNF, these results did not reflect in the stem productivity.

The results suggest that inoculation of the sugarcane crop seems to be more successful mainly in crops cultivated in soil classes with low fertility. In addition, the commercial variety used also influenced the interaction with the inoculated bacteria. The better understanding of the plant-bacteria interaction, the selection of endophytic diazotroph strains as well as sugarcane varieties, need to be exploited to obtain the maximum benefit from BNF.

ACKNOWLEDGEMENTS

RESUMO

Resposta da inoculação com bactérias diazotróficas endofíticas em duas variedades micropropagadas de cana-de-açúcar

Experimentos anteriores estimaram que a cana-de-açúcar pode obter até 60% do N acumulado via fixação biológica de nitrogênio (FBN). Neste trabalho, os efeitos da inoculação da mistura de cinco espécies de bactérias diazotróficas endofíticas foram testados em duas variedades de cana-de-açúcar micropropagadas, sob condições de campo. Após 17 meses de crescimento, a produtividade e a FBN apresentaram influência do genótipo vegetal e da localidade de cultivo. As maiores contribuições via FBN foram observadas no solo de menor fertilidade, para ambas variedades de cana-de-açúcar. Pequenos aumentos de produtividade foram observados para a variedade SP 701143 nos solos de baixa e média fertilidade. Por outro lado, a inoculação na variedade SP 813250 apresentou decréscimo de produtividade nos três tipos de solo testados.

Palavras-chave: inoculação com bactérias endofíticas, fixação biológica de nitrogênio, bactérias promotoras do crescimento vegetal, cana-de-açúcar.

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Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria

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Abstract

It is well described that the beneficial interactions between plants and bacteria are genotype and site specific. Brazilian sugarcane varieties can obtain up to 70% of their nitrogen requirement from biological nitrogen fixation (BNF), and this contribution is related to the Brazilian breeding and selection processes, by example of the variety SP70-1143. In this study the effect of two inoculation mixtures containing diazotrophic bacteria in our earlier pot experiment was evaluated with two sugarcane varieties, a known responder, SP70-1143, and a newly selected variety, SP81-3250, to investigate the sugarcane genotype effect and the role of the mixtures. The sugarcane varieties SP70-1143 and SP81-3250 were grown under commercial field conditions at three sites with contrasting soil types: an Alfisol, an Oxisol and an Ultisol that means a low, medium and high natural fertility respectively. The stem yield and BNF contribution in response to bacterial inoculation were influenced by the strain combinations in the inoculum, the plant genotype, and the soil type and nitrogen fertilization, confirming the genetic and environmental influence in PGP-bacteria interactions. Inoculation effects on the BNF contribution and stem yield increased in the variety SP70-1143 grown in the Alfisol without nitrogen fertilization for three consecutive crops, and it was equivalent to the annual nitrogen fertilization. The plants grown in the Oxisol showed small increases in the productivity of the variety SP70-1143, and in the Ultisol the sugarcane plants presented even decreases in the stem productivity due to inoculation with diazotrophic bacteria mixtures. The results demonstrate the feasibility of the inoculation technology using diazotrophic bacteria in micropropagated sugarcane varieties grown in soils with low to medium levels of fertility. In addition, the results also indicated that specific plant – bacteria – environment combinations are needed to harness the full benefits of BNF.

Introduction

Brazil is the largest sugarcane producer in the world, with the crop occupying more than 5 million hectares and generating up to one million direct jobs (web data, www.unica.com.br). Production is still growing due to the increasing demand for ethanol for export. The annual application of nitrogen-fertilizer for Brazilian sugarcane is around 50 kg N ha^{-1}, with a cost near US\$ 500 t^{-1} (web data, www.udop.com.br). If N-fertilizer application could be reduced by one half (less 125,000 t N y^{-1}) due to the biological

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nitrogen fixation (BNF), producers could save estimated US\$ 62.5 m y^{-1}. This approach could significantly reduce cost of bio-energy. The BNF contribution of Brazilian sugarcane varieties was first evaluated using $^{15}N_2$ isotope incorporation in the plant tissues (Ruschel et al., 1975). The potential of BNF contribution was reported to be up to 70% of total nitrogen incorporated by some sugarcane varieties (Urquiaga et al., 1992). The success of Brazilian sugarcane varieties in obtaining BNF contributions for its nitrogen demand are believed to be due to the historical characteristics of cane breeding programs in Brazil, mainly driven to develop varieties which produces well in low fertility soils, thus indirectly selecting BNF-associative varieties (Baldani et al., 2002).

No nitrogen-fixing species have yet been definitively identified to provide nitrogen to grasses. Nevertheless, studies using plant growth-promoting bacteria (PGP-bacteria) as inoculants have shown promising results with improvements in plant nitrogen status and productivity in various cropping systems (Boddey et al., 2003; Kennedy et al., 2004; Okon and Labandera-Gonzalez, 1994). Among the essential factors needed to obtain an optimum response to PGP-bacteria inoculants, plant and bacterial genes seem to play the major role because of the strong associative status of the relationship (Assmus et al., 1995; Bashan and Holguin, 1997; Benizri et al., 2001; Olivares et al., 1997; Sturz and Nowak, 2000; Van Peer et al., 1990), but this genes may be under the regulation of environmental conditions. Actually, the relationship between sugarcane and diazotrophic bacteria has not been definitively understood. In a recent search for N_2-fixing endophytes in Japanese sugarcane varieties, Ando et al. (2005) found that *Bradyrhizobium*, *Serratia*, and *Klebsiella* are promising candidates for predominant endophytic diazotrophs in sugarcane using nifH gene segments were amplified with degenerate primers from DNA extracted from stems of sugarcane without culturing them. It has been suggested that the endophytic habitat provides a better environment for the PGP-bacteria rather than the rhizosphere, because the direct provision of nutritional elements, and the low O_2 environment needed for optimum nitrogenase expression. In return, the bacteria may provide the host plant with biologically fixed nitrogen and other growth-promoting molecules (Baldani

et al., 1997; Döbereiner et al., 1995). However the contribution of rhizosphere and soil bacteria should not be underestimated, because of the high diversity and population levels (Baldani and Baldani, 2005).

The inoculation practice of PGP-bacteria in non-legume plants has been adopted in some countries, and comprises a promising agribusiness (Bashan, 1998; Kennedy et al., 2004). Unfortunately, the improvement of productivity achieved with such practices still presents high variation (from zero up to 30%) and low reproducibility for several crops (Dobbelaere et al., 2003; Okon and Labandera-Gonzalez, 1994). The widespread use of PGP-bacteria inoculants as a habitual agricultural practice requires a more critical assessment because of the high variability observed in the productivity of plants grown at different sites and in different crop rotations. On the other hand, the substitution of pesticides and fertilizers that require high energy inputs in their manufacture is highly desirable, as already demonstrated in the Brazilian soybean crop (Alves et al., 2003). Positive responses to inoculation with diazotrophic bacteria have been demonstrated in sugarcane plants grown under greenhouse and field conditions (Mirza et al., 2001; Muñoz-Rojas and Caballero-Mellado, 2003; Muthukumarasamy et al., 1999; Oliveira et al., 2002; Sevilla et al., 2001). On the other hand, studies to evaluate the BNF potential of uninoculated Brazilian sugarcane varieties grown in the field point out the importance of favorable environmental conditions (soil and climate) for the best beneficial effects (Boddey et al., 2003). Besides the BNF contribution to sugarcane by the diazotrophic bacteria, such bacteria may also promote plant growth due to the production of phytohormones (Bastián et al., 1998; Maheshkumar et al., 1999; Paula et al., 1991; Sevilla et al., 1998) and protection against pathogenic bacteria (Muthukumarasamy et al., 2000; Piñon et al., 2002).

Is now well recognized that the best effect, or a compatible interaction (as defined for pathogenic relationships) of the plant growth-promoting bacteria in non-legumes is associated with the interactions of the genotypes of the host plant and the bacteria and the environment (G×E interactions) (Gyaneshwar et al., 2002; Kennedy et al., 2004). In sugarcane, the influence of plant

variety, bacterial strain and nitrogen amendment on the association have already been demonstrated (Muñoz-Rojas and Caballero-Mellado, 2003). Indeed, the soil type showed a stronger influence in the rhizosphere microbial density and community structure than did different maize cultivars (Chiarini et al., 1998), but not in conifers (Chanway et al., 2000). Nevertheless, abiotic soil factors were demonstrated to control the activity of introduced bacteria as observed for *Azospirillum* (Bashan et al., 1995; Van Veen et al., 1997).

The diversity of the diazotrophic colonizers of sugarcane, associated with the low response to nitrogen fertilization in tropical areas, led this crop to be considered as a model in the BNF studies with non-legume in Brazil (Baldani and Baldani, 2005; Boddey et al., 2003). Positive results obtained in studies of sugarcane inoculation with PGP-bacteria (Muthukumarasamy et al., 1999; Oliveira et al., 2002) encourage and justify research to identify the best bacterial species as inocula, strategies and methodologies to introduce selected strains in the commercial sugarcane varieties. The aim of this work was to evaluate the response of two sugarcane varieties grown in three soil types in the field to inoculation with two mixtures of selected diazotrophic strains.

Material and methods

Soil types

Inoculated plants were grown in three contrasting soil types representative of major sugarcane producing soils in Brazil:

(1) High soil fertility – An Ultisol located at Jaú (São Paulo State); (2) Medium soil fertility – An Oxisol located at Piracicaba (São Paulo State); (3) Low soil fertility – An Alfisol located at Seropédica (Rio de Janeiro State). The chemical analysis of those soils is presented in Table 1.

The three experimental sites were amended with phosphorous and potassium fertilizers as recommended for sugarcane based on soil analysis, and also micronutrients in the form of fritted trace elements, FTE (1.0 kg ha^{-1}) in each growth season. The three soil types received one application of dolomite one month before planting of the micropropagated plants. Three rates of nitrogen fertilizer (ammonium sulfate) were applied as treatments: 0, 25.0 and 50.0 kg N ha^{-1} (none, half and the entire nitrogen dose as recommended by COPERSUCAR, a Brazilian cooperative association of 91 companies of industrial producers and sugarcane suppliers), and 0, 50.0 and 100.0 kg N ha^{-1} in the ratoons. The plants were irrigated at each site during the initial 2 months of growth to ensure establishment in the field. The experimental units were plots of 30 m^2 with plants distributed in five rows of 6 m with 1.5 m between rows and 0.5 m between plants.

Sugarcane varieties

Two micropropagated sugarcane varieties were tested based on contrasting breeding characteristics. The variety SP70-1143 has been recommended for cropping on low fertility soils, and was used as a reference variety since Urquiaga et al. (1992) demonstrated its high BNF contribution. The variety SP 813250 is newer and was bred for cropping on medium to high fertility soils (Table 2). COPERSUCAR (www.copersucar.com.br) bred both sugarcane varieties and provided about 9000 axenic micropropagated plants of each variety.

Inoculum and inoculation

The Embrapa Agrobiologia Culture Collection (BR 465-RJ, km 47 – CP 74.505, CEP 23.890-000 – Seropédica, RJ, Brazil) provided the five species of diazotrophic bacteria used in this

Table 1. Chemical analysis of the soils at the three experimental sites evaluated

Soil site	pH (water)	Al (cmolc/dm^3)	Ca + Mg (cmolc/dm^3)	Ca (cmolc/dm^3)	P (mg/dm^3)	K (mg/dm^3)	N (%)
Ultisol	4.7	1.1	8.4	1.8	15.0	78.0	0.1
Oxisol	4.9	1.2	2.1	0.8	11.0	44.0	0.1
Alfisol	5.0	0.2	6.5	0.6	5.0	38.0	0.0

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Table 2. Characteristics of sugarcane varieties used in this study

	SP70-1143	SP81-3250
Soil fertility	Low	Medium
Crop season	Middle	Beginning/middle
Cross origin	IAC48-65 X unknown	CP70-1547 X SP71-1279
Productivity	High	High
Ratoon growth	Optimum	Optimum
Maturity	Medium	Medium
Sucrose Tenor	Medium	High
Fiber Tenor	High	High
Smut	Resistant	Susceptible
Rust	Susceptible	Resistant
Borer	Intolerant	Resistant
Spittlebug	Intolerant	Susceptible

Table 3. Diazotrophic species, bacterial strains, sugarcane variety source and code adopted in this work

Species	Strains*	Sugarcane variety source	Bacterial code
Gluconacetobacter diazotrophicus	BR 11281	Roots – *Saccharum* sp.	Gd
Herbaspirillum seropedicae	BR 11335	Roots – SP70-1143	Hs
Herbaspirillum rubrisubalbicans	BR 11504	Stems – SP70-1284	Hr
Azospirillum amazonense	BR 11115	Stems – CB45-3	Aa
Burkholderia tropica	BR 11366	Plantlets – SP71-1406	Bk
Mixture 1 – Gd + Hs + Hr			
Mixture 2 – Gd + Hs + Hr + Aa + Bk			

*Bacterial number at the Bank of Diazotrophic Bacteria Culture Collection of Embrapa Agrobiologia.

work (Table 3). The inoculants were prepared by growing each strain overnight in 5 mL of Dyg's liquid media (Oliveira et al., 2002) and using it as a inoculum produced a larger volume (100 mL of culture of each strain in Dyg's liquid medium). Samples were counted in a Neubauer chamber and normalized to 10^9 cells mL^{-1} using sterile Dyg's medium. The inoculation mixtures were obtained by mixing equal volumes of each normalized inoculum, to reach the bacterial mixtures as presented in Table 3.

Flasks of 250 mL capacity containing 50 mL of modified MS medium were prepared for sugarcane inoculation (Reis et al., 1999). Four to six rooted plantlets were inoculated with 0.1 mL bacterial mixture (2.0×10^6 cells mL^{-1}) in the modified MS. After inoculation, the plants were incubated *in vitro* for 120 h (5 d) at 30 °C and 12 h photoperiod (50 μmol m^{-2} s^{-1} of active photosynthetic light). The plants were acclimatized in a nursery for 60 d under 26–30 °C temperature and daily watered, in trays contain-ing the commercial substratum Plantmax® (Eucatex, www.eucatex.com.br) routinely used by COPERSUCAR to propagate sugarcane. Nitrogen was added twice (ammonium nitrate) in a 10 mg L^{-1} solution spread over the leaves, at the 45th and at the 60th day of the acclimatization.

Sampling and BNF contribution

Plant biomass was harvested after 15 (Ultisol and Oxisol) or 17 months (Alfisol). The ratoon biomass was harvested after 12 months at all three sites. At the Alfisol site, an additional ratoon was harvested (3rd cut, not presented in the Figure 1 for better comparisons). The natural abundance of the ^{15}N isotope (δ^{15}N) present in the inoculated plants and the δ^{15}N of the uninoculated treatments were analyzed to determine the BNF contribution. In this case, 10 samples of the leaf index (leaf + 3) were collected 9 months after growth in the field for both the plant and ratoon crops, sampling plants growing in the middle

rows of plots that had not received nitrogen fertilizer. The collected leaves were washed with distilled water, dried and milled for isotopic analysis using a Delta Plus mass spectrometer (Finnigan MAT, Germany). The proportion of plant nitrogen derived from air (Ndfa) obtained by the inoculated treatment was calculated using the following equation:

$$\% \text{Ndfa} = \frac{100 \times (^{15}\text{N control plant} - (^{15}\text{N test plant}))}{(^{15}\text{N control plant})}$$

Statistical analysis

The statistical design was a factorial of 3×3 with four replicates (inoculation treatment $\times$ nitrogen rate) for each variety grown at each experimental site. Comparisons of the means were made using LSD test at 5% level of confidence.

Results

At all experimental sites, the sugarcane variety SP81-3250 demonstrated higher productivity than SP70-1143. The ratoon crop of SP81-3250 was also higher than SP70-1143 except in the Alfisol, although dry matter yield decreased in the ratoons of both varieties at all sites (Figure 1). Indeed, the dry matter decreased in the ratoons of both varieties at the three sites. Interactions between the inoculation treatments and the nitrogen rates were significant. Inoculation promoted increases as well as decreases in the productivity of the sugarcane, with regard to the interaction of the soil classes, sugarcane varieties and nitrogen rates. The inoculants showed better growth-promoting effects in the soils with lower and medium fertility, and without nitrogen fertilizer.

Variety SP70-1143

Increases in the stem yields of the sugarcane variety SP70-1143 in response to inoculation with the mixtures of the bacteria were observed in the Alfisol soil (poorest fertility), without nitrogen fertilizer amendment. The increases were up to 18.1, 10.2 and 7.1% for the three consecutive cuts in plants inoculated with Mixture 1, while Mixture 2

increased biomass up to 27.8, 17.9 and 31.4%, respectively. The highest nitrogen fertilizer dose increased the stem yield up to 31.9, 18.3 and 13.1% for the three cuts (Figure 1a). The observed BNF contribution was in agreement with the biomass results at the Alfisol site. Inoculation with Mixture 1 increased plant nitrogen up to 18.6 and 22.7% for the plant crop and the first ratoon, respectively. The Mixture 2 response was higher, reaching 31.3 and 38.3% of the total nitrogen in the plant crop and in the first ratoon, respectively (Table 4). Nevertheless, interactions with nitrogen fertilization and the inoculum mixtures showed no yield increases, and sometimes the productivity of stems became lower than the uninoculated nitrogen-fertilized plants, as observed for the other two sites.

The effect of inoculation with the bacterial mixtures and nitrogen fertilization in the stem productivity at the Oxisol site was lower, compared with that observed at the Alfisol site. No statistically significant differences were detected for any treatment. In the ratoon crop, the stem yield was similar to the controls (Figure 1c). The $\delta^{15}\text{N}$ estimate of the BNF contribution in the Oxisol was zero, for both the plant crop and the ratoon crop (Table 4). In contrast, an increase of 9% was obtained by the addition of the higher nitrogen dose (50 kg N ha^{-1}), but only for the plant crop (Figure 1c). If compared to the Alfisol, the combination of inoculation and nitrogen fertilization in the Oxisol seems to give an additive effect for the plant crop yield, where the highest productivity (11.8% increases) was observed for the combination of Mixture 2 and 25 kg N ha^{-1}.

When the variety SP70-1143 was grown in the Ultisol, a slight non-significant decrease in the stem yield was observed with the PGP-bacteria inoculation. In the plant crop, the productivity decreased up to 8 and 6.8% with the inoculation of Mixtures 1 and 2, respectively (Figure 1c). In the ratoon crop, the response was also negative and the decreases reached up to 7.6 and 5.1% with Mixtures 1 and 2, respectively. Also, the nitrogen fertilization with one half of the recommended dose negatively influenced the stem productivity of uninoculated plants, with up to 5.9 and 8.7% for the plant and the ratoon crops, respectively (Figure 1c). On the other hand, the full nitrogen dose showed a non-significant

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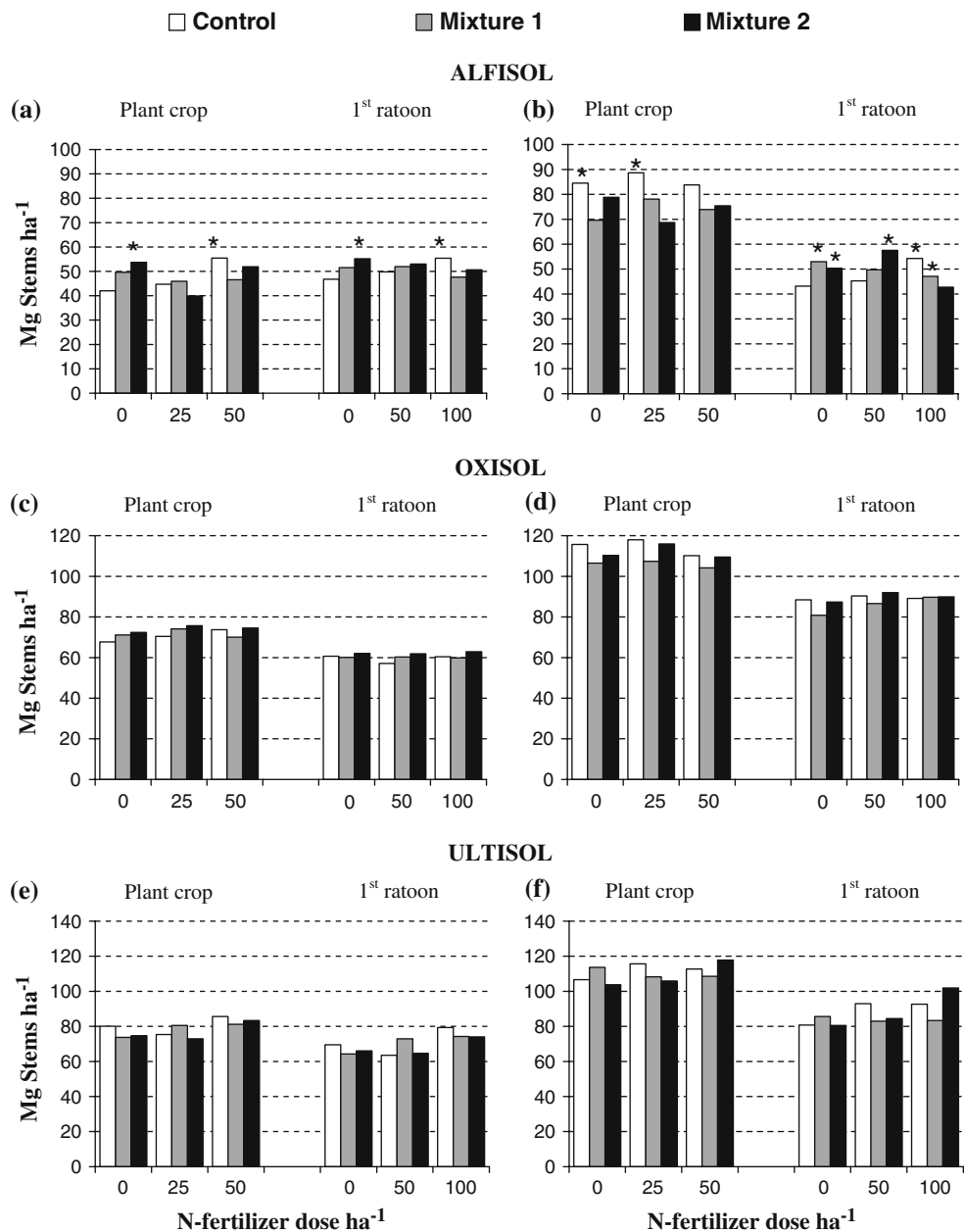
Figure 1. Sugarcane yield in response to PGPB inoculation and nitrogen fertilization. Statistics differences by LSD test at 5% level of confidence are shown as * above vertical bars. Contrasts should be considered for each nitrogen dose. Mixture 1 and Mixture 2 is described in accordance to Table 3. The data are means from four plots. (a) Variety SP70-1143; (b) variety SP81-3250; (c) variety SP70-1143, (d) variety SP81-3250; (e) variety SP70-1143; 5 (f) variety SP81-3250.

increase in the stem yield of up to 6.9 and 14.1% for the plant and ratoon crops, respectively. Considerable BNF contributions up to 23.7 and 13.4% for the plant crop inoculated with the Mixtures 1 and 2, respectively, but not corresponded to the cane yields. No BNF contribution was observed in the ratoon crop inoculated with either mixture (Table 4).

Table 4. Biological nitrogen fixation contributions of two diazotrophic bacteria mixtures inoculated in two micropropagated sugarcane varieties grown in three soil types

Soil types	Sugarcane variety	Inoculation treatments				
		Control	Mixture 1[a]		Mixture 2[b]	
		$\delta^{15}N$	$\delta^{15}N$	BNF estimate	$\delta^{15}N$	BNF estimate
Plant crop						
Ultisol	SP70-1143	6.23	4.75*	23.72	5.39	13.44
	SP81-3250	6.26	6.09	2.77	5.89	2.27
Oxisol	SP70-1143	4.20	4.56	−8.39	4.39	−3.03
	SP81-3250	5.17	5.07	2.45	5.23	−0.53
Alfisol	SP70-1143	5.38	4.83	18.62	4.40*	31.28
	SP81-3250	5.60	5.38	3.39	3.84*	21.39
Ratoon crop						
Ultisol	SP70-1143	6.23	6.64	−6.58	7.65	−22.79
	SP81-3250	6.26	7.70	−23.00	7.35	−17.41
Oxisol	SP70-1143	4.20	5.42	−29.05	5.11	−21.67
	SP81-3250	5.17	6.49	−25.53	5.99	−15.86
Alfisol	SP70-1143	5.38	4.16*	22.68	3.32*	38.29
	SP81-3250	5.60	3.21*	42.68	3.69*	34.11

[a/b]According to Table 3. *Significant LSD test at 5% level of confidence, obtained by comparison of uninoculated plants $\delta^{15}N$.

Variety SP81-3250

The plant crop of the variety SP81-3250 showed no response to nitrogen fertilization at the Alfisol site. In addition, a decrease of 17.6% (significant) and 6.7% of plant crop productivity was observed in response to inoculation with Mixtures 1 and 2, respectively. A negative effect on the stem productivity of the plant crop was also observed with the combination of PGP-bacteria inoculation and nitrogen fertilization (Figure 1b). Interestingly, and similar to the observed results for the variety SP70-1143 at the Ultisol site, the BNF measured by the $\delta^{15}N$ analysis demonstrated a significant contribution of up to 21.4% in the plants inoculated with Mixture 2 (Table 4).

The response of the ratoon crop to inoculation and nitrogen fertilization at the Alfisol site were quite different to the plant crop productivity, although the stem yield had diminished by one half. In this case, there were significant increases in the productivity of up to 22.7 and 24.7% with Mixture 1, and 16.6 and 16.7% with Mixture 2, for the two consecutive ratoons. A positive effect was also observed with nitrogen fertilization, with the stem yield increasing up to 4.9 and 18.3% with addition of 50 kg N ha^{-1} and 25.8 and 8.1% with 100 kg N ha^{-1} for the

first and second ratoons, respectively (Figure 1b). The combination of nitrogen fertilization plus the inoculum mixtures promoted stem yield increases of the two ratoon crops, mainly the plants inoculated with Mixture 2 and fertilized with 50 kg N ha^{-1} (Figure 1b). Indeed, the BNF contribution in the ratoon crop was higher in plants inoculated with Mixture 1 than Mixture 2, with the contribution being up to 42.7 and 34.1%, respectively, contrasting with that observed for the plant crop (Table 4).

At the Oxisol site, variety SP81-3250 showed similar responses in stem yield productivity to that observed for variety SP70-1143, with no significant effect of either nitrogen fertilization or inoculation with the bacterial mixtures (Figure 1d). Decreases in the stem productivity of up to 8.0 and 8.6% were observed with Mixture 1, and up to 4.7 and 1.3% with Mixture 2 for both the plant crop and the ratoon crop, respectively. No BNF contribution in the plant crop or in the ratoon crop was observed with the $\delta^{15}N$ analysis.

Sugarcane plants of the variety SP81-3250 grown in the Ultisol showed no effect of nitrogen fertilization or the two inoculation mixtures applied in the plant crop. In the first crop year, a small increase (up to 8.5%) in the stem yield with half of the recommended N dose was

observed, while the entire dose increased the stem yield up to 5.7%. Mixture 1 increased the stem productivity up to 6.6% while Mixture 2 decreased the productivity up to 2.7% (Figure 1f). In the ratoon crop, the productivity of stems also decreased and reached up to 75% of the plant crop yield. The productivity of the nitrogen-fertilized ratoon increased up to 15.1 and 14.8% with the use of one half and the entire nitrogen dose, respectively. Mixture 1 increased the stem productivity up to 6.0%, while Mixture 2 had no effect on the ratoon (Figure 1f). The BNF estimation resulted in small contributions of 2.8 and 2.3% for the plant crop inoculated with Mixtures 1 and 2, respectively, and no contribution was estimated in the ratoon (Table 4).

Discussion

In this work, we observed the influence of the soil type, inoculation mixture and nitrogen fertilization level in the yield response and BNF contribution of two sugarcane varieties. A compatible interaction was observed in the Alfisol soil type, where the inoculation with the bacteria showed the best performance among the tested soil classes. This finding is supported by the results of stem yield and BNF contribution (Figures 1 and 2, Table 4), where Mixture 1 was the best inoculum for the variety SP81-3250, while Mixture 2 was the best inoculum for the variety SP70-1143. The variability of the BNF contribution among different sugarcane varieties grown at the same experimental site was previously reported (Urquiaga et al., 1992), as well as different BNF contributions for the same sugarcane variety grown at different sites (Boddey et al., 2003). In addition, the incidence of spittlebugs which can be used to track C4 grasses that exhibit associative nitrogen fixation, as pointed out by Thompson (2004), were not covered in this work. However, the most responsive sugarcane variety SP70-1143 is intolerant to spittlebugs, while the least responsive variety SP81-3250 is susceptible (Table 2).

The measured BNF contributions of sugarcane grown in the Alfisol were in agreement with the productivity, meaning higher BNF contributions associated with higher stem yields. On the other hand, the sugarcane varieties grown in soils with higher natural fertility (Oxisol and Ultisol), showed a lower response to the inoculation mixtures as did the nitrogen fertilization, and therefore resulted in positive or negative effects. In such soil classes, the estimation of the BNF contribution was not always correlated with the stem yield (Figure 1d, Table 4), suggesting that BNF may not be the unique plant growth-promoting effect mediated by inoculation with the mixtures. Disturbances in the soil $\delta^{15}N$ by continuous experiments under nitrogen-fertilized conditions might indeed underestimate the BNF contributions at the Oxisol and the Ultisol sites. The available nitrogen in the Oxisol and Ultisol soils could also inhibit the BNF contributions of diazotrophic bacteria to the N nutrition of the sugarcane. In fact, Kennedy et al. (2004) and Muñoz-Rojas and Caballero Mellado (2003) pointed out that the BNF process could not be the major role played by the associative bacteria in promoting the growth of sugarcane plants.

The productivity results obtained from the Oxisol and Ultisol experiments suggest that the nitrogen available in the soils was sufficient for the development of the sugarcane varieties, minimizing the growth-promoting effects and BNF contribution of the inoculated mixtures. The lower response to nitrogen fertilization with respect to the stem yield, even when 100 kg N ha^{-1} was applied in the ratoon crops, supports this hypothesis. However, we point out that the inoculation of the plants grown on the Alfisol, increased the stem yield of both varieties SP70-1143 and SP81-3250 in the ratoon crops to a degree similar to or higher than the nitrogen fertilizer added annually.

The negative effect of nitrogen fertilization on the population of sugarcane associative diazotrophic bacteria such as G. diazotrophicus and Herbaspirillum, as well as its inhibitory effect on the BNF in sugarcane, has been previously reported (Fuentes-Ramírez et al., 1999; Reis et al., 2000). The nitrogen fertilization levels used in this study should have depleted the plant growth-promoting potential of the inoculation mixtures at all three experimental sites, by inhibiting the plant-PGP-bacteria compatible

interaction. Indeed, increases in the stem yield in response to nitrogen fertilization combined with the inoculants were without a defined behavior, and confounded the statistical analysis (Figures 1a–1f).

Our main goal was to discover if the response to inocula mixtures was related to the sugarcane variety and edaphic conditions, as well as to the nitrogen availability to the plants. Further studies concerning other variables such as the colonization of micropropagated plants by the native diazotrophic bacteria as well as the temporal expression of plant genes responsible for a compatible association over contrasting crop conditions, may help to explain some of the results obtained in the present study. Nevertheless, the persistence of the beneficial inoculation effects over three consecutive harvests at the Alfisol site, with the lower responses to mineral nitrogen fertilization in both sugarcane varieties at all the tested soil classes, encourage us to recommend the adoption of the inoculation technology in sugarcane in the near future. Actually, a multidisciplinary breeding program envisaging sugarcane varieties able to obtain their nitrogen supply mainly from an association with efficient diazotrophic strains, should be initiated to support the continuous needs for new varieties by the sugarcane industry.

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Spatial extent of riverine flood plumes and exposure of marine ecosystems in the Tully coastal region, Great Barrier Reef

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Abstract.   Tully River flood plume monitoring data for 11 events (1994–2008) were used to determine what physical characteristics of the floods (size of flood, direction of plume movement, shape of hydrograph) most influence the flood plume water quality and areal extent. During some events, the maximum area influenced by the Tully flood plumes extended into the Coral Sea. Areal extents depended on wind direction and discharge volume, with large extents more likely during light or northerly winds. Strong gradients in water quality existed away from the Tully mouth during the wet season and the adjacent marine ecosystems were regularly exposed to land-derived material. Flood plumes were grouped into three plume types: primary, secondary and tertiary plumes, based on water-quality characteristics (suspended solids, coloured dissolved organic matter and chlorophyll). The number of reefs and seagrasses exposed to plume waters varied from year to year, and was dependent on the characteristics of the event. Over the 11 years, out of the major 37 reefs and 13 seagrass meadows identified in the Tully marine area, between 11 (30%) and 37 coral reefs (100%) and most of the seagrass meadows were inundated by either a primary or secondary plume every year.

Introduction

River run-off is the principal carrier of eroded soil (sediment), nutrients, pesticides and chemical pollutants from the land into the coastal and inshore waters of the Great Barrier Reef (GBR) lagoon (Furnas 2003). On average, $\sim$70 km^3 of freshwater is discharged each year by rivers and streams into the GBR lagoon (Furnas 2003). Most of this run-off is delivered in discrete, short-lived flood events during the 5-month summer wet season, forming distinct flood plumes in the coastal zone that sometimes reach far out into the lagoon. In the wet season, the estuaries of the GBR coast are dominated by river run-off, and the 'estuarine' mixing zone, where the salinities range from 0 to 36, is located in the marine environment (Dagg *et al.* 2004), which is quite different to many temperate rivers (Eyre 1998).

Riverine plumes and the materials they carry have always had an impact on the GBR during these short-term events. However, elevated concentrations of nutrients, suspended sediments and pesticides, owing to changes in land use over the past 200 years of European settlement, are now potentially affecting the health of coastal and inshore ecosystems (Furnas 2003; Brodie and Mitchell 2005; Fabricius 2005; Schaffelke *et al.* 2005). The large quantities of sediment, nitrogen, phosphorus and significant amounts of pesticides lost from agricultural systems are easily measurable in rivers as they discharge into the GBR in flood conditions (Devlin and Brodie 2005; Lewis *et al.* 2009). The flood waters of rivers draining catchments dominated by agriculture typically have, for example, up to 30-fold higher concentrations of dissolved inorganic nitrogen (nitrate and ammonium) than rivers with undeveloped catchments.

Land run-off in many systems is seen as a source of contaminants that can have a negative impact on coastal ecosystem health and productivity. Increased turbidity and herbicide concentrations can negatively affect the growth and abundance of coastal and inshore seagrasses (Schaffelke *et al.* 2005; Waycott *et al.* 2005). In addition to physical disturbance, water quality is an important driver of coral reef health at local (reviewed in Fabricius 2005), regional (van Woesik *et al.* 1999; Fabricius *et al.* 2005), and GBR-wide scales (De'ath and Fabricius 2008). The effects of various water-quality constituents are manifold, including disturbance by sedimentation, light reduction by increased turbidity, reduced calcification rates by excess inorganic nutrients and inhibition of photosynthesis by herbicide exposure, and generally affect early life-history stages more than adult corals (e.g. Fabricius 2005; Negri *et al.* 2005; Cantin *et al.* 2007). Increases in freshwater discharge, sediment load and nutrients have been linked with a decline in live coral cover (Restrepo *et al.* 2006) and an increase in the areas of deoxygenated water in summer (Malakoff 1998; McKee *et al.* 2004). Corals are phototrophic organisms and reduced light availability as a result of high turbidity or sedimentation leads to resource limitation (Fabricius 2005; Cooper *et al.* 2008). In addition, exposure of corals to elevated levels of nutrients, sedimentation and turbidity may affect certain species that are sensitive or vulnerable to these environmental conditions. This may lead, in

the medium to long term, to reduced densities of juvenile corals, subsequent changes in the community composition, decreased species richness and shifts to communities that are dominated by more resilient coral species and macroalgae (van Woesik *et al.* 1999; Fabricius *et al.* 2005; DeVantier *et al.* 2006).

The impact of flood plumes, in terms of their extent, duration and biogeochemical processes, is intrinsically linked to catchment management and reef health; however, our understanding of the drivers and consequences of plume waters is limited for the GBR. The aim of the present study was to analyse flood plume monitoring data from one GBR catchment and its associated marine area over a period of 14 years to determine what physical characteristics of the floods (size of flood, direction of plume movement, shape of hydrograph) most influence the flood plume water quality and areal extent, assuming that no major land-use changes occurred that caused changes in material loads and delivery.

Materials and methods

Data collection

Riverine plume monitoring is an essential component of the long-term monitoring of marine water quality in the GBR. Flood plume monitoring was conducted by the Great Barrier Reef Marine Park Authority (GBRMPA) from 1994 to 2002 (Devlin and Brodie 2005). Recent plume monitoring (2007 to present) has been undertaken as part of the current Reef Plan Marine Monitoring Program (Prange *et al.* 2007). This programme has monitored water quality, seagrass and coral reef status in the inshore GBR lagoon (along ~1000 km of coastline) since 2005 as part of a government initiative 'to halt and reverse the decline in water quality entering the GBR'.

Study area

The Tully and Murray catchments are located within the Wet Tropics Region of North Queensland and drain wet tropical rainforest in the upper reaches, beef grazing along the mid reaches and a large coastal floodplain with a series of interconnected wetlands that have been extensively modified to support sugarcane and banana production as well as urban centres (Armour *et al.* 2009; Kroon 2009). The considerable floodplain network transports sediments, nutrients and pesticides into the GBR, either directly through these wetlands or via the larger Tully and Murray Rivers (Bainbridge *et al.* 2009). During the wet season, the coastal and inshore areas adjacent to the Tully catchment are regularly exposed to flood waters from the Tully River, and to a lesser extent from the Herbert River via the Hinchinbrook Channel.

The Tully River is one of Australia's least variable rivers, representing the generally wet tropical climate of the region. It floods regularly, one to four times per year, with riverine discharge extending into the adjacent marine waters. The marine environment adjacent to the Tully catchment has several continental islands with well-developed fringing reefs, which are of public and economic importance for the tourism industry and recreational activities including camping and fishing (GBRMPA 2009). The coastal and inshore zone also supports intertidal and subtidal seagrass beds. The area has several inshore Marine National Park Zones ('no-take' zones that allow non-extractive

recreational use) and a large Conservation Park Zone (very limited extraction of marine resources permitted) around the greater Dunk Island area. Key benthic habitats in this area include 37 coral reefs (including coastal and inshore fringing reefs and inner midshelf platform reefs) and 14 seagrass meadows (coastal and inshore around islands).

Description of the plume events

Hydrograph and weather records for the Tully area were investigated for 11 flood events over the period 1994–2008. The information collated for each event included the hydrograph trajectory, total discharge volume, nutrient and sediment loads and prevailing wind strength and direction (Fig. 1). Flow volumes from all floods from 1972 were combined and percentiles were calculated for small, average and large flood events. We rated a flood event as 'average' when the annual discharge was within the inter-quartile range of the long-term data set, that is, from 2 122 424 to 3 607 342 ML. Small floods had a discharge less than the 25th percentile and large floods had a discharge greater than the 75th percentile (Fig. 1).

Plume extent and the environmental drivers of the extent and duration of the plume

Aerial images from 1994 to 1999 were combined with remote sensing images from 2002 to 2008 to describe the full extent of riverine plumes from the Tully River during 11 events over that period. River plumes monitored from 1994 to 1999 were mapped using aerial survey techniques. Over the monsoonal season, weather reports were closely monitored and when plumes formed, aerial surveys were conducted once or twice during the event. Plumes were readily observable as brown turbid water masses contrasting with the clearer seawater. The visible edge of the plume was followed at an altitude of 1000–2000 m in a light aircraft and mapped using a global positioning system (GPS). Where individual rivers flooded simultaneously, as often happens in the wet tropics, adjacent plumes merged into a continuous area. In these cases, efforts were made to distinguish the edge of the individual river plumes through colour differences (these efforts were only successful during 1998 and 2000). In all other years, the extents of the combined plumes were mapped. Spatial analyses using GIS techniques were applied to the aerial survey results. Flood plumes associated with Cyclone Sadie (1994), Cyclone Violet (1995), Cyclone Ethel (1996), Cyclone Justin (1997), Cyclone Sid (1998) and Cyclone Rona (1999) were plotted.

Moderate Resolution Imaging Spectroradiometer (MODIS) remote sensing Level-0 data were acquired from the NASA Ocean Colour website (http://oceancolor.gsfc.nasa.gov). Sea-WiFS Data Analysis System (SeaDAS) routines were used to process MODIS Aqua and Terra data, producing quasi-true colour images and Level-2 products for periods corresponding to high flow rates in the Tully River from 2003 to 2008 with little or no cloud cover. Chlorophyll *a* and coloured dissolved organic matter (CDOM) absorption at 412 nm were estimated using the GSM01 algorithm at 250-m resolution (Maritorena *et al.* 2002).

The highly turbid nature of the study region and the close proximity to the coastal zone mean that standard near-infrared (NIR) atmospheric corrections are inaccurate and the
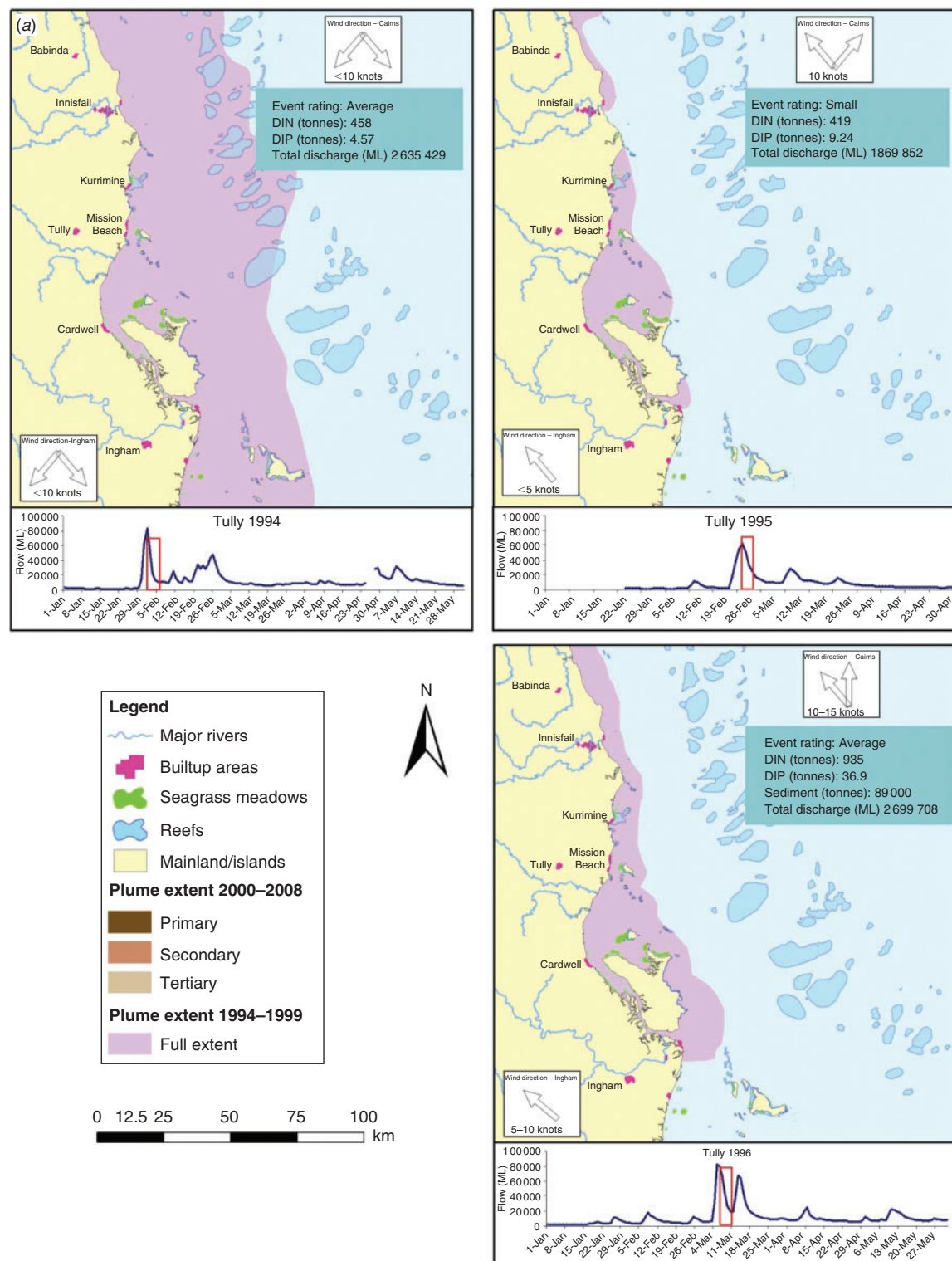
Fig. 1. (*a*) Riverine plume extents estimated from aerial surveys for the period 1994 to 1996 in the Tully marine region. Hydrographs are shown for January to May, and the red box denotes the date of the aerial flyover. Exports of sediment and nutrients are calculated for the wet season period (December–April). (*b*) Riverine plume extents extracted from aerial flyovers for the period 1997 to 2000 in the Tully marine region. Hydrographs are shown for January to May, and the red box denotes the date of the aerial flyover. (*c*) Riverine plume extents and plume types estimated using remote sensing images for the period 2003 to 2008 in the Tully marine region. Hydrographs are shown for January to May, and the red box denotes the date of the aerial flyover. Exports of sediment and nutrients are calculated for the wet season period (December–April). DIN, dissolved inorganic nitrogen; DIP, dissolved inorganic phosphorus; n/a, data not available.
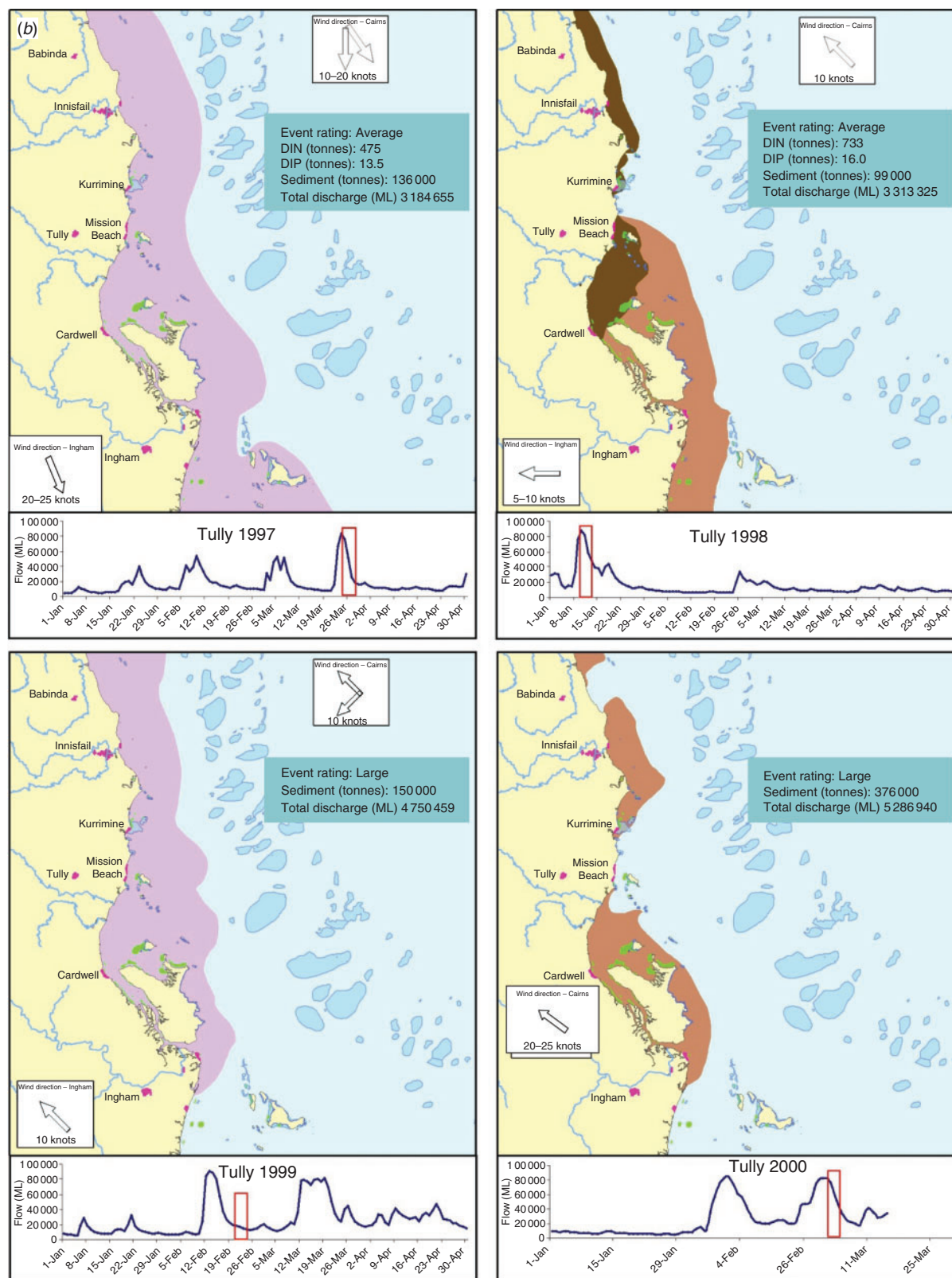
Fig. 1. (*Continued*)
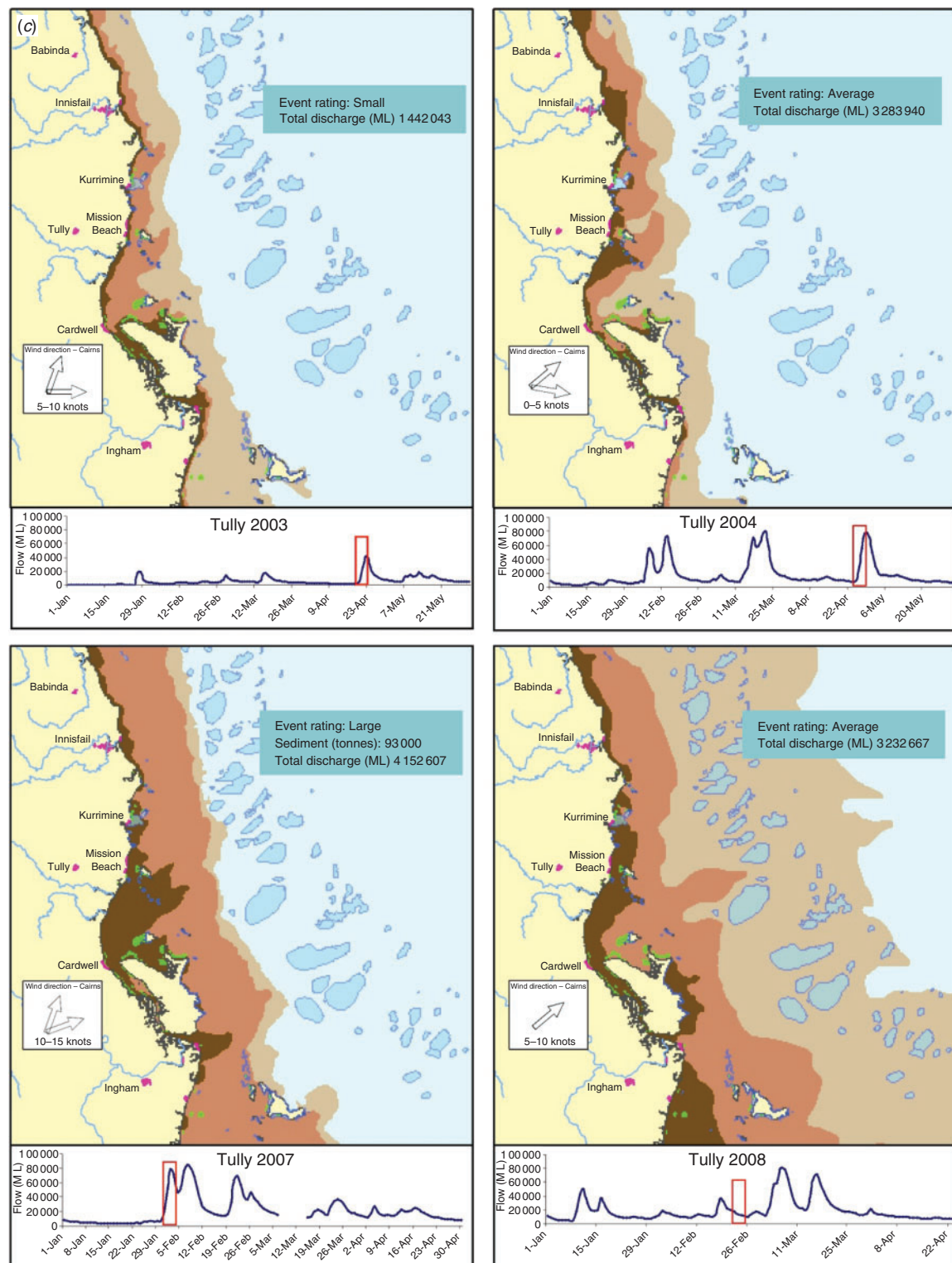
Fig. 1.   (*Continued*)

quality of the retrieved product may be reduced (Wang and Shi 2007). To alleviate this effect, the atmospheric correction described by Wang and Shi (2007) was implemented in SeaDAS. Processing filters, such as cloud and stray light masks, were not used because they may result in regions of interest containing high sediment loads being masked. Suitable images for quantifying flood plume extent were often difficult to identify because of dense cloud cover during flood periods; thus, only four events were identified between 2003 and 2008.

Single images were selected on the basis of their image quality and transposed from geo-referenced true colour images and/or CDOM measurements into GIS shape files.

The MODIS imagery was re-referenced to conform to Geocentric Datum of Australia (GDA), map grid of Australia (MGA) projection. This was simply done by applying the imagery geographic coordinate values to the MGA-94 projected values (metres) until a simple bilinear solution (i.e. universal transverse mercartor (UTM)) was achieved. If a more rigorous algorithm (i.e. cubic) was applied over the image then the true spherical projection was achieved.

The derived CDOM absorption at 412 nm combined with careful examination of the quasi-true colour and chlorophyll *a* images provided the information used to define river plume 'type' and extent. A combination of high CDOM absorption and high sediment concentrations apparent in the quasi-true colour imagery defined the boundaries of 'primary plumes'. Regions with high CDOM absorption and high chlorophyll *a* concentrations, but reduced sediment loads, were identified as 'secondary plumes'. 'Tertiary plumes' were defined by low chlorophyll *a* concentrations and low CDOM absorption values. These are simple qualitative indices for separating the different stages of plume movement and extent, and further work on threshold definition is required.

For the final imagery classification and interpretation, two products were used. The initial classification method, as described above, allowed us to map the three main plume mapping densities (e.g. primary, secondary and tertiary) on the basis of CDOM absorption, and the second, the true colour images, allowed for a visual correlation of the classified values. By using both of these products, it was possible to delineate the three recognised plume classifications with a suitable degree of confidence. In areas where cloud had completely obscured the plume, an estimation of the plume extents was achieved by correlating the plume patterns from consecutive imagery periods in the following days.

A qualitative analysis was applied to each plume using the characteristics of the plume (discharge volume, certain wind conditions) to interpret the extent and direction of the plume relative to the size of the event (small, average or large based on total flow). Aerial surveys or remote sensing images of the plume extent represented only 1 day during a plume event, thus providing a single snapshot in time. However, by focusing on one catchment and combining all available plume surveys, an estimate of the overall extent of the riverine plume could be identified, driven by wind and flow patterns. A plume exposure map was calculated from the intersection of the plume image and type from both the aerial surveys (1995–2000) and remote sensing images (2003–2008) for the Tully marine area.

Water-quality sampling inside the plumes

Water samples were collected from multiple sites within the plume waters. The sampling locations were dependent on which rivers were flooding and the areal extent of the plume, but generally samples were collected in a series of transects heading out from the mouth of the Tully River. The timing of the sampling also depended on the type of event and the logistics of vessel deployment. Most samples were collected inside the visible area of the plume, although some samples were taken outside the edge of the plume for comparison. Salinity profiles were taken at all sites. Surface samples were collected at 0.5 m below the surface, with either a Niskin bottle or a plastic sampling container. Samples taken at depth were collected with Niskin bottles. The volumes filtered for all analyses depended on the turbidity of the water. Subsamples were filtered onto glass-fibre (GF/F) filters for an analysis of chlorophyll; the filter and retained algal cells were wrapped in aluminium foil and frozen. A second subsample was filtered onto a pre-weighed 0.45-μm membrane filter to determine the amount of suspended solids. Nutrient samples were collected using sterile 50-mL syringes and pre-rinsed three times with the seawater to be sampled. A 0.45-μm disposable membrane filter was then fitted to the syringe and 10-mL samples were collected in polypropylene screw-top sample tubes, pre-rinsed with filtered water. The tubes were then stored either on ice in an insulated container or in a freezer, depending on the sampling vessel. Separate samples for silicate analysis were stored at room temperature.

Analytical methods

Processing of the water samples occurred in different laboratories with comparable methods and quality-assurance techniques. The samples were analysed for concentrations of dissolved inorganic nutrients (NH_4, NO_2, NO_3, NO_2^+, NO_3, PO_4 and Si) by standard procedures (Ryle *et al*. 1982) implemented on a Skalar 20/40 autoanalyser (Skalar Analytical, Breda, The Netherlands), with baselines run against artificial seawater. Analyses of the total dissolved nutrients (total dissolved nitrogen and total dissolved phosphate) were carried using persulfate digestion of the water samples (Valderrama 1981), and were then analysed for inorganic nutrients, as above. Dissolved organic nitrogen and dissolved organic phosphate were calculated by subtracting the separately measured inorganic nutrient concentrations (above) from the TDN and TDP values. Particulate nitrogen concentrations of the particulate matter collected on the GF/F filters were determined by high-temperature combustion using an ANTEK Model 707 Nitrogen Analyser (Antek Instruments Inc., Houston, TX, USA). The filters were freeze-dried before analysis. Following primary (650°C) and secondary combustion (1050°C), the nitrogen oxides produced were quantified by chemiluminescence.

Particulate phosphorus was determined colourimetrically (Parsons *et al*. 1984) following acid-persulfate digestion of the organic matter retained on the glass fibre filters. Acid-washed glass mini-scintillation vials were used as reaction vessels. Filters were placed in the vials with 5 mL of 5% w/v potassium persulfate and refluxed to dryness on an aluminium block heater using acid-washed marbles as stoppers for the vials. Following digestion, 5 mL of deionized water was added to each vial and the

filter and salt residue was resuspended and pulverized to dissolve all soluble material. The residue in the vials was compressed by centrifugation at 3500 rev min⁻¹ and the inorganic P determined colourimetrically in aliquots of the supernatant. Inorganic and organic P standards were run with the batch of samples.

Chlorophyll *a* concentrations were determined by fluorescence following maceration of algal cells and pigment extraction in acetone (Parsons *et al*. 1984). A Turner 10-005R fluorometer was used for the analysis and was periodically calibrated against diluted chlorophyll extracts prepared from log-phase diatom cultures (Jeffrey and Humphrey 1975). The concentrations of suspended solids were determined gravimetrically from the difference between loaded and unloaded membrane filter weights after drying the filters overnight at 60°C. Wet filter salt blanks were subtracted from the resulting weight.

Assessment of exposure for key marine habitats (seagrass beds and coral reefs)

For a detailed description of the flood and non-flood (ambient) water-quality conditions in the Tully marine area, high-frequency instrument records were obtained from one site, Dunk Island (5 m depth, from October 2007 to October 2008). The Eco FLNTUSB Combination instruments (Wet Laboratories, Philomath, OR, USA) simultaneously measure *in situ* chlorophyll fluorescence and turbidity and are designed for long deployments. The data were converted from raw instrumental records into actual measurement units (μg L^{-1} for chlorophyll fluorescence, NTU for turbidity) according to standard procedures of the manufacturer. The records were quality checked using a time-series data-editing software (WISKI-TV, Kisters, Aachen, Germany). Turbidity readings were converted to total suspended solids (TSS) concentrations using an equation derived from a correlation of instrument data and TSS concentrations from concurrently collected water samples: (TSS (mg L^{-1}) = 1.3 × FLNTUSB Turbidity (NTU)) (Schaffelke *et al*. 2009).

Data analysis

Transport of the materials in the plume was investigated by mixing profiles, which relate concentrations of water-quality constituents to salinity. These profiles are commonly used to analyse processes in flood plumes, such as estimating conservative or non-conservative mixing processes (Eyre 2000). However, problems with the interpretation of these relationships may arise when the concentrations of the parameters change rapidly in the river/plume interface, such as rapid deposition of particulate matter in the lower salinity zones. Mixing profiles for dissolved inorganic nitrogen (DIN), dissolved inorganic phosphorus (DIP), TSS and chlorophyll *a* were selected to represent the movement of dissolved and particulate matter through the plumes, the uptake of dissolved inorganic nutrients and the growth of phytoplankton biomass through the plume.

Composite plume mapping

Plume exposure maps were produced using a combination of plume indices and ArcMap geoprocessing. Using the plume indices described above, each polygon was assigned a numeric (short integer) value, that is, primary plume = 3, secondary

plume = 2 and tertiary plume = 1, into an 'index' field. A combined data set was then produced by applying a UNION function (geoprocessing function in ArcMap) to all plume data sets, which produced a composite table of each plume index and an 'exposure' value was calculated by summing all the 'index' values for each polygon. The polygons were then aggregated on the basis of their new exposure value. The plume exposure value was overlaid on the selected Tully marine area to calculate the frequency of exposure for key benthic habitats.

Results

Extent of the plumes

Over the 11-year study period, the spatial extents of the flood plumes from the Tully River were highly variable from year to year (Fig. 1*a–c*). Small flood events, calculated as being below the 25th percentile of the long-term discharge record, occurred in 1995 and 2003, with limited offshore movement of the plume water. In 1995, SE winds constrained the small volume of water to the coast, whereas in 2003 the winds varied from S to SW, resulting in a larger offshore plume off the Tully marine area. There was a well-defined tertiary plume south of the Tully, most likely influenced by the southern flooding rivers Herbert and Burdekin (Fig. 1*a*, *c*).

Average floods occurred in 1994, 1996, 1997, 1998, 2004 and 2008. The 1994 and 1997 plumes covered a very large area as a result of the northerly winds. In contrast, the 1996 and 1998 floods, which had similar flows, covered a much smaller area owing to the prevailing SE winds. The W/SW winds in the 2004 flood period moved the primary plume further offshore. The 2008 flow event had a very large spatial extent with the secondary and tertiary plumes reaching into the Coral Sea. This was partly because of the prevailing SW winds, but also a result of the very large flow event of the Burdekin River in January/February 2008, leading to a combined flood plume from several rivers.

Large events, calculated as being above the 75th percentile of the long-term discharge record, occurred in 1999, 2000 and 2007. Prevailing south-easterly winds during the flood periods in 1999 and 2000 constrained the plume extents to the coast. In contrast, the large flow volume of the 2007 flood event coupled with the S/SW winds resulted in a large plume extent for both the primary and secondary plumes, which almost reached the midshelf reefs.

Water-quality gradients

The concentrations of water-quality constituents were highly variable within and between flood events (Table 1). These values are not only influenced by the size of the event and the wind direction influencing the plume extent, but are also highly dependent on the time of sampling relative to the hydrograph. For example, the 1994 flood was an average-sized event in terms of flow, but the plume was dispersed over a large area (Fig. 1*a*), resulting in only marginally elevated water-quality constituent concentrations compared with the non-flood values (Table 1). In contrast, the 1995 event was a small flood, but the plume was constrained to the coast (Fig. 1*a*) and had high concentrations of TSS, DIN and high chlorophyll at the time of sampling (Table 1). The plume of the large 1999 event, which had a total sediment export of 150 000 tonnes over that wet season, was sampled 5 days after

Table 1. Minimum and maximum concentrations of water-quality variables in samples taken in the Tully plumes from 1994 to 2007

Trigger values are from the Water Quality Guidelines for the Great Barrier Reef Marine Park (GBRMPA 2009). –, data not available; DIN, dissolved inorganic nitrogen; DIP, dissolved inorganic phosphorus; DON, dissolved organic nitrogen; DOP, dissolved organic phosphate; PN, particulate nitrogen; PP, particulate phosphate; TSS, total suspended solids

Variable	Unit	Trigger value (annual mean)	1994		1995		1997		1998		1999		2006		2007	
			Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
Salinity			30.2	35.5	2.2	32.6	2.9	23.6	0.0	23.2	6.3	38.4	3.3	32.1	7.0	31.5
DIN	μM		0.1	0.6	1.1	15.7	0.4	5.2	2.2	6.9	0.5	6.3	0.9	11.3	0.2	19.5
DON	μM		5.7	17.8	1.9	12.3	4.7	14.6	0.0	15.4	0.1	8.4	6.9	14.2	–	–
PN	μM	1.5	–	–	–	–	–	–	–	–	–	–	2.2	6.9	1.6	4.7
DIP	μM		0.0	0.0	0.1	0.1	0.0	2.5	0.1	0.4	0.0	0.3	0.0	0.2	0.0	0.4
DOP	μM		0.0	0.3	0.0	0.1	0.2	0.6	0.0	0.6	0.0	0.4	–	–	–	–
PP	μM	0.9	–	–	–	–	–	–	–	–	–	–	0.1	0.9	0.1	0.4
TSS	mg L^{-1}	2.0	0.8	1.4	4.6	26.1	–	–	5.8	39.1	0.4	14.9	1.9	19.3	0.7	7.1
Chlorophyll	μg L^{-1}	0.45	0.6	0.9	0.4	4.6	0.5	3.2	0.1	2.5	0.0	2.2	0.6	2.1	0.4	3.4

peak flow, by which time the river flow had decreased, but the flood plume was constrained to the coast by winds (Fig. 1*b*). The TSS values reached as high as 15 mg L^{-1} (Table 1), exceeding summer water-quality guideline trigger values by approximately fivefold (GBRMPA 2009).

The transport and dilution of water-quality constituents within the plumes were analysed using the mixing profiles of DIN, DIP, TSS and chlorophyll *a* against salinity (Fig. 2). Overall, DIN decreased along an increasing salinity gradient, controlled by conservative (dilution) and non-conservative (biogeochemical uptake) processes. There was, on average, a reduction of 10–20% in the DIN freshwater end-member through the salinity range, with DIN concentrations in the higher salinities (above 30) clearly elevated (1–5 μM) in comparison with non-flood levels (Furnas 2003; De'ath and Fabricius 2008). Concentrations at the freshwater end varied between events, with initial concentrations exceeding 15 μM in 1995 and 2007, in comparison to all other years where the initial concentrations ranged from 5 μM to just under 10 μM. Sampling in both 1995 and 2007 captured the 'first flush' events carrying high concentrations of newly mobilised DIN from the fertilised agriculture lands on the adjacent catchment (Bainbridge *et al.* 2009; Mitchell *et al.* 2009). The DIP showed an increase from the lower to middle salinity ranges, reflecting desorption of dissolved inorganic phosphorus from suspended particles and dilution in higher salinities.

The TSS concentrations throughout the events ranged from 0.8 to 39.1 mg L^{-1} (Fig. 2). Chlorophyll concentrations were variable, ranging from just below the detection limit to 4.6 μg L^{-1}. The average chlorophyll values at the freshwater end were low (0.2–2 μg L^{-1}), reflecting limitation of growth as a result of corresponding high TSS values and light-limiting conditions. The chlorophyll *a* maxima were measured in the 10–20 salinity range, suggesting that phytoplankton growth was optimal in the middle salinity range with low TSS concentrations, high nutrients and adequate light conditions (Fig. 2). Chlorophyll also increased slightly in the 30–35 salinity range, indicating that uptake of available inorganic nutrients and increased phytoplankton growth were still occurring in the secondary/tertiary plume areas.

Exposure of key marine habitats (seagrass beds and coral reefs) to flood plumes

In total, 147 water-quality samples were taken in the Tully marine area during flood events. Of these, 85% of chlorophyll measurements ($n = 101$) exceeded the chlorophyll water quality trigger value of 0.63 μg L^{-1} and 32% exceeded the TSS ($n = 63$) trigger value of 2.4 mg L^{-1} (GBRMPA 2009) set for the summer period. Less frequent sampling occurred for particulate nitrogen (PN) and particulate phosphate (PP), but all 31 measurements exceeded both of the water quality trigger values for PN (1.8 μM) and PP (0.11 μM).

Instrumental records from 2007 to 2008 gave a detailed picture of the water-quality conditions during both flood and non-flood conditions at Dunk Island (Table 2). During the main flood period of the Tully River (December 2007 to March 2008), the water at the Dunk Island station had a mean chlorophyll concentration of 0.59 μg L^{-1} and 38% of the mean daily
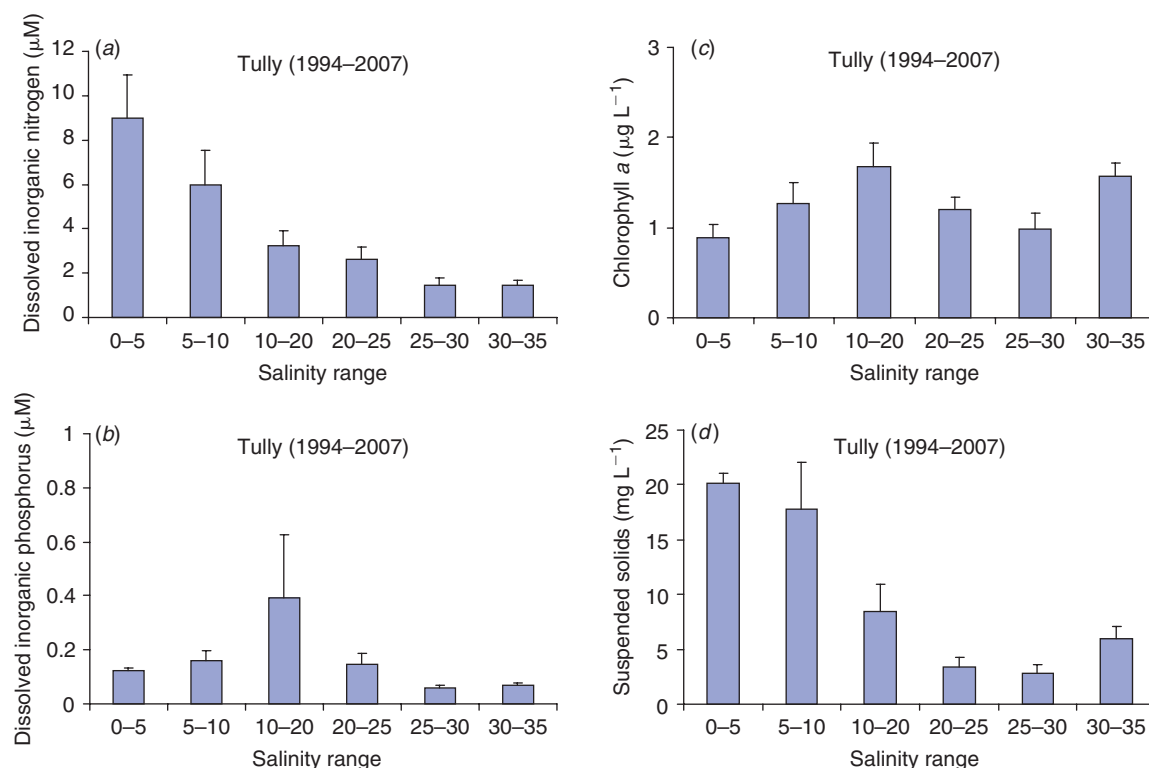
Fig. 2. Water-quality parameters in six salinity ranges summarised from mixing profiles in flood plumes from 1995 to 2007. (*a*) Dissolved inorganic nitrogen, (*b*) dissolved inorganic phosphorus, (*c*) chlorophyll and (*d*) total suspended sediment. The salinity ranges from 0 to 35 and is broken into six bands. Data are total averages over years and sampling sites (+s.e.).

chlorophyll values exceeded the summer chlorophyll trigger value for the GBR ($0.63 \, \mu g \, L^{-1}$; GBRMPA 2009). The mean chlorophyll concentration during the non-flood period was $0.34 \, \mu g \, L^{-1}$, which is close to the winter chlorophyll trigger value ($0.32 \, \mu g \, L^{-1}$; GBRMPA 2009). The suspended solids concentrations around Dunk Island were slightly elevated during the flood period (Table 2), but were very variable all year round. The mean TSS concentration during the flood period was $3.4 \, mg \, L^{-1}$, with a maximum daily mean of $23 \, mg \, L^{-1}$, reached during the March flood peak. The mean concentration during the non-flood period was $2.4 \, mg \, L^{-1}$, above the mean annual trigger value for the GBR ($2.0 \, mg \, L^{-1}$; GBRMPA 2009). Approximately 30% of the daily values exceeded this guideline trigger value in both flood and non-flood conditions.

The number of reefs and seagrasses exposed to the plume waters varied from year to year, and depended on the type of plume. Over the 11 years, a minimum of 11 reefs (30%) and a maximum of 37 reefs (100%) were inundated by either a primary or secondary plume (Fig. 3; Table 3), indicating that it is likely that at least one-third of the reefs is exposed to plume waters every year. For the years with remote sensing data available to validate plume type (1998, 2003–2008), we estimated that 6–15 reefs were inundated by primary plumes carrying high sediment loads, which is up to 41% of the inshore reefs in the Tully marine area and that 5–16 reefs (43%) were inundated by secondary plumes with elevated nutrient and chlorophyll concentrations. A smaller number of inshore reefs were inundated by a tertiary

flood plume in three flood events (Table 3). It is important to note that tertiary plume extents and the associated exposure of reefs may have been underestimated in the years when the plume extent was estimated from aerial images only (1995–2000) on the basis of a colour change between the fresh and marine waters. Out of the 14 seagrass beds within the Tully marine area, at least 13 were inundated by either a primary or secondary plume in 10 of the 11 analysed events (Fig. 1), with the exception of 2000, when only seven seagrass beds were affected (Fig. 3; Table 3).

Discussion

Riverine flood plumes regularly inundate the marine environment of the Tully area, sometimes several times per year. Using both aerial and remote sensing images, we identified that riverine plumes can extend, in certain years, much further offshore and at more frequent intervals than previously reported (Fig. 1; Devlin *et al*. 2003; Devlin and Brodie 2005; Maughan *et al*. 2008). Previously, the small number of water-quality measurements in flood plumes indicated that there was an inshore–offshore gradient for many water-quality constituents; however, the frequency and intensity of the inundation and the concentration were unknown (Devlin *et al*. 2001 Brodie and Mitchell 2005).

Classification of the riverine plumes into distinct types (primary, secondary and tertiary plumes) helps elucidate more clearly the transport of different water-quality constituents in

Table 2. Summary of the chlorophyll and total suspended solids (TSS) concentrations from deployments of WET Laboratories Eco FLNTUSB combination fluorometer and turbidity sensors at Dunk Island for 12 months in 2007–2008

n, number of daily means in the reported time series; s.e., standard error

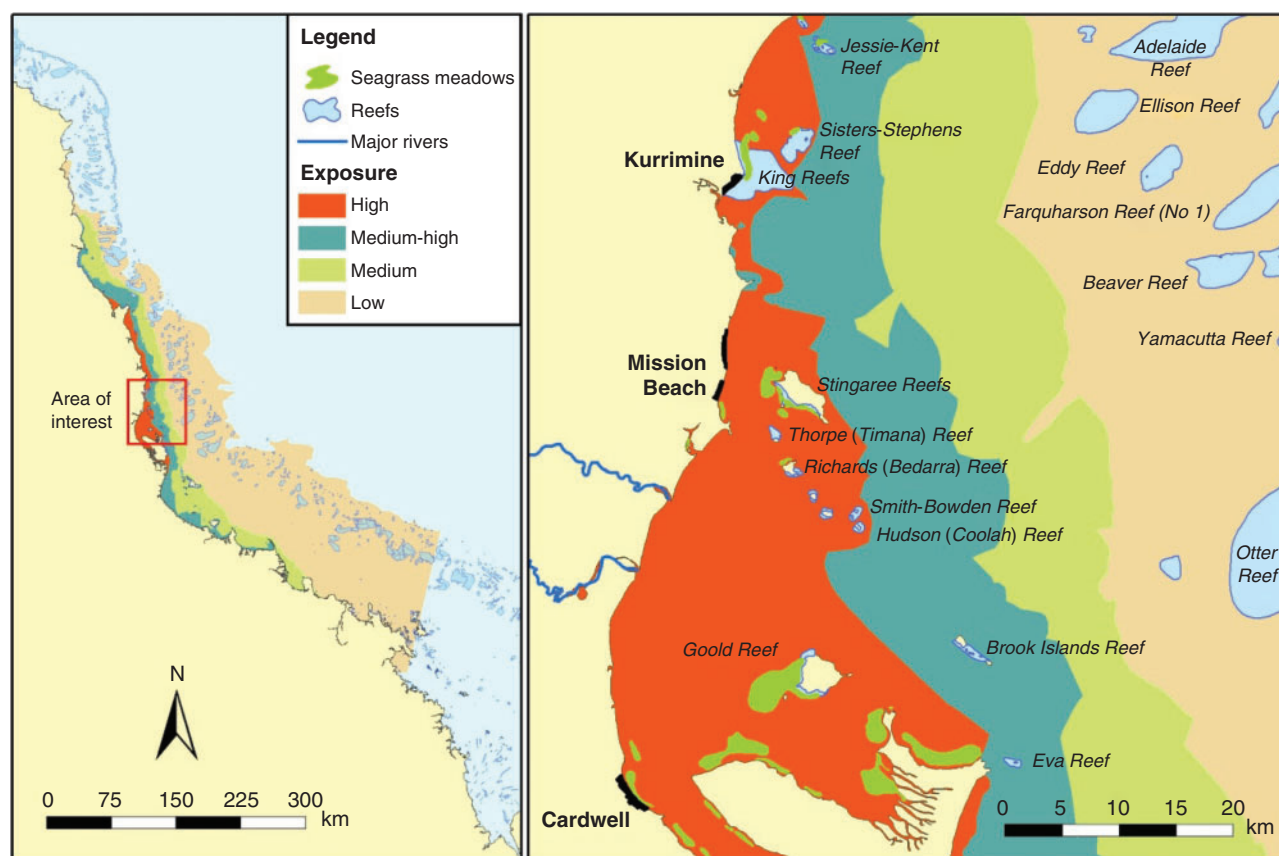|	High flow period 27/12/2007–23/04/2008			Ambient period 17/10/2007–26/12/2007, 24/04/2008–16/10/2008		
	Mean	s.e.	*n*	Mean	s.e.	*n*
Chlorophyll *a* (μg L^{-1})	0.59	0.02	86	0.34	0.01	264
TSS (mg L^{-1})	3.35	0.48	86	2.38	0.15	264

Fig. 3. Exposure of biological communities within the plume area. The colours denote the level of exposure to plume waters (high, medium-high, medium and low).

Tully River plumes by defining the spatial movement of the suspended sediments, dissolved nutrients and chlorophyll by the extent of the specific plume type.

Spatio-temporal patterns of plume water are difficult to resolve using only traditional biogeochemical methods owing to the constraints of direct sampling. This problem can be addressed by using satellite observations of visible spectral radiance regularly collected by NASA imagery Although suitable remote sensing images were only available for a limited number of days during the analysed high flow events for the Tully marine area, mainly because of heavy cloud cover (Rakwatin *et al.* 2007), the use of the remote sensing images gave far more detailed information about the plume type and the main constituents associated with that type than a composite aerial plume image, which can only be assessed visually.

The prevailing wind at any point during the high flow event was the dominant factor controlling the movement, extent and direction of the Tully plume. It has been previously reported that the prevailing and often strong SE winds constrain plume waters to the coast with a northwards movement, whereas at low wind speeds plumes move in a northerly direction from the river mouth as a result of Coriolis forcing and can spread well offshore

Table 3.   Exposure of marine ecosystems to flood plumes
Thirty-seven coral reefs and 13 seagrass meadows were identified in the Tully marine area Data are the number of reefs or seagrass meadows that were inundated by flood plumes at the time that aerial or remote sensing imagery was taken to assess the flood plume extent. In 1994–1997 and 1999, plume extents were based on aerial surveys and delineation between primary and secondary plumes was not possible, thus the full aerial extent is defined as a secondary plume

Plume type	1994	1995	1996	1997	1998	1999	2000	2003	2004	2007	2008
					Coral reefs						
Primary plume	0	0	0	0	12	0	0	6	12	15	14
Secondary plume	37	19	24	24	5	24	11	16	7	7	9
Tertiary plume	0	0	0	0	0	0	0	3	5	0	14
					Seagrass meadows						
Primary plume	0	0	0	0	10	0	0	5	7	11	9
Secondary plume	13	11	13	13	3	13	7	8	5	3	4
Tertiary plume	0	0	0	0	0	0	0	0	1	0	0

(Chao 1988*a*; Wolanski 1994; Devlin *et al*. 2003; Devlin and Brodie 2005). If the wind forcing is opposed to the Coriolis forcing in direction, that is, northerly or north-easterly winds, the overall plume movement may be to the south. The extent of the transport of dissolved and particulate nutrients is also related to the intensity and duration of the rainfall event and the flow during the different stages over the river discharge hydrograph (rising, peak, falling waters). For example, a large first flush event in a wet season in the Tully catchment, such as those sampled in 1998, 2003 and 2007, would export very high loads of dissolved and particulate nutrients into the GBR lagoon owing to the mobilisation of the inorganic material stored in the agricultural soils.

Tully flood plumes move in response to prevailing weather conditions over the coastal shelf with the plume itself constituting an estuary with mixing processes from the freshwater end (mouth of the river) to the seawater end (end of plume). Constituents act differently within the plume water. For some constituents, the plume water is a simple mixing interface between the rivers and the lagoon. For others, the river and the corresponding plume act as an open-ended system in which biological and chemical removal takes place, substantially reducing the amount of constituent that reaches the reef (Dagg *et al*. 2004). Cycling processes within plumes for different constituents are markedly different and hence plume cycling can not only change total nutrient loads, but can also modify the ratios of one nutrient to another, which holds implications for the biological responses to plume waters.

The transport of dissolved inorganic nitrogen was controlled primarily by dilution, with elevated concentrations moving large distances (>20 km) offshore. The removal of DIN appears to be dominated by conservative mixing, indicating that the physical processes (dilution) are operating over shorter time frames than the biogeochemical processes Although there was a substantial decrease in the DIN concentrations through the salinity gradient, our within-plume sampling data indicate that dissolved nitrogen moved further offshore than suspended solids and at elevated concentrations compared with baseline values. Thus, there is a greater potential for the uptake of DIN by phytoplankton over large areas of the Tully marine area.

In contrast to the movement of DIN, average concentrations of DIP increased in the mid salinity range, suggesting that

desorption of inorganic P from particulate P is occurring at these salinities. Davies and Eyre (2005) report on a similar process in the Daintree estuary, with low concentrations of DIP at low salinities, most likely assimilated by phytoplankton and increasing in the middle estuary, originating from desorption of inorganic P from suspended sediments as the pH increases through the estuary. This can be an important mechanism for the transport of phosphate to the ocean in other rivers; for example, in the Amazon River more than half of the phosphate reaching the ocean is released from particulate matter during plume mixing (DeMaster and Pope 1996).

The highest values of TSS were measured in the freshest parts of the plumes, with values close to ambient in the higher salinities, suggesting deposition of the heavier particulate matter close to the coast. In the initial mixing zone, water velocity is reduced and most of the river-derived particulate matter settles from the plume.

The non-conservative profile of chlorophyll along salinity gradients within plumes reflected the complex relationship between phytoplankton growth and nutrient and light availability (Cloern 2001). Pelagic and benthic algal and microbial communities rapidly take up the nutrients exported by flood plumes into the GBR lagoon waters (Alongi and McKinnon 2005), leading to short-lived phytoplankton blooms and transient events of higher level organic production (McKinnon and Thorrold 1993; Furnas *et al*. 2005). This is shown in the salinity range between 10 and 25, where the highest chlorophyll concentrations were measured, with suspended sediment levels being sufficiently low to allow enhanced phytoplankton productivity, fuelled by the elevated nutrients from the plume waters (McKinnon and Thorrold 1993). Removal of inorganic nutrients across the plume-water fronts at a salinity of ~26 has also been noted in the Yantze River plume (Tian *et al*. 1993) and the Annan River (Davies and Eyre 2005).

A risk assessment based on the prevailing movement of river plumes from all major GBR rivers identifies coral reefs at high risk of exposure to flood plumes; these coral reefs are mainly located to the north/north-east of rivers draining catchments with a high proportion of fertilised agriculture (Maughan *et al*. 2008). During the northerly and/or offshore winds in 1994, 1997 and 2008 (Fig. 1*a–c*), riverine plumes moved far offshore, reaching

the mid and outer shelf reefs and even the Coral Sea, potentially exposing offshore marine ecosystems to materials transported by the flood plumes. In contrast, the prevailing south-easterly winds keep plumes confined to the coastal and inshore areas, but, owing to limited dilution and dispersal, expose ecosystems in these areas to elevated concentrations of nutrients, suspended solids and other material transported in the run-off.

Instrumental water quality records at Dunk Island, which has seagrass and coral reef habitats, showed that over the course of 1 year, the concentrations of suspended solids (measured as turbidity) were often elevated, whereas chlorophyll concentrations were relatively low for most of the year, but showed a clear flood signal. Elevated sediment and nutrient levels decrease in a matter of weeks after a flood event by sedimentation, biological uptake, dilution and dispersal. Material in the GBR inshore waters remains in the coastal zone until transported out of the GBR lagoon over weeks to months, primarily via the northern and southern ends of the reef (Luick *et al.* 2007; Wang *et al.* 2007) or after being assimilated into the inshore food web through biological uptake until it is eventually removed from the system by remineralisation or burial (Alongi and McKinnon 2005). Wind and tide-driven turbidity events are common in the GBR lagoon and are important drivers of the underwater light climate that shapes coastal benthic ecosystems such as seagrass meadows and coral reefs (Larcombe *et al.* 1995; Alongi and McKinnon 2005; Cooper *et al.* 2008). Terrestrial fine sediment transported into the Tully coastal area by flood plumes may be easily resuspended for prolonged periods of time (Wolanski *et al.* 2008), especially after large flood events, leading to frequent spikes in turbidity.

A comparison of plume data to water-quality guidelines (GBRMPA 2009) shows that a large proportion of the measured data exceeds trigger values for TSS, chlorophyll, PN and PP. The inshore coral reefs and seagrass beds adjacent to the Tully catchment are likely to be affected by these elevated concentrations, at least during the weeks of exposure. The longer-term impacts of flood plumes are currently not well understood, but are the subject of ongoing research. These impacts include, for example, recurrent resuspension of settled material leading to periodically elevated TSS concentrations over long time periods or ongoing high nutrient availability from foodweb cycling. Our estimates of the exposure of marine ecosystems to flood plumes showed that coastal and inshore coral reefs and seagrass beds in the Tully marine area were inundated every year by primary plumes and were exposed to intermittently high sediment and high nutrient concentrations during flood plumes, and potentially high loads of sedimenting particles.

The major adverse effect on corals is decreased light availability as a result of high water turbidity and short-term or intermediate smothering by high sedimentation during flood events or because of resuspension of terrigenous fine sediments by wind and waves.

Our assessment of 11 flood events from 1994 to 2008 showed that as a result of the regular high rainfall and associated flooding, the marine ecosystems adjacent to the Tully catchment are regularly exposed to elevated concentrations of nutrients, suspended sediments and other land-derived materials, such as herbicides. Knowledge about the overall catchment loads and sources of land-derived materials as well as the relationships to various land uses in the Tully area is continually improving (e.g. Armour *et al.* 2009; Bainbridge *et al.* 2009; Mitchell *et al.* 2009; Wallace *et al.* 2009). The effects of excess nutrients and sediments in the marine environment are also increasingly understood (e.g. De'ath and Fabricius 2008). However, less well known are the physical and biogeochemical processes transporting and transforming land-derived materials in the marine environment, as well as the hydrodynamics of the GBR inshore area that control, for example, residence times. The missing links between catchment and marine processes hamper the implementation of management options for specific water-quality constituents. A primary use for the results of the present study will be to set targets connecting end-of-river loads of particular materials to an intermediate end-point target, such as chlorophyll (Brodie *et al.* 2009), and, in the future, to an ecological end-point target, such as a composite indicator for coral reef health (Fabricius *et al.* 2005).

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Plant Growth-Promoting Effects of Diazotrophs in the Rhizosphere

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Plant Growth-Promoting Effects of Diazotrophs in the Rhizosphere

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ABSTRACT: Because of their ability to transform atmospheric N_2 into ammonia that can be used by the plant, researchers were originally very optimistic about the potential of associative diazotrophic bacteria to promote the growth of many cereals and grasses. However, multiple inoculation experiments during recent decades failed to show a substantial contribution of Biological Nitrogen Fixation (BNF) to plant growth in most cases. It is now clear that associative diazotrophs exert their positive effects on plant growth directly or indirectly through (a combination of) different mechanisms. Apart from fixing N_2, diazotrophs can affect plant growth directly by the synthesis of phytohormones and vitamins, inhibition of plant ethylene synthesis, improved nutrient uptake, enhanced stress resistance, solubilization of inorganic phosphate and mineralization of organic phosphate. Indirectly, diazotrophs are able to decrease or prevent the deleterious effects of pathogenic microorganisms, mostly through the synthesis of antibiotics and/or fungicidal compounds, through competition for nutrients (for instance, by siderophore production) or by the induction of systemic resistance to pathogens. In addition, they can affect the plant indirectly by interacting with other beneficial microorganisms, for example, *Azospirillum* increasing nodulation of legumes by rhizobia. The further elucidation of the different mechanisms involved will help to make associative diazotrophs a valuable partner in future agriculture.

KEY WORDS: biocontrol, BNF, nutrient uptake, PGPR, phytohormones, stress resistance, vitamins.

I. INTRODUCTION

The rhizosphere is the narrow zone of soil surrounding the root that is under the immediate influence of the root system. This zone is rich in nutrients when compared with the bulk soil, due to the accumulation of a variety of organic compounds released from roots by exudation, secretion, and deposition (Curl and Truelove, 1986). Because these organic compounds can be used as carbon and energy sources by microorganisms, microbial growth and activity is particularly intense in the rhizosphere. This is reflected by the number of bacteria that are found around the roots of plants and that is generally 10 to 100 times higher than in the bulk soil (Weller and Thomashow, 1994). Plant-associated bacteria that are able to colonize roots are called rhizobacteria and can be classified into beneficial, deleterious, and neutral groups on the basis of their effects on plant growth. Beneficial rhizobacteria that stimulate plant growth are usually referred to as Plant-Growth-Promoting Rhizobacteria or PGPR (Davison, 1988; Kloepper *et al*., 1989), a group that includes different bacterial species and strains belonging to genera such as *Acetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Herbaspirillum,* and *Pseudomonas* (Weller and

Thomashow, 1994; Glick, 1995; Probanza *et al.*, 1996).

Diazotrophic bacteria, by their ability to convert N_2 into ammonia, which can be used by the plant, also belong to the PGPR. Because of their competitive advantages in a C-rich, N-poor environment, diazotrophs may become selectively enriched in the rhizosphere (Döbereiner and Pedrosa, 1987), putting themselves in a good position to promote plant growth. Although bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium, Mesorhizobium,* and *Azorhizobium* are known for their capacity to fix atmospheric nitrogen in a symbiotic relationship with the roots of leguminous plants, they are usually not considered as PGPR in this highly specific symbiotic interaction. However, Rhizobiaceae also have the ability to form nonspecific associative interactions with roots of other plants without forming nodules (Reyes and Schimidt, 1979). They were found to attach to the surface of monocots in the same manner as they attach to those of dicot hosts (Shimshick and Hebert, 1979; Terouchi and Syõno, 1990). Furthermore, they are able to stimulate the growth and increase the yield of these nonlegumes both in greenhouse and field experiments (Biswas *et al.*, 2000; Yanni *et al.*, 2001), and therefore they can also be considered as PGPR in these cases. The subject of this review is restricted to plant growth promotion by free-living or associative N_2–fixing bacteria. The *Rhizobium*-legume and *Frankia*-non-legume symbioses will not be included. For recent publications on these topics see HussDanell (1997), Schwencke (1998), Schwencke and Caru (2001), Spaink *et al.* (1998), and Subba Rao and Rodriguez-Barrueco (1998). *Rhizobium* will only be discussed when used as PGPR with non-legumes.

Rhizosphere diazotrophs were mainly isolated in the 1960s to 1970s, but their contribution to the nitrogen nutrition of plants is still under debate. This controversy is discussed in this review. Unlike the rhizobia-legume symbiosis, where the biologically fixed N can meet the needs of the host plant, BNF contributions by associative diazotrophs to their hosts are usually considered to be low. Given the fact that the nitrogenase, the enzyme converting N_2 into NH_3, is inhibited in the presence of combined forms of N, such as nitrate or ammonia, it is unlikely that organisms that benefit plant growth by fixing nitrogen, can do so in soils where high amounts of nitrogen fertilizer are added, as it is usually the case in intensive agricultural systems. However, in the case of *Azospirillum*-inoculated plants, positive responses of plants to inoculation were also found under high N nutritional levels (Reynders and Vlassak, 1982; Kapulnik *et al.*, 1983), indicating that plant responses are not only due to N_2 fixation activity in the rhizosphere, but that other mechanisms are clearly involved.

In addition, grain grasses such as wheat, maize, and rice are still the most important plants for the nutrition of the world's population. Although grain legumes are the main protein source in many developing countries, the total world area cultivated with these plants is only about 25% of the area that is used for cereal grasses (Food and Agricultural Organization of the United Nations, 2001; http://www.fao.org/). The study of the associative interactions between diazotrophs and nonleguminous plant species and the understanding of the mechanisms involved therefore remain of agricultural importance.

This article gives an overview of the different mechanisms by which associative diazotrophs have been found to promote plant growth, and special attention is paid to the experiments clarifying these mechanisms. A main obstacle during the preparation of this article was the frequently observed lack of information on the nitrogen-fixing capacity of the microorganisms involved. Many PGPR were isolated by screening for one selected trait, for example, P-solubilization or biocontrol activity, while other characteristics like N_2–fixation were not investigated. In the case that the diazotrophic character of the organism was not confirmed, the reports were not included in this review. Therefore, it is likely that more information on plant growth-promoting mechanisms by associative diazotrophs is available than cited here.

II. BIOLOGICAL NITROGEN FIXATION

The first associative diazotroph was reported by Beijerinck in 1925 under the name *Spirillum*

lipoferum. However, it was only about half a century later, after the discovery of the highly specific *Azotobacter paspali–Paspalum notatum* association and the rediscovery of *Spirillum lipoferum* (now called *Azospirillum*) by the group of Döbereiner (Döbereiner *et al*., 1972; Döbereiner and Day, 1976), that scientists became increasingly interested in diazotrophic bacteria associated with graminaceous plants. Because the benefit of nitrogen fixation from nodulated legumes to agriculture was already established at that time, it was expected that the associative diazotrophs would favor nonleguminous plants in the same way. Several genera of bacteria have now been reported to contain diazotrophs, which may be loosely or more intimately (endophytes) associated with plants, including *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus,* and *Pseudomonas*. An extensive phylogenetic classification of nitrogen-fixing organisms was made by Young in 1994. While the capability of these organisms to fix nitrogen *in vitro* can be demonstrated easily, efforts to quantify nitrogen fixation in natural associations with plants have produced widely varying results. In the past 30 years many crop-inoculation studies, coupled to acetylene reduction measurements, N balance and ^{15}N isotope dilution experiments, have been conducted with root-associated bacteria to determine whether the bacteria supply significant amounts of nitrogen to cultivated plants (Boddey *et al*., 1999; James, 2000).

The acetylene reduction assay (ARA) is the most widely used method because of its simplicity and low cost. A major drawback of this assay is that it only measures nitrogenase activity and reveals no information on whether the fixed N is incorporated into the plant (Boddey, 1987; Boddey *et al*., 1995). N balance experiments have the disadvantage that the plant N is not necessarily derived from the air but might also result from improved nutrient uptake by the inoculated plant. The most useful methods for examining N_2 fixation in the field and in large greenhouse experiments are still the ^{15}N isotope dilution and ^{15}N natural abundance techniques (James, 2000).

Using these methods it was reported that certain Brazilian sugar cane varieties can derive 50 to 80% of plant N from BNF, equivalent to 150 to 170 kg N ha^{-1} y^{-1} (Lima *et al*., 1987; Boddey *et al*., 1991, 1995; Urquiaga *et al*., 1992; Döbereiner *et al*., 1993; Boddey and Döbereiner, 1995). However, the amount of nitrogen fixed is highly variable and dependent on plant genotype and environmental conditions (Boddey *et al*., 1991). In large experiments comparing up to 70 rice varieties, it was estimated in one experiment that the amount of nitrogen derived from air (Ndfa) ranged from 0 to 20.2%, and in the other experiment that an equivalent of 16 to 70 kg N ha^{-1} crop^{-1} was fixed (App *et al*., 1986; Shrestha and Ladha, 1996). A substantial number of studies conducted at the International Rice Research Institute in the Philippines suggest that on the whole 20 to 25% of the total nitrogen needs of rice can be derived from associative fixation (App *et al*., 1980; Ladha *et al*., 1986, 1987; Watanabe *et al*., 1987; Roger and Ladha, 1992). Using the ^{15}N isotope dilution technique, it was estimated that Kallar grass may fix up to 26% of its N content (Malik *et al*., 1997). For the batatais cultivar of *Paspalum notatum,* this was nearly 11% (Boddey *et al*., 1983). While in the above-mentioned examples, the contributions of biological N_2 fixation are of agronomical significance, most studies on wheat have shown little or no N_2 fixation by this crop, even when inoculated with *Azospirillum* or other diazotrophs (Lethbridge and Davidson, 1983; Kapulnik *et al*., 1985a; Boddey, 1987; Chalk, 1991; Bremer *et al*., 1995). One study on N_2 fixation with maize suggested that some cultivars fix up to 60% of their N after inoculation with appropriate strains of *Azospirillum* (Garcia de Salamone *et al*., 1996), while other cultivars showed decreased grain yield and plant N accumulation (Garcia de Salamone and Döbereiner, 1996). On the whole, greenhouse studies with maize, sorghum, and Setaria did not show substantial N_2–fixation in *Azospirillum* inoculated plants (Okon and Labandera-Gonzalez, 1994).

In these kinds of studies no proof has been given for the organism(s) responsible for BNF. It was never shown that growth stimulation was caused by the direct transfer of fixed nitrogen from the diazotroph to its plant partner. *Acetobacter diazotrophicus* and *Azotobacter paspali* were found to be predominantly present in sugar cane and *Paspalum notatum,* respectively (Döbereiner *et al*., 1972; Li and MacRae, 1991;

Dong *et al*., 1994), and thus assumed to be responsible for the N contribution to their host plant. However, the simple isolation of a diazotroph from a plant provides no conclusive evidence that BNF contributed to plant growth promotion. Inasmuch as no differentiated structures are present on the root system in associative plant-bacterium interactions and a wide diversity of PGPR can be isolated from the rhizosphere of a single plant, it is often impossible to determine which organism is actually responsible for plant growth promotion or N_2 fixation. In the case of wetland rice, interpretation is even more difficult because a proportion of this N may be derived from free-living N_2–fixing cyanobacteria in the flood water or heterotrophic N_2 fixers in the soil (Eskew *et al*., 1981).

To provide direct evidence that the plant benefits from the N_2 fixed by the assumed diazotroph, plant inoculation experiments with nonnitrogen fixing (Nif⁻) mutants as negative controls are required, coupled with careful ^{15}N-based balance studies. With the use of such mutants in inoculation experiments, it becomes clear that in most of the cases BNF is not involved in the plant growth promotion. Nif⁻ mutants of *Azospirillum, Azoarcus* sp. strain BH72 or *Pseudomonas putida* GR12–2 have been shown to be still capable of stimulating plant growth (Lifshitz *et al*., 1987; Morgenstern and Okon, 1987b; Bashan *et al*., 1989; Hurek *et al*., 1994). No ^{15}N isotope dilution or nitrogen balance experiments have been done with these Nif⁻ mutants. The fact that BNF is apparently not involved in plant growth promotion by these strains cannot be simply attributed to the absence of nitrogenase expression. Using a translational *nifH-gusA* fusion, it was observed that *Azospirillum nif* genes are expressed during the association with wheat roots (Vande Broek *et al*., 1993). On the other hand, some host specificity of BNF has been reported. When the *nifK* mutant of *Azoarcus* sp. strain BH72, which has a Nif⁻ phenotype, was used to inoculate rice seedlings in a gnotobiotic system, the same increase in plant biomass and total protein content was found as after inoculation with the wild-type strain, strongly suggesting that N_2 fixation was not involved in the observed plant growth promotion (Hurek *et al*., 1994). Nevertheless, immunogold labeling as well as reporter gene studies revealed high nitrogenase gene expression levels of the endophyte *Azoarcus* sp. BH72 inside roots of rice seedlings, suggesting that environmental conditions inside rice roots are permissive for endophytic nitrogen fixation in bacterial microcolonies in the aerenchyma (Egener *et al*., 1999). However, when this Nif⁻ mutant was inoculated onto Kallar grass plantlets, these plants showed significantly lower dry weight and accumulated less nitrogen than those inoculated with the wild-type strain *Azoarcus* sp. BH72 (Hurek *et al*., 1998; Hurek *et al*., 2002). This indicates that in the case of Kallar grass, *Azoarcus* may fix N_2 *in planta* and transfer the fixed N to the host plant. Additional proof for this was given by the fact that abundant BH72 *nifH* transcripts were retrieved from the roots of plants inoculated with the wild-type strain but not from noninoculated control plants or plants inoculated with the Nif⁻ mutant strain, indicating that nitrogen fixation by *Azoarcus* sp. BH72 and not by any other diazotrophic bacteria had provided combined nitrogen to the plant. Furthermore, both wild-type and mutant *Azoarcus* sp. BH72 could not be reisolated using established protocols, which led the researchers to conclude that *Azoarcus* sp. contributes fixed nitrogen to the plant in an unculturable state. The mechanism by which this transfer occurs has still to be determined. It may be direct transfer or simply an indirect process via death and mineralization of the bacteria.

Until now, there is only one other case where thorough proof has been given for direct N transfer by the diazotroph to the host plant and that is for *Acetobacter diazotrophicus* associated with sugarcane (Sevilla *et al*., 2001). The wild-type strain and a *nifD* mutant of *Acetobacter diazotrophicus*, unable to fix nitrogen (Nif⁻), were used to inoculate sterile sugarcane plantlets prepared from meristem tissue culture. Sugarcane plants inoculated with the wild-type strain generally grew better and had a higher total N content 60 days after planting than did plants inoculated with the Nif⁻ mutant or uninoculated plants (Sevilla *et al*., 2001). These results indicate that the transfer of fixed N from *A. diazotrophicus* to sugarcane might be a significant mechanism for plant growth promotion in this association. When N was not limiting, growth enhancement was

observed in plants inoculated with either wild-type or Nif⁻ mutants, suggesting the additional effect of a plant growth-promoting factor provided by *A. diazotrophicus*. IAA and gibberellins have been identified in cultures of *A. diazotrophicus* (Fuentes-Ramirez *et al*., 1993; Bastián *et al*., 1998). The production of these growth factors may be secondary to the effects of nitrogen fixation. A $^{15}N_2$ incorporation experiment demonstrated that *A. diazotrophicus* wild-type strains actively fixed N_2 inside sugarcane plants, whereas the Nif⁻ mutants did not (Sevilla *et al*., 2001). As in this case, no plant-specific compound was analyzed for ^{15}N content to prove that significant combined or fixed N was transferred from bacteria to plant material, the authors conclude that the question of whether the $^{15}N_2$ was incorporated only into bacterial mass present inside the plants remains unanswered.

One problem with which most of the studies with root-colonizing diazotrophs suffer is that the amount of fixed N_2 supplied to their host plants appears to be very low (Rao *et al*., 1998). This has been attributed to the fact that free-living diazotrophs do not, contrary to symbiotically living *Rhizobium* spp., excrete N from their cells (Kleiner, 1984). In the case of associative diazotrophs, the fixed nitrogen remains mainly in the bacterial cells and is released to the host only at a later stage of plant growth after death and decay of the bacterial biomass (Rao *et al*., 1998). This process is inefficient, and perhaps delayed, when compared with the active release of the immediate products of N_2 fixation by living bacteria, as occurs in legume nodules (Mylona *et al*., 1995). This might in part explain the poor performance of the associative system. Also, much of the "plant-associated" N_2 fixation reported from ^{15}N isotope dilution studies could be due to this process (James, 2000). *In vitro*, NH_4^+-excreting mutants of *A. brasilense*, although physiologically disadvantaged, can supply more N to a host than wild-type strains (Christiansen-Weniger and Van Veen, 1991; Christiansen-Weniger and Vanderleyden, 1994). In the case of *A. diazotrophicus*, Cojho *et al*. (1993) demonstrated that this bacterium is able to excrete part of the fixed nitrogen into the medium. By using an amylolytic yeast to mimic the plant, they showed that 48% of the total nitrogen fixed by the bacteria was transferred to the yeast, making *A. diazotrophicus* a good candidate for actual contribution of fixed N_2 to its host.

Wood *et al*. (2001) suggest that the inability of the host plant to release sufficient carbon in the rhizosphere is a major constraint in the development of associative N_2–fixing systems. In a laboratory co-culture model using wheat plants inoculated with an ammonium-excreting strain of *A. brasilense*, they found a 48–fold increase in the amount of newly fixed N_2 that was transferred to the shoot tissue when malate was added to the co-culture. They further suggest that wheat plants with an increased release of photosynthate to the rhizosphere offer perspectives for the development of agricultural systems that are more self-supporting for nitrogen nutrition.

Still, when comparing the bacterial numbers present in both associative and symbiotic bacterium-plant interactions, it remains doubtful that associative bacteria will ever be found to contribute substantial amounts of BNF to their plant host in nature. Even when inoculated at large numbers initially (up to 10^7 cfu per seed), the number of associative bacteria rapidly decreases until in most cases about 10^3 to 10^5 cfu g⁻¹ plant root is reached (Jacoud *et al*., 1999; Burdman *et al*., 2000). Compared with rhizobia, which are present at about 10^7 to 10^8 cfu g⁻¹ plant root, this number would be insufficient to provide the plant with sufficient amounts of fixed N.

III. PRODUCTION OF PLANT GROWTH-PROMOTING SUBSTANCES

In the recent decades there has been increasing evidence that besides N_2–fixation, synthesis and export of phytohormones by the N_2–fixing bacteria may play an important role in the observed plant growth promotion. Phytohormones, also called plant growth regulators (PGRs), are well known for their regulatory role in plant growth and development. PGRs are organic substances that influence physiological processes of plants at extremely low concentrations. Because the concentration of hormonal signals is critical to the regulation of various physiological processes in plants, local changes of phytohormone levels can

lead to characteristic changes in plant growth and development. In 1979, production of auxins, cytokinin-like and gibberellin-like substances was proposed for *A. brasilense*, since the increased number of root hairs and of lateral roots observed after inoculation with this bacterium could be mimicked by the application of a mixture of indole-3–acetic acid (IAA), kinetin, and gibberellic acid GA₃ (Tien *et al*., 1979). Moreover, in several other studies the increased plant growth observed after inoculation with *Azospirillum* was proposed to be due to bacterial phytohormone production (Okon and Kapulnik, 1986; Harari *et al*., 1988). Indirectly, a role for phytohormones in the plant growth promoting effect was suggested when Nif⁻ mutants of different diazotrophs appeared still to be able to stimulate plant growth (see section on BNF). Barbieri *et al*. (1986) showed that a Nif⁻ mutant of *A. brasilense* Sp6, which is a producer of IAA, yielded a very similar plant response as the wild-type strain, that is, increase in the number and length of the lateral roots. Similarly, early seedling root growth of canola and lettuce was significantly promoted by the inoculation of seeds with certain strains of *R. leguminosarum*, including nitrogen- and nonnitrogen-fixing derivatives (Noel *et al*., 1996).

A. Auxins

Most of the attention has been focused on the role of the phytohormone auxin. The most common and best characterized and at the same time physiologically most active auxin in plants is indole-3–acetic acid (IAA), which is known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants (Cleland, 1990; Hagen, 1990). The capacity to synthesize IAA is widespread among soil- and plant-associated bacteria. It has been estimated that 80% of bacteria isolated from the rhizosphere can produce the plant growth regulator IAA (Patten and Glick, 1996). Several IAA biosynthetic pathways, classified according to their intermediates, have been reported in bacteria (Patten and Glick, 1996), and in the case of *Azospirillum* IAA biosynthesis was studied extensively (Prinsen *et al*., 1993; Costacurta *et al*.,

1994; Vande Broek *et al*., 1999; Lambrecht *et al*., 2000). A survey of the IAA biosynthesis pathways utilized by plant-associated bacteria reveals that pathogenic bacteria such as *Pseudomonas syringae*, *Agrobacterium tumefaciens*, and *Erwinia herbicola* synthesize IAA predominantly via the indole-3–acetamide (IAM) pathway. Synthesis by this route is generally constitutive. PGPR such as *Rhizobium*, *Bradyrhizobium*, and *Azospirillum* species synthesize IAA mainly via the indole-3–pyruvic acid (IPyA) pathway, which may be subject to more stringent regulation by plant metabolites (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996). By using HPLC and/or GC-MS, the presence of IAA and related compounds in the growth medium could be demonstrated for many diazotrophs, including *Acetobacter diazotrophicus* (Fuentes-Ramirez *et al*., 1993; Bastián *et al*., 1998), *Azospirillum* spp. (Crozier *et al*., 1988; Zimmer and Bothe, 1988; El-Khawas and Adachi, 1999), *Azotobacter* (Pati *et al*., 1995), *Herbaspirillum seropedicae* (Bastián *et al*., 1998), *Klebsiella pneumoniae* (El-Khawas and Adachi, 1999), *Bradyrhizobium elkanii* (Minamisawa *et al*., 1996), *Rhizobium leguminosarum* bv. phaseoli (Atzorn *et al*., 1988), and *Paenibacillus polymyxa* (Lebuhn *et al*., 1997). *Paenibacillus polymyxa*, described as a N₂–fixing species (Achouak *et al*., 1999), was formerly named *Bacillus polymyxa* but has been reclassified by Ash *et al*. (1993).

The first evidence for the role of IAA in the observed plant growth promotion was obtained by attempts to mimic the effect of the bacterium on root growth by the direct application of IAA onto the roots. It could be shown with wheat that inoculation with *A. brasilense* Cd and the application of pure IAA to the roots both increased root length, number of lateral roots, and number of root hairs (Harari *et al*., 1988; Martin *et al*., 1989). Similar experiments with *A. brasilense* (Kolb and Martin, 1985; Jain and Patriquin, 1985; Morgenstern and Okon, 1987b) and *P. polymyxa* (Holl *et al*., 1988) provided strong evidence for IAA production by these diazotrophs and the responsibility of this hormone for the observed effects on plants. An example of the *in vivo* phytohormonal activity of *A. brasilense* was given by Inbal and Feldman (1982), who reported induction of normal development in growth hormone-deficient dwarf mutants of summer wheat by inoculation.

A relatively straightforward way to directly monitor the effects of bacterially synthesized auxin is to compare plants treated with wild-type PGPR strains and with mutant strains that either do not produce or overproduce auxin. A Tn5–induced mutant of *A. brasilense* Sp6 producing a very low amount of IAA showed a reduced ability to promote wheat root system development in terms of both number and length of lateral roots and of the distribution of root hairs when compared with the wild-type strain (Barbieri and Galli, 1993). An IAA-overproducing mutant of *A. brasilense* (selected for resistance to 5–fluoro-tryptophan) had a greater effect on root hair development than the wild-type strain at low inoculum concentrations, but was more inhibitory at higher bacterial densities (Harari *et al.*, 1988). By using different genetically modified strains, the contribution of auxin biosynthesis by *A. brasilense* in altering root morphology was evaluated in a plate assay (Dobbelaere *et al.*, 1999). Inoculation with increasing concentrations of the wild type strains *A. brasilense* Sp245 and Sp7 resulted in a strong decrease in root length and increase in root hair formation (Figure 1). This effect was abolished when inoculating with an *ipdC* mutant of *A. brasilense* producing only 10% of the wild-type IAA level (Figure 2). The *ipdC* gene encodes a key enzyme in the IPyA pathway of IAA synthesis by *A. brasilense*. On the other hand, the observed auxin effect was enhanced further by adding tryptophan (Trp), a precursor for IAA synthesis, to the plates and could be mimicked by replacing the *Azospirillum* cells by a particular concentration of IAA. Together these results confirm the important role of IAA produced by *Azospirillum* in altering wheat root morphology.

A tryptophan auxotrophic *Rhizobium* mutant did not promote seedling root growth of canola and lettuce to the same extent as the parent strain, indicating that also in the *Rhizobium*/non-legume interaction the plant growth regulator IAA might be involved (Noel *et al.*, 1996). However, the authors were not able to fully complement these mutations by adding exogenous tryptophan to the growth medium.

In addition to IAA, bacteria such as *P. polymyxa* and azospirilla also release other compounds in the rhizosphere that could indi-rectly contribute to plant growth promotion like indole-3–butyric acid (IBA), Trp and tryptophol or indole-3–ethanol (TOL) (Fallik *et al.*, 1989; Lebuhn and Hartmann, 1994; Lebuhn *et al.*, 1997; El-Khawas and Adachi, 1999). IBA is a compound that is widely used in agriculture as a commercial promoter of root initiation in cuttings (Nickell, 1982). Trp may enhance endogenous IAA synthesis in the plant root. Direct uptake of applied Trp by plant roots followed by conversion into IAA within their tissues has already been proposed by Martens and Frankenberger (1994). In addition to the synthesis of IAA, plant growth promotion by *P. polymyxa* strains was proposed to be due to its production of TOL (Lebuhn *et al.*, 1997). TOL is formed after the reduction of indole-3–acetaldehyde (IAAld), a side reaction of the IAA pathway. It is easily taken up by plants, serves as an IAA storage form, and can be converted into the active phytohormone, IAA, by plant TOL-oxidase and O_2 (Sandberg, 1984).

B. Cytokinins

The isolation and quantification of cytokinins in nonpathogenic soil bacteria in general and diazotrophic bacteria in particular has received little attention. Because cytokinins are a diverse group of labile compounds that are usually present in small amounts in biological samples, they have often been difficult to identify and quantify. Usually, cytokinin-like activity is reported using bioassays (Nieto and Frankenberger, 1990b). The effects of exogenously applied cytokinins on plants are numerous, the most notable of which is enhanced cell division, but also root development and root hair formation are reported (Frankenberger and Arshad, 1995). Plants and plant-associated microorganisms have been found to contain over 30 growth-promoting compounds of the cytokinin group. One study indicated that as many as 90% of the microorganisms found in the rhizosphere are capable of releasing cytokinins when cultured *in vitro* (Barea *et al.*, 1976). Using bioassays, radio-immunoassays, or HPLC-UV spectrometry, cytokinin production was shown in *Azotobacter* spp. (Barea and Brown, 1974; Azcón and Barea, 1975; Gonzalez-Lopez *et al.*, 1986; Martinez-Toledo *et*

FIGURE 1. Effect of inoculation with *A. brasilense* Sp245 on root development and morphology of 1–week-old wheat seedlings. (A) Effect on root length. The left root system represents an uninoculated control. The other root systems are taken from wheat seedlings inoculated with 10^6, 10^7, 10^8, and 10^9 cfu ml^{-1} from left to right, respectively. (B) Effect on root hair formation and root diameter. Left root tip represents an uninoculated control. The middle and right root tip are taken from seedlings inoculated with 5×10^7 and 5×10^8 cfu ml^{-1}, respectively. (Reproduced from Dobbelaere *et al.* (1999) with kind permission from Kluwer Academic Publishers.)

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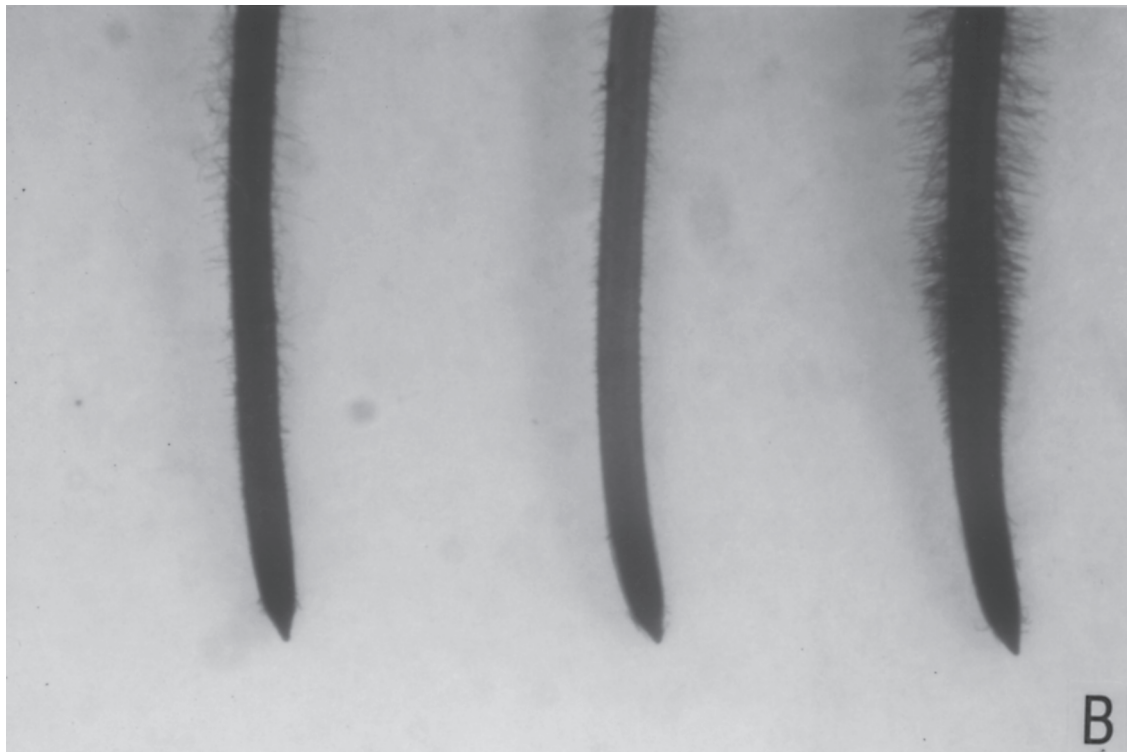
FIGURE 2. Effect of inoculation with *A. brasilense* Sp245b, an *ipdC* mutant strain, on root development and morphology of 1-week-old wheat seedlings. (A) Effect on root length. The left root system represents an uninoculated control. The other root systems are taken from wheat seedlings inoculated with 10^6, 10^7, 10^8, and 10^9 cfu ml^{-1} from left to right, respectively. (B) Effect on root hair formation and root diameter. Left root tip represents an uninoculated control. The middle and right root tip are taken from seedlings inoculated with 10^7 and 5×10^8 cfu ml^{-1}, respectively. (Reproduced from Dobbelaere *et al*. (1999) with kind permission from Kluwer Academic Publishers).

al., 1988; Nieto and Frankenberger, 1989), *Azospirillum* spp. (Horemans *et al.*, 1986; Cacciari *et al.*, 1989), *Rhizobium* spp. (Phillips and Torrey, 1970; Upadhyaya *et al.*, 1991), and *Paenibacillus polymyxa* (Timmusk *et al.*, 1999).

However, genes and enzymes involved in the biosynthesis of bacterial cytokinins have so far only been characterized in phytopathogens, that is, *A. tumefaciens*, *E. herbicola,* and *P. syringae*, and even in these organisms knowledge of the biosynthetic pathways is rather limited (Arshad and Frankenberger, 1998). Therefore, no direct evidence for a role of cytokinin production in plant growth promotion, by using mutant strains producing no or more cytokinin, has been provided. Indirectly, a possible involvement of cytokinins in plant growth promotion was demonstrated by using a *Rhizobium leguminosarum* mutant that is auxotrophic for adenosine, a precursor for cytokinin biosynthesis. This mutant did not promote early seedling root growth of canola and lettuce to the same extent as the parent strain, suggesting that cytokinin might be involved in the observed promotion by the wild-type strain (Noel *et al.*, 1996). So far, the mutation could not be fully complemented by adding exogenous adenosine to the growth systems.

Nieto and Frankenberger (1990a, 1991) studied the effect of the cytokinin precursors adenine (ADE) and isopentyl alcohol (IA) and of the cytokinin-producing bacterium *Azotobacter chroococcum* on the morphology and growth of radish and maize under *in vitro*, greenhouse, and field conditions. The combination of ADE, IA plus the bacterium enhanced the growth of the plants to a much greater degree than when only the precursors or *A. chroococcum* were applied. The improvement in plant growth was attributed primarily to the increase in cytokinin production by *A. chroococcum* in the rhizosphere.

As cytokinins move from roots to shoots, root exposure to cytokinin could affect plant growth and development. Increases in yield and N, P, and K content of grains obtained after exogenous application of cytokinins in field trials with rice (Zahir *et al.*, 2001) support the hypothesis that bacterially supplied cytokinins to the soil can improve the growth and yield of treated plants.

C. Gibberellins

Also in the case of gibberellins (GAs), the bacterial genetic determinants have not been identified so far. Therefore, no mutants are available to demonstrate the role of this phytohormone in plant growth promotion. Recently, there appears to be renewed interest in these phytohormones because a number of recent articles report on the production of GAs by diazotrophic bacteria. Over 89 GAs are known to date and are numbered GA_1 through GA_{89} in approximate order of their discovery (Frankenberger and Arshad, 1995; Arshad and Frankenberger, 1998). The most widely recognized gibberellin is GA_3 (gibberellic acid), the most active GA in plants is GA_1, which is primarily responsible for stem elongation (Davies, 1995). Using a bioassay, HPLC or GC-MS, GA production has been demonstrated in *Azotobacter* spp. (Barea and Brown, 1974; Azcón and Barea, 1975; Gonzalez-Lopez *et al.*, 1986; Martinez-Toledo *et al.*, 1988), *P. polymyxa* (Sattar and Gaur, 1987), *Rhizobium leguminosarum* bv. phaseoli (Atzorn *et al.*, 1988), *A. brasilense* (Janzen *et al.*, 1992), *A. lipoferum* (Bottini *et al.*, 1989; Piccoli and Bottini, 1994; Piccoli *et al.*, 1996), *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Bastián *et al.*, 1998), and *Bacillus pumilus* and *Bacillus licheniformis* (Gutiérrez-MaHero *et al.*, 2001). Most likely, the GAs formed do not play a significant physiological role in the producing organism and, consequently, can be regarded as secondary metabolites (Rademacher, 1994). However, several observations suggest that these GA-producing microorganisms might induce or promote growth in the host plants through the action of the released GAs. One of the earliest studies in this respect reports on similar effects obtained by inoculation of tomatoes with *Azotobacter chroococcum* or gibberellin application (Jackson *et al.*, 1964). Fulchieri *et al.* (1993) observed that GA_3 had similar effects as *A. lipoferum* inoculation on promotion of root growth in 48-h-old maize seedlings, especially in increasing hair density in areas physiologically active for nutrient uptake and water absorption. In a similar comparative study, GA_3 application or *Azospirillum* spp. inoculation were compared for their effect on height and fresh weight of uniconazole treated

maize plants. Uniconazole inhibits internode growth in a number of plant species by blocking the three oxidation steps in the GA biosynthesis pathway (Yamaji *et al.*, 1991). Because both *A. lipoferum* and *A. brasilense* increased height at similar levels with respect to application of 0.1 μg GA$_3$ per plant, Lucangeli and Bottini (1997) concluded that GAs produced by *Azospirilum* spp. play an important role in the early stages of plant growth in Gramineae. Further, it was found that *Azospirillum* spp. could enhance shoot growth in the dwarfs *d1* of maize and *dx* of rice, which are defective for the biosynthesis of active GAs, suggesting the *in vivo* production of these phytohormones by these bacteria (Lucangeli and Bottini, 1996). A similar bioassay with dwarf alder seedlings was used to demonstrate *in vivo* GA production by *Bacillus pumilus* and *Bacillus licheniformis* (Gutierrez-Manero *et al.*, 2001).

In addition to GA production, *Azospirillum* spp. cultured in a nitrogen-free biotin-based chemically defined medium are able to hydrolyze GA glucosyl conjugates both *in vitro* (Piccoli *et al.*, 1997) and *in vivo* (Cassán *et al.*, 2001), releasing GAs and so providing another way to promote plant growth. These results suggest that the growth promotion in plants that is induced by *Azospirillum* infection may occur by a combination of both gibberellin production and gibberellin-glucoside/glucosyl ester deconjugation by the bacterium (Piccoli *et al.*, 1997).

The ability of *Azospirillum* spp. to alleviate the effects of water deficits in cereal seedlings under salt and osmotic stresses (Creus *et al.*, 1997; Hamdia and Elkomy, 1998) can also be attributed at least partly to bacterial GA production (Piccoli *et al.*, 1999). In *Zea mays*, *A. lipoferum* inoculation or GA$_3$ application significantly increased chlorophyll, K, Ca, soluble sugars, and protein contents in plants grown at NaCl concentrations generating up to –1.2 MPa of osmotic strength, when compared with controls (Hamdia and Elkomy, 1998). This would explain the better growth (greater fresh weight/dry weight) and faster elongation rate in shoots of inoculated plants. The effect might also be due to improvement of the active hairy root zone (Fulchieri *et al.*, 1993), which facilitates water absorption.

D. Other Plant Growth Regulators

Although ethylene is synthesized by many, and perhaps all, species of bacteria and fungi (Primrose, 1979), it has not been studied for diazotrophic bacteria. In addition to IAA, abscisic acid (ABA) has been detected by radio-immunoassay or TLC in supernatants of *Azospirillum* and *Rhizobium* spp. cultures (Kolb and Martin, 1985; Dangar and Basu, 1987), but no role in plant growth promotion was reported. Inasmuch as the primary role of ABA in stomatal closure is well established, as well as its uptake by and transport in the plant, its presence in the rhizosphere could be extremely important for plant growth under a water-stressed environment, such as found in arid and semiarid climates (Frankenberger and Arshad, 1995). As the authors suggest, future research should focus on this vital aspect of plant-microbial-soil interaction relative to ABA.

In addition to classic phytohormones, it has been shown that nitrite at a concentration range of 0.1 to 10 m*M* (e.g., that generated by the dissimilatory nitrate reductase of *Azospirillum*) mimics the effect of IAA in several plant tests for auxins (Zimmer *et al.*, 1988). Because nitrite alone can hardly exert phytohormonal effects, it is postulated that nitrite reacts with a substance in the plant cells and that a product formed by this reaction functions as auxin. Such a substance could be ascorbate, as the effect of nitrite could be enhanced by adding ascorbate.

When studying the role of phytohormone production in the mechanism of plant growth promotion by diazotrophs, some caution is required concerning the interpretation of the results. Since plants as well as many diazotrophs can synthesize phytohormones, one has to take into account that diazotrophic bacteria may also be able to affect plant endogenous hormone levels, irrespective of their own hormone production (Fallik *et al.*, 1989). Inoculation of maize seedlings growing in nutrient solution with *Azotobacter chroococcum* resulted in a synergistic increase in phytohormone concentration (IAA, ABA, isopentenyladenosine, zeatin riboside, and dihydrozeatin riboside) compared with the sum of hormone production by sterile plant roots and by bacterial cultures (Müller *et al.*, 1989). However, it is hard to distinguish

between the hormones synthesized by the plant in response to PGPR stimulation and the hormones synthesized by the PGPR itself (Fallik *et al*., 1989). Therefore, all possibilities, the plant synthesizing more phytohormones upon presence of the bacterium, the bacteria producing and exporting more phytohormones under the influence of plant roots, or both, remain open. In the case of auxin production by *Azospirillum*, a model was proposed for the role of IAA as a reciprocal signaling molecule in the *Azospirillum*-plant interaction (Lambrecht *et al*., 2000). In this model, bacterial growth and IAA production is promoted by the presence of root exudates and the IAA precursor Trp, resulting in larger amounts of IAA produced in the rhizosphere. In addition, as in *A. brasilense,* the *ipdC* gene is upregulated by IAA, the presence of plant-derived IAA in the rhizosphere could further enhance bacterial IAA synthesis. This then stimulates the proliferation of plant roots, nutrient uptake, and eventually plant growth, which in turn results in increased root exudation. However, this IAA amplification loop is suggested to be strictly regulated, because large concentrations of exogenously applied IAA inhibit root development. Further study with *A. brasilense* mutants that are completely deficient in IAA biosynthesis are needed to test the postulated model.

In addition, phytohormones do not act alone but rather interact with one another in a variety of complex ways (Barendse and Peeters, 1995). The complexity of multiple hormonal regulation is illustrated by the fact that each of the hormones have been found to be able to affect nearly every phase of plant growth and development (Leopold and Noodén, 1984). As an example, the balance between auxin and cytokinin levels controls cellular differentiation and organogenesis in tissue and organ culture, ranging from shoot proliferation to root formation as the ratio auxin/cytokinin increases (Wareing and Phillips, 1970; Skoog and Schmitz, 1972). Additionally, auxins and cytokinins stimulate ethylene production synergistically (Stenlid, 1982). It is clear that the effect of bacterial phytohormone synthesis on plant growth is quite complex and should be seen in view of the interaction with other phytohormones.

Finally, an interesting approach to studying the involvement of phytohormones in the plant/

bacterium interaction is the use of marker genes possibly involved in the process. Many auxin-responsive genes have been isolated from plants, having a wide difference in time of response, depending on the gene and cell type (Abel and Theologis, 1996). For early responding genes such as *GH3*, *SAUR 15A,* and *PS-IAA4/5* (Abel and Theologis, 1996; Guilfoyle *et al*., 1998) increased mRNA levels have been observed as early as 5 min after adding auxin. These primary response genes are activated without a requirement for *de novo* protein synthesis. *GH3,* isolated from soybean, is one of the best-studied early responding genes (Hagen and Guilfoyle, 1985; Li *et al*., 1999). The expression of this gene is very low or not detectable in most vegetative tissues of intact seedlings or plants, but can be strongly and rapidly induced when exogenous auxin is applied (Hagen *et al*., 1991). Induction of expression of the *GH3* promoter-*GUS* reporter gene can occur in most organs and tissues in a dose-dependent manner, indicating that auxin is a limiting factor for activiation of the *GH3* gene promoter (Li *et al*., 1999). Therefore, the *GH3–GUS* gene can be a good molecular marker for monitoring changes in either endogenous auxin concentration or cellular sensitivity to auxin in plants. Meanwhile, many *GH3–GUS* transgenic lines are available and are used intensively in physiology studies. The use of these transgenic plants will be of help to better understand the mechanism of IAA signaling from bacteria to plants. On the other hand, mutants of *Arabidopsis* that overexpress IAA (i.e., *trp2*, *trp3*; Last *et al*., 1991; Normanly *et al*., 1993) or which are altered in root formation (i.e., *rhd6*; Masucci and Schiefelbein, 1994) can be useful to monitor the effect of altered plant IAA levels on the colonizing bacteria. With these data, it should be possible to validate the previously proposed model and to establish the role of IAA in beneficial bacteria-plant interactions.

IV. SYNTHESIS OF ENZYMES THAT CAN MODULATE PLANT GROWTH AND DEVELOPMENT

A rather new and unsuspected mechanism of plant growth promotion involves the plant hor-

mone ethylene. Ethylene is a potent plant growth regulator that affects many aspects of plant growth, development, and senescence (Reid, 1987). In addition to its recognition as a "ripening hormone", ethylene promotes adventitious root and root hair formation, stimulates germination, and breaks the dormancy of the seeds (Pratt and Goeschl, 1969; Esashi, 1991). However, if the ethylene concentration remains high after germination, root elongation (as well as symbiotic N_2 fixation in leguminous plants) is inhibited (Jackson, 1991).

It has been proposed that many plant growth-promoting bacteria may promote plant growth by lowering the levels of ethylene in plants. This is attributed to the activity of the enzyme 1–aminocyclopropane-1–carboxylate (ACC) deaminase, which hydrolyzes ACC, the immediate biosynthetic precursor of ethylene in plants (Walsh et al., 1981; Yang and Hoffman, 1984). The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth (Honma and Shimomura, 1978; Klee et al., 1991). In this way the bacterium acts as a sink for ACC and as such is lowering the ethylene level in plants, preventing some of the potentially deleterious consequences of high ethylene concentrations (Glick et al., 1998).

In nature, ACC deaminase has been commonly found in soil bacteria that colonize plant roots (Klee et al., 1991; Glick et al., 1999; Belimov et al., 2001). Many of these microorganisms were identified by their ability to grow on minimal media containing ACC as its sole nitrogen source. In this way, *Azospirillum* spp., *Herbaspirillum* spp., *Azoarcus*, *Azorhizobium caulinodans*, *Gluconacetobacter diazotrophicus*, *Burkholderia vietnamiensis*, *Azotobacter* spp., *Azorhizophilus,* and *Pseudomonas* spp. were all found to be able to use ACC as the sole nitrogen source for growth (De Troch and Vanderleyden, unpublished results). An example of such an ACC deaminase containing bacterium is the PGPR *Pseudomonas putida* GR12–2 (Lifshitz et al., 1986, 1987) that stimulates root growth of a number of different plants (canola, lettuce, tomato) under gnotobiotic conditions (Glick et al., 1994, 1997; Hall et al., 1996). *Pseudomonas putida* GR12–2 was chemi-

cally mutagenized and three independent mutants that lacked ACC deaminase activity were selected. Unlike the wild type, none of these selected mutants was able to promote the growth of canola seedling roots under gnotobiotic conditions (Glick et al., 1994). It was concluded that the ACC deaminase may play a role in the mechanism that this bacterium uses to stimulate canola root elongation. However, in these experiments the mutants were created by chemical mutagenesis, and as a result it is not certain that the mutations were within the ACC deaminase structural gene per se. Indirectly, the role of ACC deaminase in the observed stimulation of root elongation was shown by introducing the ACC deaminase structural gene (acdS) from *Enterobacter cloacae* UW4 in an *A. brasilense* strain that did not show ACC deaminase activity. Inoculation of tomato and canola seedlings with *A. brasilense* cells transformed with acdS under the control of the lac promoter significantly promoted root length as compared to plants inoculated with the nontransformed strains (Holguin and Glick, 2001).

Experiments with other (nondiazotrophic) bacteria show that PGPR expressing ACC deaminase can also decrease the deleterious effects of different environmental stresses such as heavy metals and flooding on plants, probably by reducing the concentration of plant stress ethylene (Glick et al., 1997; Burd et al., 1998; Grichko and Glick, 2001). Plants respond to stress by increasing the production of ethylene in their tissues (Yang and Hoffman, 1984; Abeles et al., 1992). Flooded tomato plants treated with *A. brasilense* Cd containing the ACC deaminase structural gene (acdS) from *Enterobacter cloacae* UW4 showed lower levels of epinasty than plants treated with the untransformed wild-type strain (Holguin and Glick, 2000). Likewise, the wild-type strain *P. putida* GR12–2 and not a mutant lacking ACC deaminase activity promoted root growth of canola seedlings under conditions of salt or temperature stress (Glick et al., 1997).

Based on the proposed model for plant growth promotion by lowering the plant ethylene levels, it is predicted that any rhizosphere bacterium that actively expresses ACC deaminase can promote the elongation of seedling roots, that is, it can act as a PGPR. In agreement with this, all *Pseudomo-*

nas spp. that were able to utilize ACC as a sole source of nitrogen also displayed PGPR activity (Glick *et al*., 1995). However, the agronomic use of PGPR that promote plant growth by mechanisms that include hydrolyzing ACC may be limited largely to dicots. Monocots are less sensitive to ethylene and therefore are less responsive to these bacteria (Hall *et al*., 1996; Holguin and Glick, 2001).

The model proposed by Glick *et al*. (1998) for the lowering of plant ethylene concentrations by PGPR also includes a role for the bacterial IAA in this mechanism of plant growth stimulation. In this model, IAA synthesized by the PGPR is taken up by the plant and can stimulate either cell proliferation and/or elongation or the activity of the enzyme ACC synthase. In the latter case, ACC synthesis within the plant is increased, and a portion of this newly synthesized ACC may be exuded from the roots and taken up by the PGPR that can metabolize it. In this way, the bacterium causes the plant to synthesize more ACC than the plant would otherwise need, thereby providing the bacterium with a unique source of nitrogen (Hall *et al*., 1996).

V. INCREASED NUTRIENT UPTAKE

Several reports have suggested that PGPR stimulate plant growth by facilitating the uptake of minerals N, P and K (NO_3^-, $H_2PO_4^-$ and K^+), and microelements, by the plant. However, there is some controversy regarding the mechanism(s) that PGPR employs in the uptake of minerals. Many investigators agree that rhizosphere organisms promote uptake of minerals by roots, but there is no generally accepted explanation for the process.

On one hand, increased mineral uptake by plants has been suggested to be due to a general increase in the volume of the root system, as reflected by an increased root number, thickness, and length, and not to any specific enhancement of the normal ion uptake mechanism (Reynders and Vlassak, 1982; Smith *et al*., 1984; Kapulnik *et al*., 1985b, 1987; Morgenstern and Okon, 1987a; Gunarto *et al*., 1999; Biswas *et al*., 2000). Higher K and Fe uptake for instance are related to thicker roots (Barber, 1985) and higher P uptake to the presence of root hairs (Gahoonia and Nielsen, 1998).

On the other hand, experiments with *Azospirillum* species have suggested that this organism specifically enhances mineral uptake (Kapulnik *et al*., 1983; Lin *et al*., 1983; Murty and Ladha, 1988). It has been demonstrated that *Azospirillum*-inoculated plants take up minerals (N, P, and K) from solutions at faster rates than uninoculated controls (Lin *et al*., 1983; Kapulnik *et al*., 1985b), and, consequently, plants in the field accumulate dry matter, N, P, and K, at higher rates (Sarig *et al*., 1984; Yahalom *et al*., 1984). Several possible explanations for this phenomenon were given.

It was found that inoculation with *A. brasilense* Cd resulted in a significant increase in the proton efflux of the roots of wheat seedlings and a reduction in the membrane potential of the root cells of soybean seedlings (Bashan *et al*., 1989; Bashan, 1991). It was proposed that *A. brasilense* inoculation influences membrane activity and subsequently proton efflux in roots, probably through the release of an as yet unidentified bacterial signal (Bashan, 1990; Bashan and Levanony, 1991). Proton extrusion through membranes of root cells, which results in acidification of the rhizosphere, is proposed to be a major mechanism in the mobilization of minerals in plants (Spanswick, 1981; Marschner *et al*., 1986).

Pectinolytic activity of *Azospirillum* cells has also been proposed to contribute to an increase in mineral uptake by a so-called "sponge" effect (Okon, 1982). The pectinolytic activity might be involved in a slight hydrolysis of the middle lamellae of *Azospirillum*-colonized cortical cells without causing cell collapse, which may accelerate water and nutrient uptake by the roots (Lin *et al*., 1983; Sarig *et al*., 1984; Kapulnik *et al*., 1985b). This hypothesis is based on the observed distortion in the arrangement of cortical cells of wheat and maize roots after inoculation with *A. brasilense* (Lin *et al*., 1983; Kapulnik *et al*., 1985c), indicating a weakening of the natural adherence in the cortex tissue of inoculated roots. However, since up until now only *A. irakense* has been shown to possess substantial pectinase acitivity (Khammas and Kaiser, 1991; Bekri *et al*., 1999), this altered

cell arrangement of *A. brasilense* inoculated wheat and maize roots must probably be attributed to the phytohormones that *A. brasilense* cells produce. As mentioned before, one of the short-term effects of IAA is cell elongation (Cleland, 1990; Hagen, 1990).

In addition, the bacterial nitrate reductase has been suggested to play a role in the enhanced N assimilation by *Azospirillum*-inoculated plants. A spontaneous chlorate-resistant and nitrate reductase negative (NR⁻) mutant of *A. brasilense* Sp245 is far less effective than the parent strain (nitrate reductase positive) in enhancing dry matter yield and nitrogen incorporation in wheat plants (Baldani *et al*., 1986; Boddey *et al*., 1986; Ferreira *et al*., 1987). Strain Sp245 seemed to supply more reduced N to the shoots, resulting in enhanced dry weights and total reduced N. It was hypothesized that the parental strain aided nitrate reduction in the roots and thus decreased nitrate translocation to the leaves, while inoculation with the NR⁻ mutant caused direct translocation and reduction of nitrate in the plant foliage. However, it was found that the NR⁻ mutant was not able to colonize the wheat roots as efficiently as the parental strain (Baldani *et al*., 1986; Steenhoudt *et al*., 2001b). Furthermore, no attention was given to inoculum concentration, and the mutant was not characterized as to possible other differences from the parent strain (like simultaneous loss of plant hormone production). Therefore, no firm conclusions can be drawn. Some well-defined nitrate reductase negative mutants are available now (Steenhoudt *et al*., 2001a) that can be used in plant inoculation experiments to confirm this hypothesis. O'Hara *et al*. (1987) did not find any differences in the growth response of maize inoculated with a wild-type (denitrifying) strain and a nitrate-respiring (dissimilatory nitrite reductase negative) strain.

Recently, a gene encoding an ammonium transporter in the root hairs of tomato (*LEAMT1;2*) has been identified. Inoculation of N-depleted tomato (*Lycopersicon esculentum*) plants with *A. brasilense* or *Azoarcus* sp. induced *LEAMT1;2* expression, while *A. brasilense nifDK⁻* mutants failed to do so (Becker *et al*., 2002), indicating that the transporter responds well to bacterially produced NH$_4^+$. Quantification of *LEAMT1;2* gene expression during inoculation experiments might help to elucidate the mechanism of improved nutrient uptake.

In the case of iron uptake, it was suggested that plants can benefit from the siderophores produced by several PGPR. Although iron is one of the most abundant minerals on Earth, in the soil it is relatively unavailable for direct assimilation by microorganisms. The reason for this is that in aerobic soils Fe is found predominantly in the form of Fe^{3+}, mainly as a constituent of oxyhydroxide polymers with extremely low solubility, about 10^{-18} *M* at neutral pH (Neilands *et al*., 1987). Minimal concentrations of iron required for normal growth of plants range from 10^{-9} to 10^{-4} *M*, depending on other nutritional factors (Römheld and Marschner, 1981; Lindsay and Schwab, 1982; Schwab and Lindsay, 1983). Similarly, minimal iron concentrations for the optimal growth of many microbes are approximately 10^{-5} to 10^{-7} (Lankford, 1973). To overcome this problem, soil microorganisms secrete low-molecular-weight iron-binding molecules (siderophores) that bind Fe^{3+}, transport it back to the microbial cell, and then make it available for microbial growth (Leong, 1986; Neilands and Leong, 1986; Briat, 1992). The production of siderophores has been reported for *Azospirillum lipoferum* (Saxena *et al*., 1986; Shah *et al*., 1992), *Azospirillum brasilense* (Bachhawat and Ghosh, 1987), *Azotobacter vinelandii* (Knosp *et al*., 1984; Demange *et al*., 1988; Page and von Tigerstrom, 1988), and for rhizobia (Guerinot, 1991, 1994; Carson *et al*., 1992).

Because of the abundance of microbial siderophores in soils, along with their outstanding Fe binding capacity and chemical stability, these compounds may contribute significantly to an increased mobility of Fe in the soil and in the rhizosphere in particular, making it more available for plants. However, the direct uptake of Fe(III) microbial siderophores by plants has been the subject of controversy (Crowley *et al*., 1991; Marschner and Römheld, 1994). Although iron uptake via siderophores has been reported for certain plant species and certain siderophores (Cline *et al*., 1984; Crowley *et al*., 1988; Bar-Ness *et al*., 1991; Wang *et al*., 1993), it was generally found that plants take up bacterial

siderophores less efficiently than certain synthetic Fe chelates or than phytosiderophores (chelators produced by monocot grasses) in the case of monocots (Römheld and Marschner, 1986; Marschner *et al.*, 1989; Crowley *et al.*, 1991; Marschner and Römheld, 1994). Nevertheless, bacterial siderophores may act as an important source of Fe for higher plants in alkaline and calcareous soils (Jurkevitch *et al.*, 1986, 1988; Bar-Ness *et al.*, 1991), where iron availability is severely limited. There is only one report in which the effect of inoculation with a diazotroph on the iron absorption by plants has been investigated. In this report, *Azospirillum brasilense* was found to increase the iron absorption and translocation by sorghum (Barton *et al.*, 1986). However, additional experiments with purified siderophores or mutant strains are necessary to confirm these observations and exclude other possible mechanisms, such as improved nutrient uptake through increased root development.

From this it is clear that, except for certain calcareous soils, the synthesis of siderophores by diazotrophs is not a principal mechanism of plant growth promotion.

VI. ENHANCED STRESS RESISTANCE

Field experiments performed in the 1980s have revealed growth-promoting effects of *Azospirillum* on plants exposed to drought stress. Sarig *et al.* (1988) reported that sorghum plants inoculated with *Azospirillum* were less drought stressed, having more water in their foliage, higher leaf water potential, and lower canopy temperature than noninoculated plants. Total extraction of soil moisture by *Azospirillum*-inoculated plants was greater and water was extracted from deeper layers in the soil profile. Therefore, sorghum yield increase in inoculated plants was attributed primarily to improved utilization of soil moisture. Foliar application of a diazotrophic *Klebsiella* sp. could ameliorate drought stress effects on wetland rice, as grain yield increased, together with increased nutrient uptake and proline content (Razi and Sen, 1996). Proline is an important osmoregulator, accumulated as a consequence of drought stress. Creus *et al.* (1998) studied the

effects of *A. brasilense* Sp245 inoculation on water relations in two wheat cultivars. They found that *Azospirillum* stimulated growth of wheat seedlings grown in darkness under osmotic stress, together with a significant decrease in osmotic potential and relative water content at zero turgor, in volumetric cell wall modulus of elasticity, and in absolute symplastic water volume and by a significant rise in apoplastic water fraction parameters. These are known physiological mechanisms of adaptation that give plants the ability to tolerate a restricted water supply (Girma and Krieg, 1992). As in this hydroponic test system no nutrients were present, the improved water status of the wheat seedlings cannot be attributed to enhanced mineral uptake and consequently growth promotion. Similarly, in a hydroponic system without nutrients, *A. brasilense* Sp245 was found to partially reverse the negative effects that drought stress had on wheat seedlings, as it was observed in the growth rate of coleoptiles (Alvarez *et al.*, 1996).

Apart from alleviating osmotic stress in plants, inoculation with diazotrophs can also enhance oxidative stress tolerance. By oxidative stress it is meant the oxidative damage caused by reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Sies, 1991; Stajner *et al.*, 1995). These highly reactive oxygen species can be generated by the oxidative metabolism of normal cells and by different stress situations. Although ROS contribute in plant defense against pathogens (Mehdy, 1994; Desikan *et al.*, 1998), they are potentially harmful to plant viability (Bowler *et al.*, 1992). With the production of antioxidant enzymes like superoxide dismutase (SOD), peroxidase, and catalase the cell can neutralize and thus control free radical formation. Also, pigments such as carotenoids could be involved in scavenging singlet oxygen and thus decrease oxidative stress (Elstner *et al.*, 1994). Inoculation with *Azotobacter chroococcum* was reported to improve oxidative stress defense ability in sugar beet leaves since inoculated plants showed increased activities of superoxide dismutase, peroxidase, and catalase and increased chlorophyll and carotenoid content (Štajner *et al.*, 1997). High activities of antioxidant enzymes (especially SOD)

are linked with oxidative stress tolerance (Štajner *et al*., 1995). However, the observed effects have not been linked yet to certain traits of the diazotroph. Therefore, it is not clear whether this increase in oxidative stress tolerance is a direct effect of inoculation or rather an indirect consequence of an overall healthier plant by inoculation with *Azotobacter*.

VII. INCREASED NUTRIENT AVAILABILITY THROUGH ORGANIC AND INORGANIC PHOSPHATE (P)-SOLUBILIZATION

Frequently, solubilization of P has been postulated as a possible mechanism of plant growth promotion by PGPR (for a recent review see Rodríguez and Fraga, 1999; Richardson, 2001). P-solubilization is important for plant growth because P is an essential nutritional element for plants, but is at the same time one of the least soluble nutrient ions in the environment, with usually less than 5% of total soil phosphate being available to plants (Epstein, 1972; Brown, 1974). Phosphorus exists in nature in a variety of organic (derived from microorganisms and plants) and inorganic (originating from applied P fertilizer) forms that are insoluble to very poorly soluble (Paul and Clark, 1989). Therefore, the addition of phosphate fertilizers has become a common practice in modern agriculture. However, a large portion of the soluble inorganic phosphate applied to soil as fertilizer is rapidly immobilized by the iron and aluminium in acid soils and by calcium in calcareous soils soon after application, thus becoming unavailable to plants (Chang and Chu, 1961; Lindsay, 1979; Sanyal and De Datta, 1991; Holford, 1997). Soil microorganisms are able to solubilize insoluble mineral phosphate by producing various organic acids (Taha *et al*., 1969; Banik and Dey, 1982; Halder *et al*., 1990; Illmer *et al*., 1995; Jones, 1998). This results in acidification of the surrounding soil, releasing soluble orthophosphate ions ($H_2PO_4^{-1}$ and HPO_4^{-2}) that can be readily taken up by plants. Furthermore, they are able to solubilize organic P compounds by means of phosphatase enzymes (Greaves and Webley, 1965; Tarafdar and Junk, 1987; Garcia *et*

al., 1992). A large number of P-solubilizing bacteria (PSB) have been isolated from the rhizosphere of several crops. It was estimated that P-solubilizing microorganisms may constitute 20 to 40% of the culturable population of soil microorganisms and that a significant proportion of them can be isolated from rhizosphere soil (Kucey, 1983; Chabot *et al*., 1993). Although there is good evidence for P-solubilization by these microorganisms in pure culture (Taha *et al*., 1969; Bajpai and Sundara Rao, 1972; Banik and Dey, 1981; Chabot *et al*., 1993), it is difficult to demonstrate P solubilization in plant-microorganism systems. The production by these strains of other metabolites beneficial to the plant, such as phytohormones, antibiotics, or siderophores, among others, has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation (Suslov, 1982; Kloepper *et al*., 1989). It is argued that the increased P uptake often observed in plants treated with PGPR is an indirect byproduct of that interaction and actually reflects a better developed root system and an overall healthier plant. Indeed, in many cases there was a stimulation of plant growth by these PSB, but no effect on P uptake could be observed (Laheurte and Berthelin, 1988; Chabot *et al*., 1993, 1996a; de Freitas *et al*., 1997; Singh and Kapoor, 1998), indicating that other mechanisms than P-solubilization could be responsible for plant growth promotion.

Experiments performed with P-solubilizing diazotrophs are few, and the results obtained quite diverse, varying according to plant or bacterial species. *Bacillus megaterium* and *P. polymyxa* are able to enhance growth and yield but not the P uptake of canola, indicating that P-solubilization is not the main mechanism responsible for positive growth response (de Freitas *et al*., 1997).

Kumar and Narula (1999) used chemically induced mutants of *Azotobacter chroococcum*, isolated from the wheat rhizosphere, with higher phosphate-solubilization activity to inoculate wheat and found significantly positive effects of inoculation on percent germination and growth emergence, with the mutant strains performing better than the parent strain. However, the mutant strains also produced significantly higher amounts of IAA, suggesting that the effect of inoculation

on seed emergence is caused primarily by growth regulators. In addition to fixing nitrogen, *A. chroococcum* is known to produce growth hormones, vitamins, and antifungal substances (Martinez-Toledo *et al.*, 1988; Doneche and Marcantoni, 1992; Revillas *et al.*, 2000). These factors, in addition to P-solubilization, might have contributed to the better growth and early seed emergence of wheat. As the authors conclude, due to an increased plant size, there is an increase in P uptake. P-dissolving bacteria may then have a secondary role in making extra P available from sparingly soluble sources, especially in P-deficient soils (Barea *et al.*, 1976).

Similarly, inoculation with two strains of *R. leguminosarum* bv. *phaseoli*, strain P31 and R1, selected for their P-solubilization ability, increased lettuce dry matter yield at certain fertilizer levels but had no significant effect on P-uptake (Chabot *et al.*, 1996a). Also in this case, other mechanisms than P-solubilization are possibly involved. Both strains produce siderophores and IAA and strain P31 also produces HCN. On the other hand, inoculation of maize with the same *R. leguminosarum* strains did result in a significantly higher concentration of P in the plants than that of the uninoculated control plants at all levels of P applied. The higher total P uptake of maize indicates that in this field experiment, P-solubilization is probably a major mechanism of growth promotion.

To further investigate the role of P-solubilization activity of strain R1 in maize root colonization, Chabot *et al.* (1996b) produced two mutants with reduced P-solubilization activity by random Tn5–mutagenesis. In a rich soil with a high available-P content, dry matter of maize was significantly increased by bacterial inoculation, compared to the uninoculated control. However, both mutant strains did not increase dry matter production in the same way, with one mutant performing better than the wild type strain, the other worse, depending on the P-fertilization (Chabot *et al.*, 1998). No data on P-content were presented. The mutants were not completely negative for P-solubilization (40% less P solubilized after 16 h incubation) and were not characterized as to which gene was mutated. The presence of several genes that control P-solubilization (Goldstein and Liu, 1987) decreases the probability of obtaining completely negative mutants. Therefore, no precise conclusions can be drawn concerning the role of P-solubilization in maize growth promotion. The observation that the wild-type strain colonized roots better than the altered mutants suggests that P-solubilization might have an important role to play in rhizosphere competitiveness of P-solubilizing rhizobia. This phenotype might influence the ability of a strain to colonize plant roots.

In conclusion, a contribution of P-solubilization in plant growth promotion has been observed in some cases, but more experiments with well-defined strains or mutants in well-defined conditions and with the appropriate controls are needed to provide clear evidence.

VIII. VITAMIN PRODUCTION

Vitamins are sometimes added to the list of compounds involved in direct plant growth promotion that PGPR can produce. However, the possibility that plant growth can be improved by inoculation with vitamin-producing bacteria has received little attention. It is generally accepted that green plants under optimal growing conditions synthesize sufficient quantities of vitamins to meet their own needs, but stress, induced by, for example, drought, unfavorable temperature, or mineral deficiency, might lead to vitamin deficiency, a deficiency that could in part be the cause of the observed yield reduction. Vitamins can reverse the negative effects of mineral deficiencies, but also under normal conditions growth stimulation and yield increases were frequently observed after vitamin application (Oertli, 1987).

Azotobacter, *Azospirillum,* and *Rhizobium* strains were found to produce some or all of the water-soluble B-group vitamins niacin, pantothenic acid, thiamine, riboflavine, and biotin in defined culture media (Gonzalez-Lopez *et al.*, 1983; Dahm *et al.*, 1993; Rodelas *et al.*, 1993; Martinez-Toledo *et al.*, 1996; Sierra *et al.*, 1999; Revillas *et al.*, 2000). Nevertheless, their possible role in plant growth promotion has never been studied. There is evidence that exogenous B vitamins can be absorbed by roots, producing favorable effects on root development (thiamin is con-

sidered to be a growth factor for plant roots; Bonner and Devirian, 1939), shoot length, dry matter production, and nutrient uptake (Oertli, 1987; Mozafar and Oertli, 1992).

Martinez-Toledo *et al*. (1996) reported that *A. vinelandii*, when grown in conditions similar to its natural habitat, produced large amounts of vitamins in the presence of combined N and when a high concentration of glucose was added as a C source. Based on these data, it can be assumed that vitamins produced by *Azospirillum* and *Azotobacter* spp. may stimulate plant growth in the field under certain C:N ratios. Comparative studies with appropriate mutants are needed to verify this hypothesis.

IX. BIOCONTROL

Increased plant productivity by a mechanism of biocontrol is indirect and results from the suppression of deleterious microorganisms and soil-borne pathogens by PGPR (Schippers *et al*., 1987). This mechanism of plant growth promotion so far has been treated in a stepmotherly way as far as diazotrophs are concerned. Although different ways of biocontrol by PGPR, especially *Pseudomonas* spp., have been described extensively in the literature (Schippers *et al*., 1987; Chet *et al*., 1991; Glick, 1995; Handelsman and Stabb, 1996; Whipps, 2001), there are only few studies on biocontrol by diazotrophs, that is to say, in most cases it is not known whether the organism is N_2–fixing or not. Isolates were screened merely for antagonism toward certain pathogens, leaving other characteristics, like N_2–fixation, unchecked since not appropriate. Therefore, it is possible that biocontrol as a mechanism of plant growth promotion by diazotrophs is far more common than generally known today.

Two ways of biocontrol can be distinguished: via antagonism of the pathogen or by changing the host plant's susceptibility, for example, by induced resistance. Bacteria can antagonize soil-borne pathogens through various mechanisms such as competition, antibiosis, or parasitism (Chet *et al*., 1991; Handelsman and Stabb, 1996). Competition for nutrients between the biocontrol bacteria and the pathogen can result in the displace-ment of the pathogen. The best understood example of this mechanism is iron competition. PGPR produce high Fe^{3+} affinity siderophores that sequester iron in the rhizoplane, making it less available to certain deleterious rhizosphere microorganisms (Schroth and Hancock, 1982; Leong, 1986; Schippers *et al*., 1987; O'Sullivan and O'Gara, 1992). The latter cannot obtain sufficient iron for growth because they produce siderophores in insufficient quantities or with less affinity for iron than those from PGPR and thus are outcompeted. Several diazotrophs have been reported to produce siderophores (see section '**increased nutrient uptake**'), but no inoculation experiments have been performed to examine their role as a competitive agent against soil bacteria. *A. lipoferum* M was found to produce siderophores that exhibit antimicrobial activity against various bacterial and fungal isolates (Shah *et al*., 1992). In contrast, *A. vinelandii* siderophores charged with iron enhanced growth of the phytopathogenic bacteria *A. tumefaciens* and *Erwinia carotovora* (Page and Dale, 1986).

In the case of antibiosis, antimicrobial compounds such as antibiotics are involved. *Rhizobium leguminosarum* bv. trifolii T24 has been shown to produce an antibiotic peptide trifolitoxin (TFX) (Schwinghamer and Belkengren, 1968; Breil *et al*., 1993, 1996). Although its spectrum of activity includes bacteria that are plant and animal pathogens (Schwinghamer and Belkengren, 1968; Triplett *et al*., 1994), *R. leguminosarum* and *Rhizobium fredii* are especially sensitive to TFX (Triplett and Barta, 1987), suggesting that TFX production is rather a mechanism of limiting nodule formation by related competing strains instead of biocontrol (Triplett and Barta, 1987; Triplett, 1988, 1990). Bacteria belonging to the genus *Azotobacter* have been found to be antagonistic against *Botrytis cinerea* Pers. These bacteria synthesize an antifungal compound of low molecular weight that overall inhibits the production of conidia by the fungus (Doneche and Marcantoni, 1992). So far, this substance has not been identified. Also, *P. polymyxa* has been shown to produce antimicrobial substances active against bacteria and fungi, that is, polymyxins (Storm *et al*., 1977) and antibiotics (Kurusu and Ohba, 1987; Rosado and Seldin, 1993; Pichard *et al*., 1995;

Kajimura and Kaneda, 1996). For *A. brasilense* and *A. lipoferum* the production of bacteriocins has been reported (Oliveira and Drozdowicz, 1987; Tapia-Hernandez *et al*., 1990).

Finally, biocontrol agents can also antagonize pathogens by parasitizing them. In this case, cell walls of pathogenic fungi are degraded by a battery of excreted enzymes, including proteases, chitinases, and glucanases. Chitin is a major structural component of most fungal cell walls (Lopez *et al*., 2001). These enzymes often have antifungal activity individually and are synergistic in mixtures or with antibiotics (Di Pietro *et al*., 1993; Lorito *et al*., 1993, 1994). Mavingui and Heulin (1994) found that *Paenibacillus polymyxa* strains isolated from the rhizoplane of spring wheat have high chitinase and antifungal activity against the fungus *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all of wheat. The *in vitro* antifungal activity was not necessarily correlated with the activity of chitinase, suggesting that additional mechanisms may be important. Nielsen and Sørensen (1997) showed that *P. polymyxa* demonstrates a multitarget and medium-independent type of fungal antagonism, probably through constitutive hydrolase expression (cellulase and mannanase).

The interaction between *P. polymyxa* and *Fusarium oxysporum* was studied in detail by Dijksterhuis *et al*. (1999), who found that this interaction starts with the polar attachment of bacteria to the fungal hyphae followed by the formation of a large cluster of nonmotile cells embedded in an extracellular matrix in which the bacteria develop endospores. This formation of a bacterial nidus around hyphae was found to play an important role in the interaction and may enhance the effectiveness of any antifungal compound produced. Additionally, extreme densities of bacteria around hyphal cells may act as a nutrient sink, resulting in a weaker condition of the fungal cells.

Recently, an indirect way of biocontrol, for which no direct contact between pathogen and biocontrol agent is required, has received increasing attention. Nonpathogenic rhizosphere bacteria have been shown to reduce disease by activating a resistance mechanism in the plant called rhizobacteria-mediated induced systemic resistance (ISR). Rhizobacteria-mediated ISR resembles pathogen-induced systemic acquired resistance (SAR), in that both types of induced resistance render uninfected plant parts more resistant toward a broad spectrum of plant pathogens (Thomashow and Weller, 1995; van Wees *et al*., 1997; van Loon *et al*., 1998). On the other hand, both forms of resistance act through different signaling pathways. While induction of SAR is salicylic acid (SA)-dependent, ISR requires components of the jasmonic acid (JA) and ethylene (ET) signaling pathways (Pieterse *et al*., 1998, 2000; Yan *et al*., 2000). It is generally observed that the protection offered by ISR is significantly less than that obtained in SAR (van Loon *et al*., 2000). However, combined induction of ISR and SAR provides greater protection than each one alone, indicating that ISR and SAR can act additively in enhancing resistance to pathogens (van Wees *et al*., 2000). Studies of ISR by rhizosphere bacteria have concentrated so far on a few species, mostly *Pseudomonas* spp., and a number of dicotyledonous plants (Ryu *et al*., 2000). Notably, no ISR has yet been reported in monocotyledons (van Loon *et al*., 1998). *P. polymyxa* is the only diazotroph for which ISR has been investigated so far. Pretreatment of *Arabidopsis* plants with *P. polymyxa* induced significant resistance against *E. carotovora* (Timmusk and Wagner, 1999). Furthermore, inoculation with *P. polymyxa* induced the JA-responsive *ATVSP* (vegetative storage protein acid phosphatase; Berger *et al*., 1995), the ET-responsive *HEL* (hevein; Potter *et al*., 1993) and the SA-responsive *PR-1* (pathogenesis related; Uknes *et al*., 1992) genes, suggesting that the PGPR induces a mild biotic stress, and that this effect initiates a systemic response that results in partial protection from the pathogen after subsequent challenge. Whether the defense response against the pathogen has been triggered via the release of pectic fragments from the plant cell wall or by the action of the hydrolytic/pectinolytic enzymes produced by *P. polymyxa* is not yet established.

In addition, *P. polymyxa* strains were shown to produce benzoic acid (BA), an antimicrobial metabolite (Lebuhn *et al*., 1997). Since BA is the immediate precursor of SA, microbial BA release at the rhizoplane might also induce plant (sys-

temic) resistance mechanisms (León *et al*., 1993). The production of SA was reported for *A. lipoferum* D2 (Saxena *et al*., 1986).

Only few papers report on the use of a diazotroph in plant inoculation experiments to test its biocontrol activity. In one experiment, *P. polymyxa* was found to be an effective antagonist to the wheat root rot pathogens *Fusarium graminearum* and *Cochliobolus sativus* in both greenhouse and field experiments (El-Meleigi and Hassan, 2000). However, the mechanism of antagonism involved was not investigated. In a second paper *A. brasilense* strain Cd was reported to protect tomato seedlings against infection by *Pseudomonas syringae* pv. tomato in a greenhouse experiment (Bashan and de-Bashan, 2002). It was suggested by the authors that the mechanism of biocontrol might be the competition between both bacterial species sharing the same host plant, resulting in the displacement of *P. syringae* pv. tomato cells by *A. brasilense*. The mechanism for the displacement is not known, but it is likely that *A. brasilense* is better able to obtain nutrients or to colonize plant surfaces. Alternatively, the protective effect obtained after *A. brasilense* inoculation might be the indirect result of the plant growth promotion exerted by this strain, resulting in an overall healthier and more robust plant that is able to fend off the pathogen more readily. This is consistent with the observation that the application of *A. brasilense* simultaneous or prior to pathogen infection was needed to obtain successful protection of the tomato seedlings. Previously, it was already reported that pretreatment of the wound sites of dicotyledonous plants with viable cells of *A. brasilense* strains 94–3 and Sp7 could effectively inhibit the development of crown gall tumors induced in these plants by virulent strains of *A. tumefaciens* (Bakanchikova *et al*., 1993).

Apart from the suppression of deleterious microorganisms and soil-borne pathogens by PGPR, also the inhibition of plant parasite by PGPR has been reported. Bacteria of the genus *Azospirillum* were found to inhibit *Striga* seed germination (Bouillant *et al*., 1997; Miché *et al*., 2000). *Striga* is an obligate parasitic weed of tropical cereals and is becoming an uncontrollable pest for food-producing crops in Africa (Sallé *et al*., 1995). The

production of small lipophilic compounds by *A. brasilense* L4 was found to be involved in the inhibition of *Striga* seed germination (Miché *et al*., 2000).

X. INCREASE IN ROOT-ADHERING SOIL (RAS)

Among the abiotic factors influencing plant growth and crop yield, the soil certainly plays a major role. Soil aggregates are the basic units of soil structure and the way in which they are arranged has a wide influence on soil physical properties and hence crop growth. Soil aggregate size for instance has been found to influence emergence, early shoot growth and root growth of maize (*Zea mays* L.) (Taylor, 1974; Donald *et al*., 1987; Logsdon *et al*., 1987a,b; Alexander and Miller, 1991). Soil components may also influence the distribution and activity of microorganisms. Habitats of soil microorganisms are dependent on soil structure that implies the presence of voids of various shapes and sizes. Hassink *et al*. (1993) found that bacterial cells were more abundant in clay and loamy soils, where narrow pores predominate, than in coarse-textured soils. In the absence of a plant, *A. brasilense* was found to preferably reside in the macroaggregates of millimeter size or in the very small clay particles of micrometer size, where it sustained a high population level of over 10^6 cells g^{-1} soil fraction (Kabir *et al*., 1994). However, unlike many other soil bacteria, *Azospirillum* spp. are not limited by adsorption to soil particles, as they are able to move through the soil to target plants (Bashan, 1999). This motility in soils might play a general role in enabling *Azospirillum* spp. to access sites where more stable colonization might occur later. The main factor affecting the movement of *A. brasilense* Cd toward wheat seedlings grown in soil was soil moisture. Of secondary importance was the soil type: the coarser the soil texture, the higher the rate of migration (Bashan, 1986).

The importance of soil aggregation in crop production lies in its indirect effect on water and air relationships in the soil. The size, shape, and stability of soil aggregates control the pore size distribution, which in turn affects many soil physi-

cal properties such as retention and movement of water, aeration and temperature (Lynch and Bragg, 1985). The resistance of soil aggregates to slaking and the dispersive effects of water (aggregate stability) are important in maintaining a porous structure in arable soils. The main agencies of stabilization are organic materials (mainly carbohydrates) derived from plants by rhizodeposition or synthesized by bacteria. It is generally believed that microbial action on soil aggregation is due to the production of exopolysaccharides (EPS) (Lynch and Bragg, 1985). Experimental observations demonstrated that the amendment of soil with microbial EPS resulted in an increased soil aggregation (Martin, 1945; Cheshire, 1979). Recently, attention has been paid to the influence of microorganisms, particularly EPS-producing rhizobacteria, on the aggregation of root-adhering soil (RAS). RAS forms the immediate environment where plants take up water and nutrients for their growth. Therefore, soil structure and aggregate stability is even more important around the root system than in bulk soil. Factors that change the physical properties of RAS can be expected to modify absorption of water and minerals by plants.

Conclusive evidence has been provided for the role of an EPS (levan) produced by *Paenibacillus polymyxa* in the aggregation of RAS on wheat. Previously it was shown by Gouzou *et al*. (1993) that inoculation of wheat with *P*. *polymyxa* rhizosphere strain CF43 resulted in an increase in the amount of RAS per unit of root tissue (RT) dry mass by 57% and developed a more porous structure in the rhizosphere soil by enhancing the frequency of the aggregate size class of 0.2 to 2 mm. *P*. *polymyxa* CF43 was isolated from the rhizosphere of wheat as an efficient nitrogen-fixing bacterium (Heulin *et al*., 1994). To demonstrate that levan, a fructosyl polymer, produced by inoculated *P*. *polymyxa* strains might be responsible for the increased soil mass adhering to the roots, a *P*. *polymyxa* CF43 null mutant (SB03) was constructed in which the *sacB* gene is disrupted (Bezzate *et al*., 2000). The *sacB* gene encodes a levansucrase that synthesizes levan. The mutant and the wild-type strains were then compared for their effect on the mass of root-adhering soil. The mutation in the *sacB* gene did not result in a lower bacterial number in the rhizosphere or on the

rhizoplane of wheat plants, indicating that the *sacB* gene apparently was not involved in the colonization mechanism. The RAS/RT ratio was increased after inoculation of wheat with strain CF43 by more than 100%. In contrast, inoculation with *P*. *polymyxa* mutant strain SB03 had no effect on root-adhering soil mass, compared with the noninoculated treatment. These results strongly suggest that levan synthesis by strain CF43 is the main mechanism involved in the improvement of root-adhering soil structuration.

Inoculation experiments under different water regimes with two other EPS-producing strains, *Rhizobium* sp. strain YAS34 and *Pantoea agglomerans* NAS206, on sunflower and wheat, respectively, similarly demonstrated an improved RAS macroporosity, an increase in RAS per root dry mass, improved root and shoot growth, and even more efficient fertilizer use after inoculation (Amellal *et al*., 1998; Alami *et al*., 2000). The nitrogen fixation capacity of these strains was not investigated. Nevertheless, these data suggest that EPS-producing strains can play an important role in the regulation of the water content (excess or deficit) of the plant rhizosphere by improving soil aggregation.

A plant for which this mechanism of plant growth promotion might be extremely important is *Ammophila arenaria* (European beachgrass), a dune-stabilizing grass that is able to proliferate in the nutrient-poor conditions of sand. To obtain sufficient nitrogen for growth, it was reported that this grass is capable of nitrogen fixation through the presence of endophytic bacteria and diazotrophic bacteria in the rhizosphere (Abdel Wahab and Wareing, 1980; Dalton *et al*., 2000). The bacteria are harbored inside the root (within the plant cell walls, especially those adjacent to vascular bundles) or in rhizosheaths that cover the root system. Although not investigated, bacterial EPS production may play an important role in the establishment of these rhizosheaths.

XI. INTERACTIONS WITH OTHER MICROORGANISMS

In addition to exploiting their individual plant growth-promoting capacity, the potential of selected diazotrophs can be improved further through

dual inoculation with other microorganisms for additive and/or synergistic effects. Bacterial diazotrophs that are able to colonize the root zones of leguminous plants, for instance, could stimulate the performance of a leguminous species by affecting the process of symbiotic nitrogen fixation. Combined inoculation of *Rhizobium* with *Azospirillum* or with *Azotobacter* has been demonstrated to increase dry matter production, grain yield, and nitrogen content of several legumes when compared with inoculation with *Rhizobium* alone (Burns *et al.*, 1981; Iruthayathas *et al.*, 1983; Sarig *et al.*, 1986; Rodelas *et al.*, 1996; Burdman *et al.*, 1998, 2000). These positive results of dually inoculated legumes have been attributed to early nodulation, increased number of nodules, higher N_2–fixation rates, and a general improvement of root development (Volpin and Kapulnik, 1994; Okon and Itzigsohn, 1995). The greater number of active nodules can be expected to contribute fixed nitrogen for higher yields under field conditions. However, concomitant application of *Azospirillum* and *Rhizobium* did not always result in a promotion of nodulation, and under some circumstances even inhibited the ability of the *Rhizobium* to nodulate its host. Stimulation or inhibition was found to be dependent on bacterial concentration and timing of inoculation (Plazinski and Rolfe, 1985a,b; Yahalom *et al.*, 1991; Burdman *et al.*, 1997; Villar Arteaga and ZúHiga Dávila, 2000). Still, little is known about the mechanisms involved in these interactions. One possibility is an increase in the uptake of mineral nutrients other than N by the inoculated leguminous roots. Mineral nutrient deficiencies are a major constraint limiting legume N fixation and yield (O'Hara *et al.*, 1988). Mixed inoculations of *Vicia faba* L. with four different *Rhizobium*/*Azospirillum* and *Rhizobium*/*Azotobacter* combinations led to changes in total content, concentration, and/or distribution of the mineral macro- and micronutrients, K, P, Ca, Mg, Fe, B, Mn, Zn, and Cu, when compared with plants inoculated with *Rhizobium* alone (Rodelas *et al.*, 1999). Although application of P is known to increase the number and weight of nodules per plant (Shaw *et al.*, 1966), *Azospirillum* apparently did not promote early events of nodule initiation by means of increased P uptake (Yahalom *et al.*, 1990).

As most of the diazotrophs have been shown to produce phytohormones, the stimulation of nodulation may occur as a result of a direct response of the plant root to these compounds. Similarly to what was observed in several grasses and cereals (Okon and Kapulnik, 1986), inoculation with *A. brasilense* was found to promote root hair formation of bean or alfalfa (Itzigsohn *et al.*, 1993; Burdman *et al.*, 1996). As *Rhizobium* infection takes place by the formation of infection threads in root hairs (Long, 1989), the stimulation of a greater number of epidermal cells to differentiate into infectable root hair cells may increase the probability of infection by *Rhizobium*, thereby increasing root potential for nodule initiation (Yahalom *et al.*, 1987). Apart from their direct effect on root morphology, phytohormones may also influence the nodulation process itself (Syono *et al.*, 1976; Yahalom *et al.*, 1987). Both positive and negative effects of *Azospirillum* on nodulation and root development could be mimicked, in some cases, by the application of PGRs such as auxins and cytokinins (Plazinski and Rolfe, 1985b; Yahalom *et al.*, 1990; Itzigsohn *et al.*, 1993).

Experiments carried out in a hydroponic system showed that inoculation with *A. brasilense* increased the secretion of flavonoids by seedling roots of common bean (Burdman *et al.*, 1996). It is well known that plant root flavonoids are the inducers of nodulation (*nod*) gene expression in *Rhizobium* (Bamfalvi *et al.*, 1988; Maxwell *et al.*, 1989; Peter and Verma, 1990). Therefore, this enhanced flavonoid production in roots might be an additional factor in nodule promotion by these bacteria. In addition, the flavonoid luteolin had similar effects to those of *Azospirillum* in increasing the main root nodule number and the total nodule number of alfalfa (Itzigsohn *et al.*, 1993). In all these cases, inoculation experiments with mutant strains are needed to clearly elucidate the mechanisms involved in these dual inoculations.

On the other hand, *Rhizobium* for its part was found to act synergistically with arbuscular mycorrhizal fungi to increase lettuce biomass production (Galleguillos *et al.*, 2000). Work by Azcon *et al.* (1978) and Bagyaraj and Menge (1978) has shown a similar synergistic or additive interaction between *Azotobacter* and Arbuscular Mycorrhizae (AM) and also for *Azospirillum* a combination

with AM appeared to reinforce the inoculation effect on barley, maize, and ryegrass (Barea *et al.*, 1983; Subba Rao *et al.*, 1985). AM can improve plant growth through increased uptake of P and other mineral nutrients especially in soils of low fertility (Ning and Cumming, 2001; Rao and Tak, 2001; Smith *et al.*, 2001). Indeed, plants inoculated with *A. brasilense* and the AM and grown without fertilizer had comparable N and P content to that of noninoculated plants supplemented with N and P fertilizers (Barea *et al.*, 1983). The diazotrophs may enhance mycorrhizal development by supplying vitamins to the rhizosphere, because mycorrhizal fungi have been shown to be dependent on or stimulated by certain vitamins (Harley, 1969; Baya *et al.*, 1981; Strzelczyk and Leniarska, 1985). Thus, inoculation with mycorrhizal fungi and vitamin-producing diazotrophs could result in improved plant growth.

Petersen *et al.* (1996) showed that *P. polymyxa* caused an increase in both early and final rhizobial root populations when coinoculated with *Rhizobium etli* on *Phaseolus vulgaris*, when compared with single inoculation with *R. etli*. In contrast to the *in planta* results, population enhancements were not observed when *R. etli* and *P. polymyxa* were co-cultured *in vitro* using minimal media in the absence of the seedling. The addition of seed exudates to the growth media also failed to stimulate the population increases observed during co-release *in planta*. These results suggest that *P. polymyxa* acts indirectly (i.e., via the plant host) to increase *R. etli* populations. Similarly, in the case of *Azospirillum* and *Rhizobium* beneficial growth responses under gnotobiotic conditions were generally obtained when *Azospirillum* was applied prior or posterior to inoculation with *Rhizobium*. Again this suggests that *Azospirillum* exerts its effects via the legume, and not through direct interaction with *Rhizobium* (Burdman *et al.*, 1998).

In a field inoculation experiment with different strains of *Azotobacter chroococcum* and *Azospirillum brasilense* on a local maize variety in India, it was found that the introduced bacteria stimulated the populations of certain other beneficial groups of microbial communities, including actinomycetes and a group of bacteria able to grow on N-free medium (Pandey *et al.*, 1998).

The latter indicates that the inoculated bacteria appear to positively influence the native N-fixing bacteria present in the rhizosphere. Actinomycetes promote plant growth possibly due to antibiotic production or by providing cross-protection (Pandey *et al.*, 1998; Watve *et al.*, 2001). The obtained results suggest that the observed effects of seed inoculation on plant growth may in part be due to the stimulation of already existing plant growth-promoting rhizobacteria in and around roots.

It is clear that after inoculation with diazotrophic bacteria, a multitude of interactions with the endogenous population may take place. Apart from the above-mentioned synergistic interactions, also competitive or antagonistic interactions exist in order to enable the bacteria to survive and compete in complex microbial communities. Microcosms studies with *Azospirillum brasilense* Cd revealed that interactions with particular populations in the microbial community control the proliferation, and hence possibly also the function, of this strain (Janzen and McGill, 1995). Therefore, it is of utmost importance to test the plant growth-promoting effect of selected diazotrophs not only under gnotobiotic conditions but also in the field.

Interactions among microorganisms within communities as well as between microorganisms and their hosts are dependent on the appropriate expression of specific genes involved in these interactions. Recently, the role of *N*-acyl-L-homoserine lactone (AHL)-mediated gene expression in the ecology of complex microbial communities has received increasing attention. AHL-mediated gene regulation, or quorum sensing, is an example of a highly conserved mechanism of gene regulation in bacteria, describing a bacterial signaling system that controls gene expression in a population density-dependent manner (Fuqua *et al.*, 1996; Pierson *et al.*, 1998b; Eberl, 1999). Given the large proportion of plant-associated bacteria that produce AHL signal molecules (Cha *et al.*, 1998; Pierson *et al.*, 1998a; Steidle *et al.*, 2001) and the common usage of AHLs by a diverse range of Gram-negative bacteria (Pierson *et al.*, 1998b), it appears likely that AHL signal molecules are used not only as population density sensors in one species but also for communication

between cells of different species. Studies demonstrating AHL-mediated interpopulation signaling in the wheat and tomato rhizosphere, as reported by Pierson *et al.* (1998a) and Steidle *et al.* (2001), respectively, strongly support the view that AHL signal molecules may serve as a universal language for cross-communication between the different bacterial populations in complex rhizosphere communities and even between bacteria and their hosts. Although it is very likely that quorum sensing also plays a role in plant growth promotion by diazotrophs in the rhizosphere, to date there are no reports on this mechanism of signaling specific for associative nitrogen-fixing bacteria. Only for *Rhizobium* the presence of AHL-mediated gene regulation has been demonstrated (Gray *et al.*, 1996; Rosemeyer *et al.*, 1998; Lithgow *et al.*, 2001; Daniels *et al.*, 2002). However, it can be expected that studies screening for AHL-mediated communication, analogous to those reported for the wheat and tomato rhizosphere, will reveal the occurrence of cross-communication in rhizosphere diazotrophs via AHL molecules.

CONCLUSIONS

Taken together with all the evidences presented above, it is clear that much more is going on in the association between diazotrophs and plants than the exchange of nitrogen fixed by the bacterium for metabolites provided by the plant. Except for a few examples in which a substantial contribution of BNF to plant growth was estimated, for example, in the sugarcane–*Acetobacter diazotrophicus* association, in most cases the nitrogen is fixed rather for the benefit of the bacterium itself than for the plant, helping the diazotroph to survive and proliferate in N-poor soils. These results led to the consensus that biological N_2 fixation associated with grasses and cereals presently has little agronomical impact (Okon and Vanderleyden, 1997). Still, the use of these bacteria can be promising due to all the other characteristics they possess to promote plant growth. As can be seen from Table 1, summarizing the different specific traits of diverse diazotrophs, these organisms can promote plant growth by more than just one mechanism. At the same time,

the fact that individual diazotrophs may possess characteristics consistent with several mechanisms renders the elucidation of the precise mechanisms involved more difficult. The elimination of specific bacterial traits by site-directed mutagenesis can be extremely helpful to provide definite proof for the bacterial characteristics believed to be involved in the observed plant growth promotion and their relative contribution. However, for many mechanisms presented here such a proof is still lacking.

Comparing the amount of evidence provided for the different mechanisms that have been discussed, it can be deduced that *Azospirillum* still is the best studied diazotrophic PGPR at this moment. However, it seems that interest is shifting now to endophytes. Endophytic diazotrophs may have an advantage over root-associated diazotrophs, such as *Azospirillum* and *Azotobacter*, in that they colonize the interior (xylem vessels and intercellular spaces) rather than the surface of the plants, and hence are better placed to exploit carbon substrates supplied by the plant (Döbereiner *et al.*, 1995; Sprent and James, 1995; Triplett, 1996; James and Olivares, 1997). They establish themselves within niches protected from oxygen, which is necessary for the expression and activity of the nitrogenase, thus their potential to fix nitrogen can be expressed at the maximal level (Baldani *et al.*, 1997). These properties may be the reason for the high nitrogen fixation observed in sugarcane plants (Urquiaga *et al.*, 1992). Also, the recent findings on the nitrogen contribution to Kallar grass by unculturable endophytic *Azoarcus* sp. support this (Hurek *et al.*, 2002). Quispel (1991) has suggested that only in endosymbiotic systems the prerequisites for effective nitrogen fixation are likely to be fulfilled, so that plants can make efficient use of atmospheric nitrogen for growth.

Furthermore, it is speculated that endophytes are promising candidates as biocontrol agents against several plant pathogens, especially vascular wilt pathogens. Endophytically resident bacteria may be strategically at the right place and at the right time for suppression of the pathogens, not only with regard to antagonistic effects, such as antibiosis and competition for nutrients at infection sites, but also with regard to optimal timing of, for example, induced resistance (Hallmann

TABLE 1

Overview of the possible mechanisms involved in plant growth promotion by diazotrophs. References in bold refer to those mechanisms for which evidence was provided. Only those diazotrophs are listed for which the mechanism has been studied.

Strain		Hormones				Increased nutrient uptake	Enhanced stress resistance	P-solubilization	Vitamins	Biocontrol	Siderophores	Increased RAS	References
	BNF	auxin	cytokinin	GA	ACC deaminase								
Acetobacter diazotrophicus	x	+	+	+									**Sevilla et al., 2001**; Fuentes-Ramirez et al., 1993; Bastián et al., 1998
Azoarcus sp.	x	+											**Hurek et al., 1998, 2002**; Malik et al., 1997
Azospirillum brasilense	–	x	+	x		x	x		+	+	+		Bashan and Levanony, 1989; Morgenstern and Okon, 1987b; Crozier et al., 1988; El-Khawas and Adachi, 1999; **Dobbelaere et al., 1999**; **Barbieri and Galli, 1993**; **Harari et al., 1988**; Cacciari et al., 1989; Janzen et al., 1992; **Lucangeli and Bottini, 1996, 1997**; **Bashan, 1991**; **Creus et al., 1998**; Rodelas et al., 1993; Tapia-Hernandez et al., 1990; Bashan and de-Bashan, 2002; Bakanchikova et al., 1993; Bouillant et al., 1997; Miché et al., 2000; Bachhawat and Ghosh, 1987
Azospirillum lipoferum	+	+		x		+				+	+		Crozier et al., 1988; Bottini et al., 1989; Piccoli et al., 1996; Piccoli and Bottini, 1994; **Lucangeli and Bottini, 1996, 1997**; Murty and Ladha, 1988; Oliveira and Drozdowicz, 1987; Saxena et al., 1986; Shah et al., 1992
Azotobacter beijerinckii	+	+	+	+									Azcón and Barea, 1975; Nieto and Frankenberger, 1989

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| Strain | Hormones | | | | ACC deaminase | Increased nutrient uptake | Enhanced stress resistance | P-solubilization | Vitamins | Biocontrol | Siderophores | Increased RAS | References |
	BNF	auxin	cytokinin	GA									
Azotobacter chroococcum	+	+	+	+			x	+	+				Martinez-Toledo *et al.*, 1988; Nieto and Frankenberger, 1989; **Stajner *et al.*, 1997**; Kumar and Narula, 1999; Revillas *et al.*, 2000
Azotobacter paspali	+	+	+	+									Boddey *et al.*, 1983; Barea and Brown, 1974
Azotobacter vinelandii	+	+	+	+					+		+		Azcón and Barea, 1975; Gonzalez-Lopez *et al.*, 1986; Nieto and Frankenberger, 1989; Gonzalez-Lopez *et al.*, 1983; Martinez-Toledo *et al.*, 1996; Revillas *et al.*, 2000; Page and von Tigerstrom, 1988; Demange *et al.*, 1988; Knosp *et al.*, 1984
Bacillus pumilus	+			x									**Gutiérrez-Mañero *et al.*, 2001**
Bacillus licheniformis	+			x									**Gutiérrez-Mañero *et al.*, 2001**
Bacillus megaterium								-					de Freitas *et al.*, 1997
Bradyrhizobium elkanii	+	+				+							Minamisawa *et al.*, 1996; Biswas *et al.*, 2000
Bradyrhizobium japonicum	+		+								+		Phillips and Torrey, 1970; Guerinot, 1991
Herbaspirillum seropedicae	+	+		+									Bastián *et al.*, 1998
Klebsiella pneumoniae	+	+											El-Khawas and Adachi, 1999

TABLE 1 (continued)

Strain	BNF	Hormones auxin	Hormones cytokinin	Hormones GA	ACC deaminase	Increased nutrient uptake	Enhanced stress resistance	P-solubilization	Vitamins	Biocontrol	Siderophores	Increased RAS	References
Paenibacillus (Bacillus) polymyxa	+	+	+					-		x		x	Lebuhn *et al.*, 1997; Timmusk *et al.*, 1999; Sattar and Gaur, 1987; de Freitas *et al.*, 1997; El-Meleigi and Hassan, 2000; Storm *et al.*, 1977; Kajimura and Kaneda, 1996; Kurusu and Ohba, 1987; Pichard *et al.*, 1995; Rosado and Seldin, 1993; Mavingui and Heulin, 1994; **Nielsen and Sørensen, 1997; Dijksterhuis *et al.*, 1999; Timmusk and Wagner, 1999; Bezzate *et al.*, 2000**
Pseudomonas putida GR12-2	-	+			x	+					+		Lifshitz *et al.*, 1987; **Glick *et al.*, 1994**; Xie *et al.*, 1996
Rhizobium leguminosarum	+	x	x			+		+			+		**Noel *et al.*, 1996**; Chabot *et al.*, 1996a; Guerinot, 1991; Biswas *et al.*, 2000
Rhizobium meliloti	+								+		+		Sierra *et al.*, 1999; Guerinot, 1991
Rhizobium phaseoli	+	+	+	+									Atzorn *et al.*, 1988
Rhizobium sp.	+		+										Upadhyaya *et al.*, 1991

+ : characteristic present

x : mechanism proven/strong arguments in favour

- : characteristic present but not involved

et al., 1997; Nejad and Johnson, 2000). It may be speculated that endophytic behavior aids in the induction of resistance, because more plant cells are being contacted by the bacteria than by isolates confined to the rhizosphere (Benhamou *et al*., 1996).

Therefore, due to the identification of these endophytic associations, the possibilities of expanding BNF to cereals and other nonlegume crops are now gaining new credibility. Still, it is clear that even in these cases, other mechanisms than N_2–fixation, for example, phytohormone production and increased nutrient uptake, continue to be responsible for a major part of the observed plant growth promotion.

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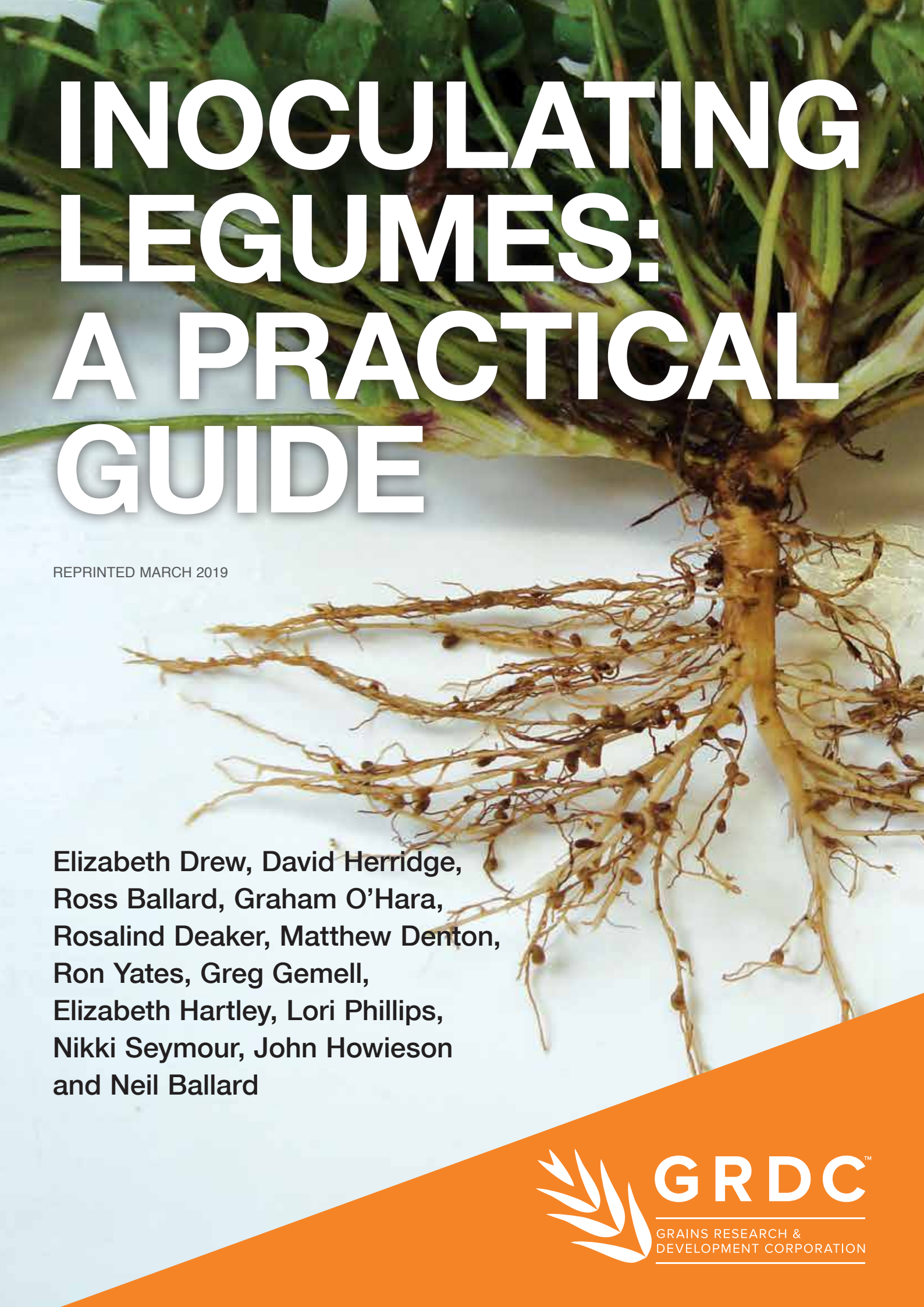
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INOCULATING LEGUMES: A PRACTICAL GUIDE

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… Each year Australian growers sow inoculated legume seed on about 2.5 million hectares, equivalent to 50 per cent of the area sown to legumes. All of the nitrogen fixed annually by legumes growing on these newly sown areas together with that fixed by the 22.5 million hectares of established and regenerating legume-based pastures can be attributed to either current or past inoculation. The total amount of nitrogen fixed by the agricultural legumes is estimated at 2.7 million tonnes annually, with a nominal value for the industry of close to $4 billion annually…

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FOREWORD

Nitrogen (N) fixed by the soil bacteria rhizobia symbiotically with Australia's pasture and pulse legumes, has a national benefit of close to $4 billion annually. This is based on nitrogen fixation rates of about 110 kilograms of N per hectare per year, legume areas of 25 million ha and fertiliser N costed to the grower at $1.25/kg, which equates to $1.55/kg plant-available N in the soil. The price of carbon-based fossil fuels, used in the production of nitrogenous fertilisers, is expected to increase substantially in the future. As this occurs, the value of legume nitrogen fixation to Australian growers will escalate.

There is an ongoing need to ensure that Australian agriculture evolves with a reliance on legumes that are effectively nodulated and that the benefits of nitrogen fixation from legumes for farming systems are maximised.

This will not occur if legume nodulation is sub-optimal, because of one or more of the following factors:

■ growers do not inoculate when they should;
■ growers use inoculation practices that do not deliver sufficient rhizobia to the developing legume seedling;
■ growers use inoculants of sub-optimal quality;
■ legume breeding programs release cultivars that are not matched with highly effective rhizobial inoculants;
■ ineffective populations of rhizobia evolve in the soil and outcompete effective inoculant rhizobia;
■ inoculant rhizobia are exposed to chemical toxicities during inoculation or soon after application to the soil; and
■ populations of soil rhizobia in regenerating pastures decline because the landscapes become hostile through soil salinity, acidity or for other reasons.

To capitalise on the potential benefits of legume nodulation and nitrogen fixation, Australian growers need to:

■ understand the role of legumes in supplying N to agricultural production systems;
■ manage legume nitrogen fixation and system N supply for maximum productivity and sustainability;
■ inoculate legumes where and when appropriate;
■ optimise inoculation outcomes through correct use of the inoculant product;
■ understand the limitations of inoculants, e.g. death of the rhizobia from exposure to toxic and dehydrating conditions;
■ have access to the most efficacious inoculant products in the marketplace;
■ understand the specific nature of the relationship between legumes and rhizobia and use the appropriate inoculant strain for a target legume-host;
■ grow the most appropriate legume in terms of environment and soil biology; and
■ manage soils to minimise plant growth-limiting factors (e.g. pathogens, heavy metals, low pH, salinity).

This handbook was written by a group of Australian experts in the field of rhizobiology and nitrogen fixation from universities and state departments of agriculture and primary industries, many of whom work within the National Rhizobium Program (NRP), to address the above issues. The NRP is a GRDC R&D program, funded in three phases between 1998 and 2012, with objectives to address the science that underpins the above issues.

The major geographic focus of the handbook is the wheat-sheep belt (essentially 100% of Australia's grain production and >50% of wool production), with a minor focus on the high-rainfall belt (about 30% of Australia's wool production).

The key audiences are growers, grower groups, commercial and government advisers, agribusiness, research agronomists, legume breeders, seed pelleters, resellers and seed merchants. It is intended that material from this handbook can be extracted and used in training workshops. Workshops would need to be tailored to the particular group. For example, the material used in workshops for individual growers/grower groups may be different for seed pelleters.

By using the handbook and/or after participating in workshops that use materials from the handbook, users should have an increased knowledge of legumes and legume nodulation in farming systems, should more effectively use inoculation as a key farm practice, and should have achieved higher farm productivity through enhanced legume nitrogen fixation and system N supply.

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FREQUENTLY ASKED QUESTIONS (FAQS)

What are legume inoculants?

Legume inoculants contain live bacteria called rhizobia and should be considered as perishable products. Rhizobia are sensitive to a range of stresses (e.g. high temperature and desiccation), which decrease their viability. A more detailed description of rhizobia is provided in Chapter 2 and guidelines for handling of inoculants in Chapters 4 and 5.

What does inoculation do?

Inoculating legume seed or soil at sowing provides a large number of effective nitrogen-fixing bacteria in close proximity to the emerging legume root to optimise nodulation and nitrogen fixation.

What are inoculant groups? Why are there different groups?

Each inoculant group has a unique strain of rhizobia that is highly effective in nodulation and nitrogen fixation for a specific cluster of legumes (also known as a legume-host group). Choosing the correct inoculant group for a particular legume host (indicated by letters) is critical for good nodulation and nitrogen fixation to occur. More information and charts of inoculant groups are provided in Chapters 2, 5 and 7.

Can I test my soil for rhizobia?

No commercial test is available for determining the presence of a particular rhizobia in a soil, but paddock history can provide a guide. If the same legume was recently grown, was well-nodulated and yielded well, the soil will likely have rhizobia for that legume. Factors that affect the persistence of rhizobia in soils are examined in Chapter 3.

How do I know if I need to inoculate my crop or pasture legume?

This will depend on the legume being sown, paddock history and soil conditions. Guidelines for assessing the need for the inoculation of a major crop and pasture species are provided in Chapter 7.

Do I need to use a sticker or adhesive with the inoculant?

Stickers are used to ensure that adequate inoculant adheres to seed. Stickers are already incorporated into peat-based inoculants for crop legumes. For pasture legumes, stickers are not contained in the inoculants and should be incorporated when inoculating seed. Stickers can also improve the survival of rhizobia. Consult the inoculant package for manufacturer recommendations. Recommended stickers should always be used. The use of sugar, oils and other sticker substitutes is not recommended.

Where can I buy inoculant?

Inoculants are sold through most rural merchandising and seed companies. Commercial manufacturers are listed in the Appendix and should be able to provide information regarding availability of inoculants and the location of retail suppliers.

Does exposure to inoculants pose a risk to human health?

Rhizobia pose no known threat to human health. Peat, liquid and freeze dried formulations contain very few other organisms and so are regarded as safe to use. Although granular formulations generally contain a low proportion of dust, they do contain other soil microbes and so gloves and face masks, similar to recommendations for handling potting mixes and soils, should be used. If in doubt, consult the manufacturer recommendations on the label.

What inoculant formulation is best?

Peat inoculants are reliable and cost-effective in most situations. Other inoculants may be easier to use or better suited to specific cropping situations. The conditions that favour the use of the different formulations are summarised in Chapters 4 and 5. Look for the Green Tick Logo to be assured that the inoculant has been independently tested and satisfies Australian inoculant quality standards.

What are the benefits of inoculation?

Inoculation is essential for nodulation where the host-legume has not previously been grown. While effective rhizobia may be present in soil where a host-legume has been inoculated and grown previously, the application of a high-quality inoculant can increase the proportion of nodules formed by the selected elite inoculant strain. Nitrogen fixation benefits resulting from inoculation are described in detail in Chapter 6.

Is there any harm from over inoculation?

As long as the extra inoculant does not cause seeder blockages, there is no harm in using higher rates of inoculation. In fact, some field trials have shown benefits from increased inoculation rates, particularly in paddocks that have not grown a pulse previously.

Can inoculation rates be reduced?

This is not recommended. Insufficient numbers of rhizobia on seed or in the soil may result in inadequate nodulation. Applying the correct rate of inoculant helps ensure prompt and effective nodulation and provides good competition against other soil rhizobia that may be less effective at nitrogen fixation.

What is the benefit of lime pelleting pasture and pulse seed?

Lime pelleting helps reduce the moisture content of the seed-inoculant mix after the application of the slurry and helps prevent clumping with small seeds. It also helps to improve survival of the rhizobia particularly where the seed comes in contact with acidic fertilisers or is sown into acidic soils.

How long can I keep inoculated legume seed?

Fresh is best! Numbers of rhizobia on seed decline rapidly in the first few hours after inoculation. Rhizobial numbers on seed are highest immediately after inoculation. We recommend that farmers sow legume seed within a day of being inoculated. A significant proportion of pasture legume seed is sold preinoculated and may be have been inoculated for several months.

Should I use starter nitrogen?

In most situations, there is no reason to use starter nitrogen when sowing legumes. There may be benefits, however, for legumes growing in soils with extremely low levels of plant-available nitrogen and for the early growth of non-legume species in mixed pastures. The nodulation of legumes is suppressed by soil-mineral nitrogen.

Are there any special fertiliser needs for legumes?

Legumes need good nutrition to grow, such as the elements phosphorus and potassium. In addition to the usual nutrients needed by plants, legumes require the trace element molybdenum (Mo), which is critical for the enzyme that is responsible for nitrogen fixation. Care should be taken when selecting an appropriate Mo fertiliser as some can be toxic to rhizobia. Application of Mo fertilisers is covered in Chapter 5.

Is dry sowing of inoculated legumes OK?

Dry sowing is not ideal. Where unavoidable, the risk of nodulation failure is minimised by deep (moisture-seeking) sowing and by limiting dry sowings to paddocks where the legume has previously grown and was adequately nodulated.

How long will the inoculant survive on seed in the soil if it does not rain?

This will depend on soil conditions, planting depth, humidity, rhizobial strain (inoculant group) and inoculant formulation. Inoculants are always best delivered on seed or directly into moist soils.

Can I mix inoculated lucerne and clover together at sowing?

Yes, different pasture species can be mixed together following inoculation. The rhizobia on the inoculated seed will not usually compete with each other to form nodules. If granular inoculants are used they must be applied at the full rate for each pasture species in the mixture.

How do I assess nodulation?

Plants are best assessed for nodulation at about eight weeks after sowing. Plants should be carefully dug from the soil intact and root systems gently washed. Nodulation can be very different on different legume species but in general numerous pink nodules near the top of the root system indicates that prompt and effective nodulation has occurred. Nodule types are discussed in Chapter 2 and descriptions of nodulation for the different legume species are provided in Chapter 7.

I forgot to inoculate, what can I do?

Inoculant is best applied at sowing. It is extremely difficult to rectify a nodulation failure after sowing. The best option would be to over-sow a granular product as soon as possible in close proximity to the original sowing furrow. Responses will decline with time, as mature roots are less likely to form nodules.

Can I spray inoculant onto the top of the soil or directly onto the legume crop or pasture?

No, it is not recommended.

Are rhizobia compatible with pesticides and fertilisers?

Rhizobia are sensitive to many chemicals, fertilisers and pesticides and exposure to them should be avoided. Fertilisers are often acidic or contain elements such as zinc that are toxic to rhizobia. Where application of both pesticide and inoculant are critical to crop establishment, the use of direct soil inoculation techniques should be considered (discussed in Chapter 5). Mixing inoculated seed with fertiliser is not recommended. Even where seed is pelleted, exposure times should be minimised.

Can large packets of inoculant be resealed and used later?

Yes, if the whole packet is not used, air should be immediately expelled, the packet carefully sealed and stored in the refrigerator at 4°C. If packets are not sealed properly, the contents will dry, which may reduce rhizobial numbers and there is a risk of contamination by other microbes. Inoculants should be used as soon as possible after opening. All inoculants should be used before the expiry date.

1 INTRODUCTION

Legumes have been used as a source of food ever since humankind first tilled the soil many thousands of years ago. From very early times, legumes were recognised as 'soil improvers'. The farmers of ancient Mesopotamia grew peas and beans in their agricultural systems because they realised that cereals, their mainstay crops, were healthier and higher yielding when grown after a legume break crop. Those legumes would have been nodulated with compatible, effective rhizobia, the group of soil organisms that infect the roots of legumes to form nitrogen-fixing root nodules.

Rhizobia live in a modified form in nodules and fix nitrogen gas (N_2) from the atmosphere. The first product of nitrogen fixation is ammonia, which is then converted to amino acids and amides within the nodules before being transported in the xylem sap to other plant parts. These products of nitrogen fixation are vital for plant growth. In return, the rhizobia are provided with habitat and supplied with nutrients and energy in the form of carbon compounds. This mutually beneficial arrangement is called symbiosis. Eventually, when the legume begins to senesce and the flow of nutrients and energy from the plant to the nodule ceases, the nodule breaks down and disintegrates and its rhizobial content is released into the soil.

Although legumes were used as rotation crops in most parts of the world through the ages, it was not until the late 19th century that the links between nodulation, nitrogen fixation and 'soil improvement' were described scientifically. Today, it is estimated that worldwide, about 40 million tonnes of nitrogen is fixed annually by 185 million hectares of crop legumes and 150 million hectares of pasture legumes. Each year in Australia, legumes are estimated to fix almost three million tonnes of nitrogen, worth $4 billion. This amount makes a substantial contribution to the estimated six million tonnes of nitrogen required annually for grain and animal production.

1.1 The practice of inoculation

Nitrogen fixation by legumes does not happen as a matter of course. Compatible, effective rhizobia must be in the soil in which the legume is growing before nodulation and nitrogen fixation can occur. When a legume is grown for the first time in a particular soil, it is highly likely that compatible, effective rhizobia will not be present. In such circumstances, the rhizobia must be supplied in highly concentrated form as inoculants.

Inoculation of legumes with rhizobia is one of the success stories of agriculture and, indeed, may be the most cost-effective of all agricultural practices. Millennia before the scientific basis of legume nitrogen fixation was understood, farmers used rudimentary means of inoculation such as the transfer of soil from paddocks growing well-nodulated legumes to others that were legume-free. As late as 1920, Australian farmers were encouraged to inoculate lucerne seed with a mixture of glue and sieved air-dried soil, the latter taken from paddocks containing well-nodulated plants of the target legume (Guthrie 1896). Inoculation of legume seeds using pure cultures of rhizobia was made possible by the groundbreaking work of German and Dutch microbiologists during the last two decades of the 19th century. Within a few years, in the marketplaces of Europe, growers had access to cultures of rhizobia for inoculating a range of legumes. Inoculation of both seed and soil were advocated. Since that time, the production and distribution of legume inoculants has become an established industry in many countries.

1.2 Inoculants and inoculation of legumes in Australia

Australian growers embraced legumes and legume inoculation from the outset. The soils that they farmed were generally low in plant-available nitrogen and the use of nitrogenous fertiliser was not an affordable option. The legumes grown, mainly pasture and forage species, had to supply nitrogen for themselves and had to be capable of effective nitrogen fixation.

In 1896, the famous agricultural chemist, Frederick Guthrie, wrote about legume nitrogen fixation in the Agricultural Gazette of New South Wales saying that "it will prove to be one of the most valuable contributions ever made by science to practical agriculture. It is of special interest to us in Australia," (Guthrie 1896).

Mr Guthrie had remarkable foresight because now, more than 100 years later, Australian farmers sow inoculated legume seed on about 2.5 million hectares, equivalent to 50 per cent of the area sown to legumes. All of the nitrogen fixed annually by legumes growing on these newly sown areas, together with that fixed by the 22.5 million hectares of established and regenerating legume-based pastures can be attributed to either current or past inoculation. The total amount of nitrogen fixed by the agricultural legumes is estimated at 2.7 million tonnes annually, with a nominal value for the industry of close to $4 billion annually (Herridge 2011).

The success of legume inoculation as a routine practice in Australian agriculture was underpinned by effective scientific research and training in the state departments of agriculture, universities and several CSIRO divisions. Centres for research on the legume-rhizobia symbiosis were established at various times in all Australian states, leading to rapid advances in knowledge and inoculant technology and putting Australia foremost in the world in inoculant development and adoption. It is timely that, in 2012, the authors, who are all involved in the discipline of rhizobiology, take time out to compile a manual that relates scientific theory to the practical aspects of the legume-rhizobia symbiosis.

1.3 This handbook

We envisage that this handbook will sit on a shelf, a desk or a counter within the reach of those needing information for their own purposes or who are giving advice to growers. We hope that it will be a one-stop shop for information on rhizobia and legume inoculation. It is also intended that this handbook will be a comprehensive resource for agronomists and other agricultural scientists in the preparation of seminars and training workshops for growers and advisers.

Names, postal and email addresses of all the contributors are provided at the front of this handbook. Users of the handbook should feel free to contact the authors directly about issues that might need clarification or elaboration. Authors will undertake to respond to all enquiries.

We hope that you enjoy the handbook and find it a valuable resource.

2 RHIZOBIA AND THE RHIZOBIA-LEGUME SYMBIOSIS

- Rhizobia are bacteria that live in the soil, on plant roots and in legume nodules.

- Rhizobia only fix nitrogen when inside a legume nodule.

- There are many species of rhizobia.

- Rhizobia species are host (legume) specific. This means different legume species require different rhizobial species to nodulate and fix nitrogen.

- Rhizobia need nutrition, water and aeration for growth.

- Rhizobia in inoculants are killed by heat (>35°C), desiccation, extremes of pH and toxic chemicals.

2.1 What are rhizobia?

Rhizobia, also known as root-nodule bacteria, are specialised soil bacteria that are prominent members of microbial communities in the soil and on plant roots. Due to their unique biological characteristic they are able to establish mutually beneficial associations with the roots of legume plants to fix atmospheric nitrogen. The availability of this fixed or reactive nitrogen can make the legume independent of soil/fertiliser nitrogen resulting in increased agricultural productivity.

This association results in the formation of specialised structures on the legume roots, known as root nodules. Within the root nodules the rhizobia absorb carbohydrate from the plant and in return fix atmospheric nitrogen for use by the plant. The nitrogen (N_2) is fixed by the rhizobia into ammonia (NH_3) that is then transferred to the plant and assimilated into organic compounds for distribution via the xylem part of the vascular system – the same part that transports water and nutrients from the soil to the shoots. Legumes are unable to fix atmospheric nitrogen by themselves, although they can absorb mineral nitrogen from the soil. Rhizobia only fix nitrogen when inside the root nodules.

Rhizobia are microscopic single-celled organisms. They are so small, being one millionth of a metre in length, that they can only be seen through a microscope. Many thousands of cells of rhizobia would fit on the head of a pin.

Although all rhizobia appear very similar, they are genetically diverse and markedly different organisms. There are about 90 named species of rhizobia, and scientists are discovering and describing about 10 new species each year. Most of these new species are being discovered as scientists explore the biodiversity of our planet with the majority of new discoveries associated with native legumes not used in agriculture. Given that there are more than 18,000 species of legumes, it is not surprising that we are continually discovering new rhizobia. At present in Australian agriculture we only use as inoculants a small number of

species of rhizobia that fix nitrogen with the legumes we grow. As new legume genera and species with potential for agricultural use are developed, there will be new species of rhizobia available as inoculants.

Rhizobia can have thread-like flagella that allow them to move through water films in soil and on plant roots.

Each species of rhizobia comprise many thousands of genetically unique forms (strains) that vary in important characteristics that influence their interaction with the legume and adaptation to soil conditions. Commercial inoculants contain single strains of rhizobia that provide optimum nitrogen fixation with the target legume and adaptation to soils where the legume is grown.

Rhizobia can be considered to be 'probiotic' bacteria for legumes – beneficial bacteria that are not pathogenic to humans, animals or plants, and can only benefit the specific legumes they nodulate.

> Rhizobia are 'probiotic' bacteria that fix nitrogen in the nodules on legumes.

2.2 Specificity of rhizobia

The relationships between particular rhizobia and particular legumes are very specific – hence different inoculants are produced for the various legumes grown in Australian agriculture.

> Only specific rhizobia will nodulate and fix nitrogen with a particular legume host – this is why we have different inoculants.

An inoculant or inoculation group is a cluster of legumes nodulated by the same species of rhizobia (Table 2.1). Different inoculation groups are nodulated by distinctly different rhizobia. For example, lupins are nodulated by the slower-growing acid-tolerant *Bradyrhizobium* spp., whereas the medics are inoculated by the fast-growing, acid-sensitive *Sinorhizobium* spp. The groupings provide a practical framework when considering if inoculation is needed based on the type of legume previously grown in a paddock, and for choosing the correct inoculant for the particular legume to be sown. Inoculants are produced and marketed commercially according to these inoculant groups. More detail of inoculants and inoculation can be found in Chapters 4, 5 and 7.

2.3 What do rhizobia need to prosper?

Rhizobia only exist as vegetative living cells (i.e. they cannot form survival structures like spores) and this makes all rhizobia very sensitive to environmental stresses. They can easily be killed by exposure to stresses such as heat, extreme pH and toxic chemicals.

As with all bacteria, rhizobia will grow when the conditions are suitable, i.e. when they are provided with food (carbon and other nutrients) and water at a suitable pH (Table 2.2). Rhizobia are aerobic organisms and need oxygen for respiration, just like us. Temperature also markedly affects rhizobia. Being single-celled microscopic organisms, rhizobia are always at the same temperature as their immediate surroundings. They have no insulation or ability to protect themselves from heat.

The conditions listed in Table 2.2 (substrate, air, water, pH and temperature) are what inoculant manufacturers try to optimise when they produce inoculants.

Rhizobia are killed in soil and on seed by heat (some die at 35°C), desiccation, extreme acidity or alkalinity, and the presence of toxic chemicals such as fertilisers, fungicides and heavy metals (Table 2.3). These stresses must be avoided when handling inoculants to ensure a maximum number of rhizobia remain alive, and are able to colonise the soil and legume roots in sufficient number to make nodules.

The acidity or alkalinity of water and other additives used during the inoculation process can determine whether rhizobia live or die. All rhizobia survive well at neutral pH (7.0), although different species vary in their sensitivity to pH (Table 2.4).

In soils below pH 5, aluminum and manganese toxicity become additional stresses that can kill rhizobia. Moderate soil salinity is usually not a practical limitation to the growth and survival of rhizobia. It is the legume that is more sensitive to salinity stress.

2.4 The process of nodulation

> Rhizobia need adequate nutrients, moisture, temperature, pH and aeration for growth and survival.

Nodulation always begins with the colonisation of the legume roots by rhizobia. The earlier the colonisation of seedling roots, the sooner root nodules develop and the rhizobia begin to fix nitrogen. A specific sequence of events and optimal conditions are required for nodulation to occur, which can be within days of plant germination.

Nodule formation on legume roots is the result of a highly regulated process. This infection process is under the genetic control of both rhizobial and plant genes, and a high degree of genetic compatibility between partners is essential for the development of nodules containing highly effective rhizobia. This strong genetic compatibility is one of the key features of the elite inoculant strains currently available to Australian farmers.

TABLE 2.1 Some of the legume inoculant groups used in Australian agriculture and their rhizobia (see Chapter 7 for a complete list of the inoculant groups).

Taxonomy of rhizobia	Commercial inoculant group	Legumes nodulated
Sinorhizobium spp.	AL	Lucerne, strand and disc medic
	AM	All other annual medics
Rhizobium leguminosarum bv. *trifolii*	B	Perennial clovers
	C	Most annual clovers
Bradyrhizobium spp.	G[1]	Lupin, serradella
	S[1]	Serradella, lupin
Mesorhizobium ciceri	N	Chickpea
Rhizobium leguminosarum bv. *viciae*	E[2]	Field peas & vetch
	F[2]	Faba beans & lentil
Bradyrhizobium japonicum	H	Soybeans
Mesorhizobium ciceri bv. *biserrulae*	Biserrula special	Biserrula
Bradyrhizobium spp.	P	Peanuts
Rhizobium sullae	Sulla special	Sulla
Bradyrhizobium spp.	I	Cowpeas, mungbeans
Bradyrhizobium spp.	J	Pigeon peas

1 Both inoculant groups G and S can be used for lupin and serradella
2 Although group E is recommended for pea/vetch and group F for faba bean/lentil, if required group E can also be used for faba beans/lentils and group F used for peas/vetch

TABLE 2.2 Rhizobia are living organisms with simple needs for growth and survival.

Requirement	Comment
Food and energy	Usually carbohydrates (sugars such as glucose)
Mineral nutrients	Essential macro and micro nutrients
Water	Rhizobia can only grow in moist conditions
Temperature	Preferred range is 15 to 30ºC
pH	Preferred range is pH 6.0 to 7.5
Air	Rhizobia are aerobes and need oxygen for respiration

TABLE 2.3 Harsh environmental conditions kill rhizobia.

High Temperatures above 35°C will kill most rhizobia	
Acidity and alkalinity	pH sensitivity of rhizobia varies (see Table 2.4)
Toxic chemicals	Fungicides, solvents, alcohols and disinfectants kill rhizobia
Inorganic chemicals	High levels of heavy metals (Zn, Cu, Co) kill rhizobia

TABLE 2.4 Sensitivity of key rhizobia to pH, where red is sensitive and green is optimal.

Rhizobia	Host legume	pH 4	pH 5	pH 6	pH 7	pH 8
Bradyrhizobium spp.	Cowpea, mungbean, lupin, serradella	light green	green	green	orange	red
Bradyrhizobium japonicum	Soybean	orange	light green	green	green	orange
Rhizobium leguminosarum bv. *trifolii*	Clovers	orange	light green	green	green	green
Rhizobium leguminosarum bv. *viciae*	Pea, faba bean, lentil, vetch	red	orange	green	green	green
Mesorhizobium ciceri	Chickpea	red	orange	green	green	green
Sinorhizobium spp.	Medics	red	red	orange	green	green

An essential feature of nodule formation is the exchange of specific signal chemicals between the legume root and rhizobia. In other words, the two partners need to have a conversation with each other and 'communicate' in a language they both understand and then modify their behaviour to form a root nodule. Often, many species of rhizobia are present in the soil around legume roots but, because the rhizobia and plant are unable to communicate, there is no nodule formation.

While the rhizobia are the partner that fixes the nitrogen in this symbiosis, the legume plants generally determine the pathway of infection, and subsequently the type of root nodule that develops.

Nodule initiation can occur in three different ways:
i) via infection of the plant root hairs;
ii) via crack entry at breaks in the roots where lateral roots emerge; and
iii) between epidermal (root surface) cells.

For any specific combination of legume and rhizobia, infection will only occur by one of these processes. However, the majority of agricultural legumes grown in Australia are infected via root hairs (Table 2.5).

2.5 Root-hair infection

The rhizobia colonise the root surfaces including root hairs, and in response to chemicals released by the legume root, the rhizobia in turn manufacture specific compounds (Nod factors). These are released into the rhizosphere (area surrounding the root) and the legume responds. The rhizobia induces formation of an infection thread that grows back down the inside of the root hair, providing a channel for their entry into the root cortical cells and multiplication. The root cortical cells in the immediate region of the infection grow and divide repeatedly, ultimately forming an outgrowth (nodule) on the root. Once the rhizobia have reached these cells they are 'released' into specialised compartments where they change into bacteroids and then begin to fix nitrogen. It is important to note that infection of root hairs is most likely to occur while plants are young. Anything that affects normal root hair development may impede nodulation.

For nitrogen fixation to occur, two unique compounds are produced in the nodules:

1. **Nitrogenase** produced by the rhizobia – this is the enzyme that facilitates the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3), i.e. nitrogen (N_2) fixation. The enzyme requires molybdenum (Mo) to function optimally, which is why this micro-element is often added as a fertiliser when legumes are sown

2. **Leghaemoglobin** produced by the plant – this compound provides the characteristic pink/red colour of healthy nodules, and is essential for nitrogen fixation to occur.

The function of the leghaemoglobin in the nodule is similar to that of haemoglobin in our blood. Both compounds act as oxygen-transport molecules making sure the right concentration of oxygen is available for the rhizobia. Excess oxygen adversely affects the nitrogenase enzyme and stops nitrogen fixation. The colour of nodules is often used as an indicator of active nitrogen fixation as the presence of leghaemoglobin (pink colour) is a prerequisite for the process. In contrast, white nodules lack leghaemoglobin and cannot fix nitrogen. Green nodules usually indicate non-functional senesced nodules, with the green colour being a breakdown product of leghaemoglobin.

2.6 Nodule types

There are two basic types of nodules on agricultural legumes – determinate and indeterminate. The legume plant alone governs which type of root nodule occurs, irrespective of the species of rhizobia.

Determinate nodules are generally spherical, less than five millimetres in diameter and lack distinct internal zones. If the internal colour of these nodules is white or green rather than pink then they are unlikely to be fixing nitrogen. Soybeans,

TABLE 2.5 Types of infection processes used by rhizobia to make root nodules for common legumes grown in Australian agriculture.

Legume	Infection pathway
Soybean	Root hair
Chickpea	Root hair
Pea	Root hair
Faba bean	Root hair
Clovers	Root hair
Medics	Root hair
Biserrula	Root hair
Serradella	Root hair
Lupin	Between epidermal cells
Peanut	At lateral root junctions
Stylosanthes	At lateral root junctions

peanuts, serradella, lotus, navy beans, cowpeas and pigeon peas are legumes that form determinate nodules.

Indeterminate nodules can keep growing throughout the season and can remain functional to meet the nitrogen demand of the crop. These nodules can develop lobed finger-like projections to give a coralloid appearance. Internally they have distinct zones and grow from the outside tip, a region called the meristem. Although some part of the nodule may go green during the growing season, if the tip is pink the nodule should still be fixing some nitrogen. Peas, faba beans, lentils, chickpeas, lucerne, medic, clover, biserrula and sulla are legumes that form indeterminate nodules.

2.7 Other important symbioses that fix nitrogen

i) *Acacia* (wattles) are a group of legumes that form nodules in association with rhizobia. Unlike all the agricultural legumes, *acacia*s are native to Australia and their rhizobia already reside in the soil. The *acacia* rhizobia are very similar to the lupin and soybean rhizobia; however, there is (perhaps fortunately) no overlap (cross infection) between them.

ii) *Casuarina* are non-legume trees that can also fix nitrogen with very special and unusual soil bacteria. These bacteria are called *Frankia*. They grow as long filaments and appear more like fungi than bacteria.

2.8 Causes of poor nitrogen fixation – legume and rhizobia incompatibility

Although scientists expend a considerable amount of time and effort selecting elite strains of rhizobia, and provide these to the inoculant manufacturers for use in commercial inoculants, we cannot always control which strain of rhizobia is successful in forming the nodules on the growing legume. In many situations there are already rhizobia resident in the soil that can nodulate the legume in preference to the applied inoculant rhizobia. These resident strains may always have been present (unlikely), they may have colonised the soil after agricultural settlement (very likely), or they may have arisen from genetic changes of inoculant rhizobia after being introduced into the soil (also very likely). So, in these situations the quest to form a nodule becomes a competition between the applied inoculant rhizobia and other strains of soil rhizobia. The quality of the inoculant and its survival during the process of inoculation is critical in this competition. This is covered in more detail in later chapters, particularly Chapters 4 and 5.

Scientists are just beginning to understand how resident strains of rhizobia evolve in the soil, and probably the best understood scenario in Australia is that of biserrula, an annual pasture legume and its inoculant rhizobia. At the time biserrula was introduced experimentally to Western Australia from the Mediterranean Basin in 1994, there were no rhizobia in Western Australian soils capable of nodulating it. All sown biserrula were inoculated with an elite strain. Within seven years we noticed that a small proportion of nodules formed on biserrula regenerating in the field were small and green, and occupied by rhizobia that differed considerably

from the original inoculant. Research since then has led us to understand that the original inoculant strain for biserrula has shared its nodulation genes with bacteria that were already in the soil in Western Australia, but were not biserrula rhizobia. These bacteria were able to nodulate biserrula only when they received the genes for nodulation, but they do not have all the other genes required for high levels of nitrogen fixation.

Hence, the evolution of rhizobia like these in soil can significantly impair nitrogen fixation of legumes because they can successfully out-compete the highly effective inoculant rhizobia to form nodules but, once in the nodules, cannot fix nitrogen.

The only way we have of managing this is to periodically re-inoculate sown or regenerating biserrula with high numbers of the highly effective inoculant rhizobia (hoping to out-compete the soil rhizobia). More long-term research is underway to identify strains of rhizobia that do not share their nodulation genes with soil bacteria; such strains would be ideal for use as inoculants.

3 NUMBER AND NITROGEN FIXATION CAPACITY OF RHIZOBIA IN SOILS

- Many soils have developed communities of rhizobia that are able to nodulate the legumes used in agriculture.

- The number of rhizobia in soil is influenced by legume use and soil properties, particularly pH.

- Different legumes and their rhizobia have different tolerances to soil pH.

- Where the legume host has not been grown recently or where soil conditions are stressful to short and long-term survival of the rhizobia, there is a good likelihood of response to inoculation.

- Communities of rhizobia in soil tend to become more diverse with time and often less effective at fixing nitrogen, compared to commercial inoculant strains.

- Some legume species readily form less effective symbioses with soil rhizobia, while other legume species do not.

- Inoculant strains, when applied at high numbers, can compete with background soil rhizobia. This provides the opportunity to introduce effective strains.

3.1 Introduction

Before European settlement, Australian soils lacked the rhizobia needed for the pulse and pasture legumes that are now commonly grown in farming systems. However, after more than a century of legume cultivation, many soils have developed large and diverse communities of these introduced rhizobia.

Rhizobia become established in soils in several ways. Many were introduced as high quality inoculants. Others arrived accidently with the movement of dust, soil and seed around the country and some have evolved via genetic exchange with other bacteria in the soil (see Chapter 1). However, because rhizobia are legume specific and their persistence is affected by soil characteristics and cultural practices, their diversity, number and nitrogen fixation capacity can vary greatly.

This chapter examines some of the factors leading to this variability and its implications for nodulation and nitrogen fixation by different legumes.

3.2 How do we know if a soil has the right rhizobia?

The legume history of the soil provides some guide. If a legume species, or others very similar to it, has not been grown in a paddock, then it is unlikely the rhizobia for that legume will be present in the soil in high numbers.

Conversely, where there has been a recent history of well-nodulated legumes in a paddock, there is a reasonable chance the rhizobia that nodulated the legume will remain in the soil.

Some extension materials suggest that inoculation is not necessary if the legume host has been grown in any of the previous four years. The problem with this simplistic rule is that it fails to recognise that the level of nodulation of the previous crop can affect the current population of rhizobia in the soil and that many soils are not conducive to the survival of large numbers of rhizobia because of factors such as extremes of soil pH and low clay content. Also, the communities of rhizobia that develop under legume cultivation often become less effective at fixing nitrogen over time.

3.3 How many soil rhizobia are needed for prompt nodulation?

The number of soil rhizobia needed for prompt nodulation lies somewhere between 100 and 1000 rhizobia per gram of soil.

We say this for two reasons. First, when commercial inoculants of rhizobia are applied at recommended rates, they add the equivalent of about 100 rhizobia per gram of soil to a 10 centimetre depth. This results in prompt nodulation. Second, the evidence from many field and greenhouse experiments is that there is poor nodulation once the number of rhizobia in soil is less than 100 per gram.

High numbers of rhizobia result in prompt nodulation and plants tend to have many nodules on the tap root, close to the top of the root system (Figure 3.1).

Low numbers of soil rhizobia can result in delayed nodulation and smaller numbers of nodules on the roots.

3.4 Measuring the number of rhizobia in soil

First it is necessary to point out that soils often contain several species of rhizobia. For example, it is common

to find clover, lucerne and field pea rhizobia in the same paddock, if all those legumes had been grown before.

A laboratory-based plant nodulation test is used to determine the number of rhizobia in soil. The legume of interest is inoculated with a sequence of dilutions of the collected soil (Figure 3.2). After four weeks plant growth, the number of plants with nodules in each of the different soil dilutions is used to calculate the number of rhizobia in the original soil sample (called a most-probable number calculation). While this test is not available to growers, it has been used by researchers to quantify numbers of rhizobia in thousands of Australian paddocks.

The test is generally used with soils collected from the top 10 centimetres of the profile, because this is where most rhizobia are concentrated and thus where most nodulation of annual legumes occurs.

Rhizobia are also found deeper in the soil profile and play an important role in nodulating annual legumes towards the end of their growth and in nodulating perennial legumes such as lucerne. These rhizobia are seldom measured.

The number of rhizobia also vary within a growing season, particularly when a legume host is grown (Figure 3.3). Numbers start to increase at the break of the season as soils become wetter and the legume host germinates. The rhizobia are stimulated to multiply in the immediate vicinity of the root (rhizosphere). They can quickly multiply to levels of 10,000 per gram of soil.

Once the rhizobia have infected the root they multiply and

change into bacteroids that are able to fix nitrogen (which they cannot do in the free living form). The root cells infected with rhizobia collectively form the nodules.

When annual legumes set seed, their nodules begin to shut down as carbohydrates that provide energy to the nodules are diverted to seed development. Eventually the nodules senesce and the rhizobia are released back into the soil. Measures of rhizobial numbers at this time can exceed one million per gram of soil.

Rhizobial numbers may then decline to less than 100 per gram of soil over the next few months if soil conditions are unfavourable, or persist at a level of many thousands under more benign conditions.

Rhizobia are sensitive to desiccation and so tend to be at their lowest number at the end of hot dry summers in temperate regions. Hence, soil samples collected close to the start of the growing season provide a good conservative guide to the number of rhizobia available for legume nodulation.

3.5 What numbers of rhizobia persist in soils?

Where soil conditions are favourable, rhizobia are able to survive in the soil for many years, even in the absence of their legume host. In this state, the rhizobia are known as saprophytes (microorganisms that live on dead or decaying organic matter). They can also live in or near the rhizospheres of non-leguminous plants and utilise their root exudates. Even so, in the absence of a legume host, numbers will progressively decrease (Figure 3.4).

Surveys of soils provide a snapshot of the number of rhizobia at a given time and reveal that many soils support large numbers of rhizobia. It is not unusual to measure more than 1000 rhizobia per gram in the top 10 centimetres of soil at the end of summer. A million rhizobia per gram have been measured in some instances. Figure 3.5 shows how the numbers of rhizobia for three pulse and two pasture legumes vary in Australian soils.

FIGURE 3.1 Example of prompt and abundant nodulation on a pea root collected from a paddock containing an adequate number of rhizobia.
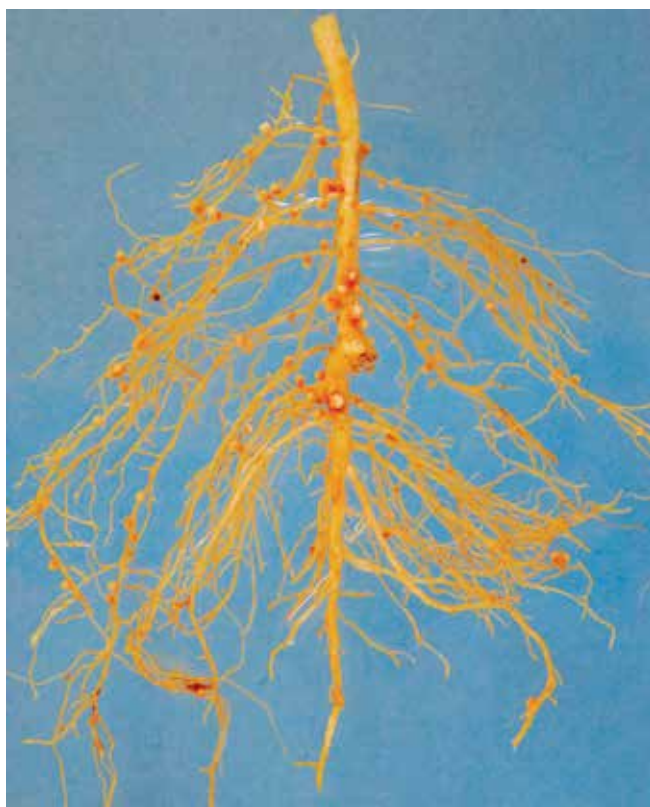
FIGURE 3.2 Method for counting rhizobia in soil. Plants are inoculated with different soil dilutions and the frequency of nodulation is measured.

FIGURE 3.3 Hypothetical scenarios of changes in the number of rhizobia through the seasonal cycle of an annual legume in southern Australia (Mediterranean climate).

Number of rhizobia per gram of soil (0 to 10cm)

Number of rhizobia pre-sowing

Plant germination multiplication of rhizobia in rhizosphere

Nodule senescence and release of rhizobia

Rhizobia surviving as saprophytes

Inoculum level available to nodulate regenerating pasture

— Benign soil — Stressful soil
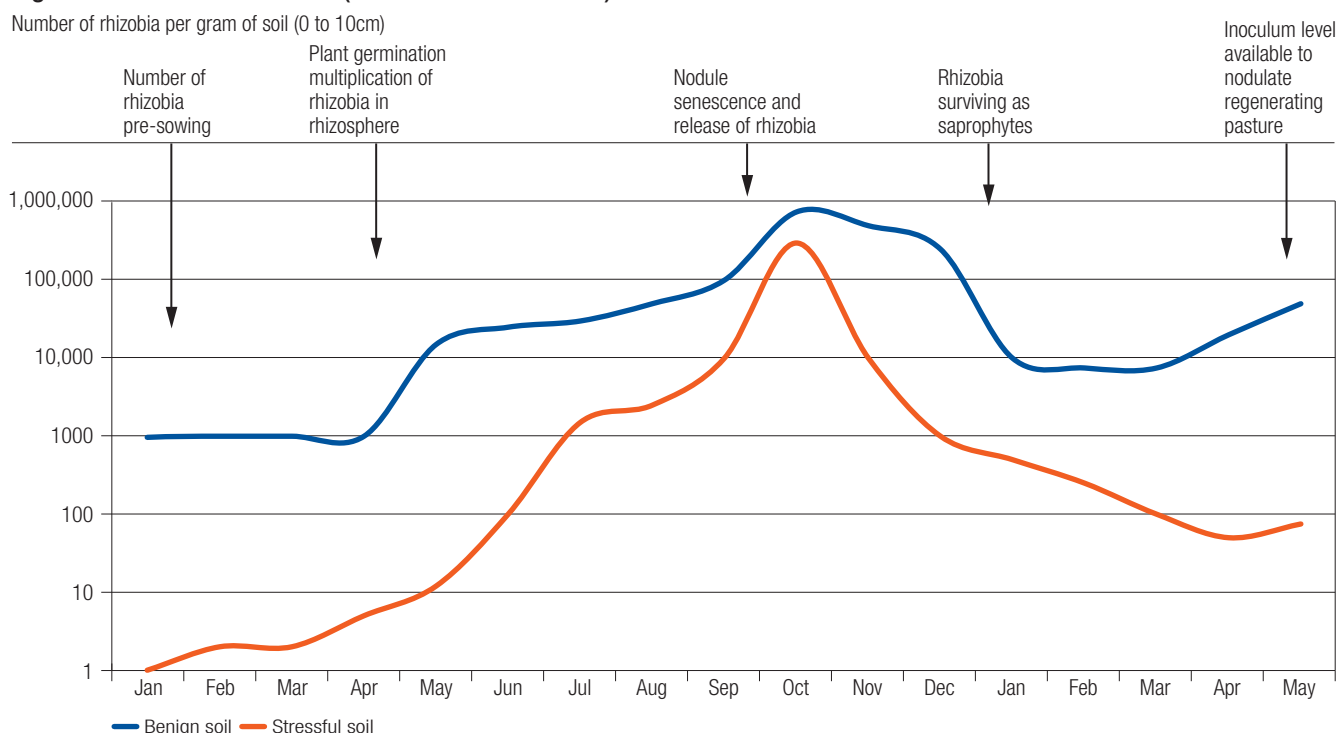
Rhizobia for the pasture legumes (medic and clover) are abundant, with more than 60 per cent of soils containing 1000 or more rhizobia per gram. Large areas that grow sown, regenerating and naturalised pasture legumes (at least 25 million hectares across the country) aid the multiplication and survival of these rhizobia.

Rhizobia for the pulse legumes are less abundant. For peas, chickpeas and lupin, more than 25 per cent of soils contained less than 100 rhizobia per gram. Understanding why some soils support fewer rhizobia is important to making sensible decisions about further inoculation.

3.6 Factors affecting the survival of rhizobia in soil

Regional (local) influences can strongly affect the occurrence of rhizobia in soil. These regional effects reflect both historical differences in legumes use as well as differences in the physical and chemical characteristics of the soils.

3.6.1 Influence of host legume

At a regional level, the more widely a legume has been grown, the more likely soils will contain the compatible rhizobia. For example, all the chickpea soils without rhizobia

FIGURE 3.4 Relationship between years of absence of the host crop and number of rhizobia in a relatively favourable soil.

Number of rhizobia per gram of soil

Years since previous host crop
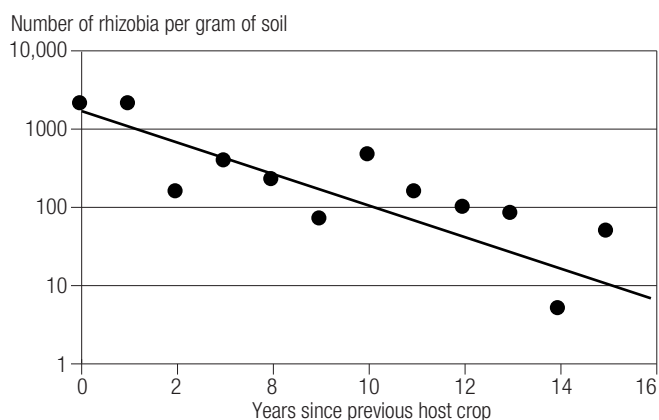
SOURCE: Pea and lupin data from Evans 2005; Ballard et al. 2004; Fettell et al. 1997; Slattery and Coventry 1989; Drew et al. 2012

FIGURE 3.5 Percentage of soils classified according to number of pea, chickpea, sub-clover, medic or lupin rhizobia they contain.

Percentage of soils

pea (n=176) chickpea (n=41) subclover (n=236) medic (n=480) lupin (n=100)

■ not quantified ■ not detected ■ 1 to 100 ■ 101 to 1000 ■ >1000
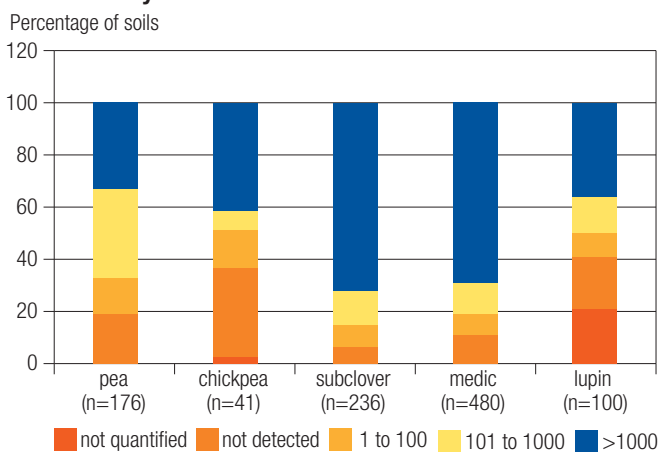
SOURCE: Chatel and Parker 1973; Slattery and Coventry 1989; Fettell et al. 1997; McInnes 2002; Howieson and Ballard 2004; Ballard et al. 2004; Evans 2005; Elias 2009; Drew et al. 2011, 2012

TABLE 3.1 Optimal pH (in calcium chloride) for a range of key legumes (most acid-tolerant at top and the least acid-tolerant at the bottom).

Legume species	Optimal pH range
Lupin and serradella	4.5 to 7.0
Peanut	4.5 to 7.0
Mungbean	5.0 to 7.5
Soybean	5.0 to 7.5
Subclover	5.0 to 8.0
Burr, murex, sphere medic	5.5 to 8.0
Pea/faba bean/lentil	5.5 to 8.0
Chickpea	6.0 to 8.5
Lucerne	6.0 to 8.5
Strand and barrel medic	6.5 to 8.5

shown in Figure 3.5 were from South Australia, where chickpeas are not usually grown. The remaining soils were from an area in New South Wales where they are commonly grown. Rhizobia for chickpea were abundant in most of these soils.

Pasture legume rhizobia often occur in high numbers in soils. This is likely due to the naturalisation and constant presence of subclover and medic in many soils. Even so, there are some species within the clovers and medics that do not consistently nodulate with the soil rhizobia. An example is the recently commercialised gland clover (cv. Prima). A combination of limited usage and a specific rhizobial requirement means that inoculation of this species is needed even where there are rhizobia that nodulate other annual clovers.

Such nodulation specificity is not common and cultivars within a legume species almost always behave similarly in terms of their rhizobial requirement.
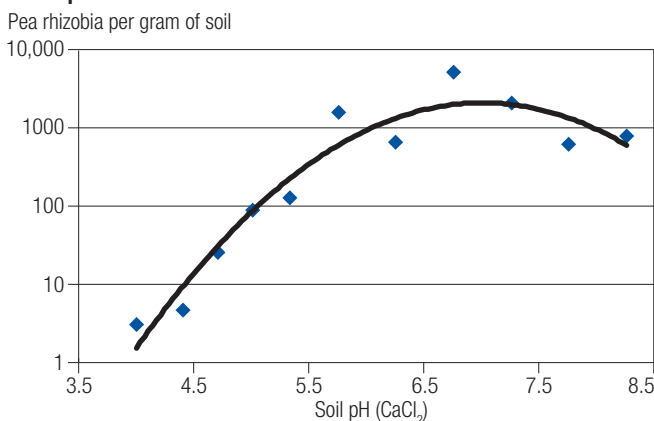
Soils vary widely in the number and type of rhizobia they support.

Soil properties and legume use are major factors affecting numbers of rhizobia in soil.

3.6.2 Influence of soil type

Soil chemical and physical properties affect the survival of rhizobia, especially pH, texture (clay content) and organic matter.

Soil pH is the best understood. It affects both the survival of the rhizobia and the formation of nodules. Different symbioses have different pH preferences. Although the rhizobia tend to be a little more sensitive to pH extremes than the legumes, understanding the pH preferences of the host legume will provide a reasonable insight into the pH preferences of the legume-rhizobia symbiosis.

The preferred pH range of some of the more common pulse and pasture legumes is shown in Table 3.1. Narrow-leaf lupin and serradella rhizobia are highly tolerant of soil acidity. They readily form nodules at pH 4.5, but can experience nodulation problems where soil pH exceeds 7.0.

Field pea rhizobia are moderately sensitive to soil acidity. Data from several surveys of pea rhizobia across Australia have been combined in Figure 3.6 to provide a good example of the relationship between soil pH and the number of pea rhizobia in those soils. Below pH 5.5 (determined in calcium chloride), the number of rhizobia is generally less than 100 per gram of soil, the threshold below which there is a good likelihood of a response to inoculation. Hence on acidic soils, frequent inoculation is recommended for peas, faba beans and lentils.

Lucerne and its rhizobia are sensitive to soil acidity with rapid decreases in nodulation measured below pH 5.0 (Figure 3.7).

The strand and barrel medics that are assigned to the same inoculant group as lucerne (AL) are similarly sensitive

FIGURE 3.6 Relationship between soil pH and the number of field pea rhizobia in soils with a history of field pea.

Pea rhizobia per gram of soil

SOURCE: Drew et al, 2012

FIGURE 3.7 Correlation between soil pH (0 to 10 cm) and the percentage of one-year-old lucerne plants with nodules.

Plants with nodules (%)

$R^2 = 0.94$

SOURCE: Unpublished data from Nigel Charman and colleagues
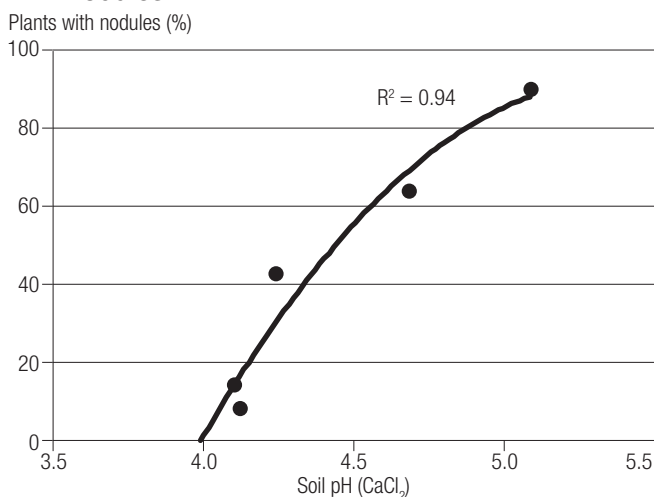
to soil acidity. Burr, sphere and murex medics are more tolerant of acid soils, with increased tolerance attributable to the selection and use of an acid-tolerant strain of rhizobia (WSM1115, group AM inoculant) selected for use with these medics.

> Soil pH affects survival of the rhizobia and the nodulation process.
>
> Different legume symbioses have different tolerances to extremes of soil pH.

The effects of acidity in the field are not always as obvious as shown in the lucerne example in Figure 3.7. In a subclover pasture, moderate acidity results in fewer but larger nodules. It is not until nodule mass falls below the level needed to supply the plant with adequate nitrogen that the effects of the acidity become obvious. At this point the legume content of the pasture can decline rapidly.

In some cases the acidity stresses are avoided by the rhizobia. Large numbers of rhizobia and adequate nodulation have been measured in regenerating subclover pastures, even though the pH (calcium chloride) of the bulk soil is less than 4.5. This is attributed to the survival of the rhizobia in small niches in the soil, often associated with soil organic matter. When these soils are disturbed as a result of cropping or at pasture renovation, the number of rhizobia are reduced when they are displaced from these niches that provide protection. There is a moderate likelihood of responses to inoculation on these soils when pastures are renovated, even though nodulation constraints may not have been apparent previously.

The relationship between soil organic matter or clay content and rhizobia is less understood and has been shown to improve the survival of clover and pea rhizobia in soil. It is also worth noting that most commercial inoculants produced for growers use peat (high organic matter) or clay as a carrier, because rhizobia are known to survive well in them.

3.6.3 Other factors

The extensive use of herbicides in farming systems is known to affect the legume-rhizobia symbiosis. However, their impact seems mostly detrimental to the plant, rather than to the growth, survival or effectiveness of the rhizobia. Even where rhizobia are present in high numbers, the damage to legume root systems by some herbicides (e.g. Group B herbicide residues in both acidic and alkaline soils in low-rainfall regions) can effectively halt nodulation.

Desiccation is also detrimental to the survival of rhizobia. Rhizobial numbers can decline by the end of a dry summer. Soils that experience long dry summers and are subject to higher temperatures may have fewer rhizobia, particularly where clay content is low or other soil stresses are present.

3.7 Diversity of soil rhizobia

There is nearly always more than one strain of a rhizobial species in a soil. Molecular methods make it possible to 'barcode' the strains that form nodules (Figure 3.8) and has shown that different nodules on a plant are often formed by different strains.

In some cases, more than 10 different strains of rhizobia can form nodules on a single legume plant growing in the field. Sometimes it is obvious that different strains of rhizobia occupy different nodules because the nodules differ in their appearance (Figure 3.9).

The spectrum of strains is also likely to differ from soil to soil. A common observation of strains in different soils and

FIGURE 3.9 Example of different nodule types on pea inoculated with field soil.

White

Pink

White

FIGURE 3.8 Different strains are shown as different 'barcodes'. Many different strains can be isolated from the nodules of a single subclover plant.

Commercial strains   Rhizobia strains isolated from a single soil
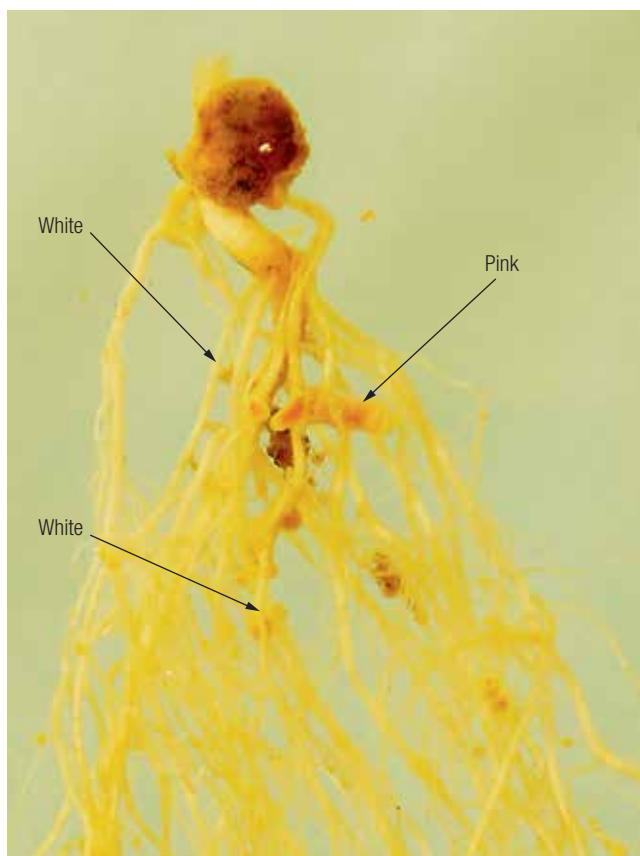
also within soils is that few are identified as the strains that have been used in commercial inoculants. In some instances this may simply be the result of inoculants not being used or not properly applied.

However, even where inoculants have been correctly used, the diversity of rhizobial communities in the soil tends to increase soon after legume introduction. This is often, but not always, associated with an increase in the number of less effective strains within the community.

The recent introduction of the pasture legume biserrula and its rhizobia into Australian farming systems has provided a unique opportunity to study the evolution of rhizobial communities. Studies have shown that the development of strain diversity can be rapid (years not decades) and is associated with the transfer of symbiotic genes to other members of the soil microbial community.

The presence of ineffective rhizobia is not always detrimental because the legume plant has some influence over nodulation. In some situations the plant is able to foster occupancy of its nodules by the more effective strains from within the rhizobial community. In other situations the plant can increase nodule number in order to satisfy nitrogen demand. Ineffective rhizobia are therefore most likely to become problematic where the rhizobial community is dominated by ineffective strains and where opportunities for continued nodulation are limited, as may be the case in stressed soils.

It is likely that about 50 per cent of legumes sown each year will be reliant on soil rhizobia for nodulation, because they are either not inoculated or because the inoculant rhizobia is present in low numbers on the seed (as in many preinoculated seeds, see Chapters 4 and 5). Most regenerating pastures are nodulated by existing soil rhizobia.

Even where inoculation is practiced and inoculants applied well, the soil rhizobia will compete and can form a significant proportion of nodules. It is therefore important to consider their nitrogen fixation capacity.

> Communities of soil rhizobia are complex, comprising many strains.
>
> It is common to find 10 different strains forming the nodules on a single plant.
>
> Soil rhizobia are rarely identified as the same strains used in inoculants.

3.8 How well do the soil rhizobia fix nitrogen with legumes?

So far we have considered the number and diversity of rhizobia. Their function or capacity to fix nitrogen is just as important. Nitrogen fixation capacity is the result of the legume-rhizobia partnership, not just the rhizobia. Therefore it is possible that the same community of rhizobia may fix less or more nitrogen with different legume genotypes.

The terms effective and ineffective are commonly used to describe differences in nitrogen fixation capacity. Here, the term effective is used where the shoot weight of plants

FIGURE 3.10 **Plants growing in N deficient potting media are inoculated with a suspension of soil to determine effectiveness of the rhizobia in that soil. Plant growth provides a measure of the nitrogen fixation capacity of the soil rhizobia.**

resulting from an inoculation treatment (rhizobia) is at least 75 per cent that of plants inoculated with a highly effective strain of rhizobia. Symbiotic capacity is deemed moderately effective when shoot weight is between 50 and 75 per cent and ineffective when below 50 per cent.

The effectiveness of soil rhizobia is commonly measured using a 'whole soil' inoculation method (Figure 3.10) or by inoculating plants with individual strains of rhizobia isolated from nodules.

Data for symbiotic effectiveness of soil rhizobia is more limited than for population number, especially for the tropical legumes (e.g. soybeans, mungbeans and peanuts). Even so, it is apparent that while the symbioses formed by the commonly grown legumes and soil rhizobia are seldom grossly ineffective, they are often less effective compared to the inoculant strain for the legume.

For example, the effectiveness of the symbioses formed

FIGURE 3.11 **Distribution of soils according to the effectiveness of their subclover rhizobia relative to an effective inoculant strain.**

Number of soils

Effectiveness	Number of soils
0–10%	1
11–20%	1
21–30%	4
31–40%	3
41–50%	5
51–60%	9
61–70%	6
71–80%	7
81–90%	3
91–100%	4
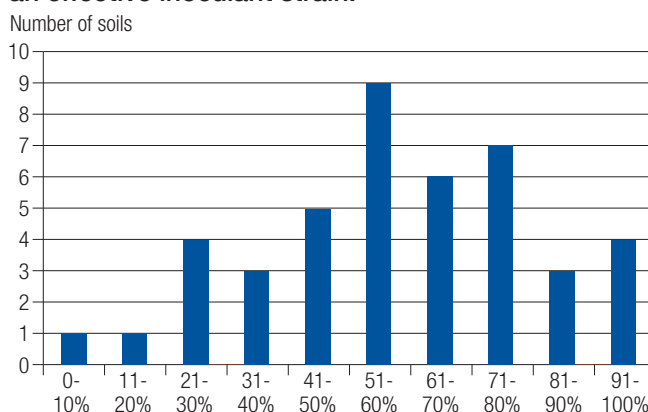
SOURCE: Drew and Ballard 2010, Drew et al. 2011

TABLE 3.2 Mean symbiotic capacity of temperate legumes with soil rhizobia relative to effective inoculant strains and distribution of the communities of soil rhizobia based on their classification as effective, moderately effective or ineffective.

Legume	Mean nitrogen fixation capacity (%)	Percentage distribution of soil rhizobia communities based on their symbiotic capacity		
		Effective ≥ 75%	Moderately effective 50 to 75%	Ineffective ≤ 50%
Field pea	78	68	23	11
Chickpea	60	25	40	35
Yellow serradella[A]	>75	-	-	-
Subclover	58	19	49	32
Strand medic	62	36	34	30
Burr medic	36	15	21	64
Lucerne	84	89	11	0
Biserrula	>75	92	-	8

A determined using individual strains isolated from soils.

SOURCE: Bowman et al. 1998; Brockwell 2001; McInnes 2002; Ballard et al. 2003; Charman and Ballard 2004; Ballard et al. 2004; Elias 2009; Drew and Ballard 2010; Drew et al. 2011, 2012.

between subclover and the rhizobia in 43 soils ranged from eight per cent to 99 per cent of that formed between subclover and the commercial inoculant strain (WSM1325). Most commonly, the communities of soil rhizobia were 51 to 60 per cent as effective as the inoculant strain (Figure 3.11). Thirty-two per cent were classed as ineffective.

Mean nitrogen fixation capacity of soil rhizobia with a range of different temperate legumes is shown in Table 3.2. The higher prevalence of ineffective symbioses for burr medic compared to strand medic and lucerne (all *Medicago*) highlights the differences in symbiotic competence between legume species.

Among the annual clovers, symbioses tend to be similar or less effective (e.g. arrowleaf clover) compared to subclover.

For field peas the majority of rhizobial communities are classified as effective. Faba beans, lentils, vetch and lathyrus, all nodulated by the same rhizobia, are likely to be similar to field peas, since we are not aware of data or anecdotal evidence to suggest otherwise. The same can be said for narrow-leafed lupin, which is nodulated by the same rhizobia that form effective symbioses with serradella.

While differences in rhizobial persistence can be linked to frequency of legume cultivation and soil properties such as pH, reasons for variation in symbiotic effectiveness are not well understood. Variation in symbiotic effectiveness is therefore difficult to predict. Generally, stressful environments exerting greater selection pressure may increase the diversity of the rhizobia at the expense of nitrogen fixation capacity.

Many soils contain rhizobia that are less effective than inoculant strains.

Some legume species are more readily compatible with a range of soil rhizobia than other legumes.

3.9 Dealing with soil rhizobia

Where large and persistent populations of rhizobia are present in the soil, a competitive barrier for the introduction of new strains of inoculant rhizobia is created. This is not a problem where the soil community is effective with the legume host. But where the soil rhizobia are not effective, high nodule occupancy by an effective inoculant strain is desirable to optimise nitrogen fixation potential. Rhizobia persist in many soils well above the threshold needed (100 rhizobia per gram) for prompt nodulation and often at numbers far greater than can be introduced through inoculation. However, rhizobia in the soil are diffusely distributed, while those applied to seed as inoculum are in close proximity to the root and able to rapidly multiply to the levels needed to achieve effective nodulation.

Studies investigating the success of applied inoculants show that if the rhizobia per seed are numerically equivalent to the number of rhizobia per gram of soil, then the inoculant strain is able to form sufficient nodules to improve plant nitrogen fixation and growth (Figure 3.12).

For example in Figure 3.12, a growth response to inoculation is only apparent in a soil containing 1000 rhizobia per gram when the number of rhizobia applied as inoculant exceeds 1000 per seed.

This and similar studies form the basis of quality guidelines that specify minimum inoculation standards of 1000 cells per seed for subterranean clover and similarly sized pasture legumes.

FIGURE 3.12 Ineffective soil rhizobia (across the bottom are the log number rhizobia per gram soil) are overcome when equivalent numbers of inoculant rhizobia are applied to the seed (shown as log number per seed).

Earlier in this chapter we state that it is common where a legume species has been grown that the number of soil rhizobia can exceed 1000 rhizobia per gram. Responses to inoculation would only be likely where the minimum standards for inoculant on seed are exceeded.

As Australian inoculants are mostly produced in sterile peat and meet minimum standards of one thousand million (1×10^9) cells per gram peat at manufacture, seed standards are easily surpassed when recommended rates of inoculation and methods of application are followed, and the seed is promptly sown.

For the pulse legumes, where seed size is larger, the number of rhizobia applied per seed is also larger (refer to application rates in Chapter 5). For field peas the recommended standard is 100,000 rhizobia per seed. High numbers of rhizobia on seed combined with the annual re-sowing of pulse crops provide a good opportunity to introduce effective inoculant strains into the soil.

However, these opportunities are less frequent for regenerating pastures and nodule occupancy by inoculant strains declines with time.

While the benefits of effective strains introduced through inoculation will be important to pasture establishment, occupancy by the applied inoculant will be temporary and possibly insignificant where the pasture phase extends past a few years.

Research to manage suboptimal populations of rhizobia in soils continues. New inoculant formulations that provide competitive and stable strains of rhizobia, higher numbers of rhizobia or allow more strategic placement of the inoculant strain are being tested.

For annual pasture species that have a propensity to form ineffective symbioses with soil rhizobia, the development of varieties that can be effectively nodulated by a large proportion of soil rhizobia is being investigated to provide a long-term solution.

3.10 Concluding comments

After more than 100 years of legume cultivation, many Australian soils have developed substantial populations of rhizobia able to nodulate commonly grown agricultural legumes. However, suitable rhizobia may still be absent from the soil if the legume has not been grown previously, or where the soil is not conducive to long-term rhizobial survival. Soil acidity often affects persistence of the rhizobia. Medic, lucerne and pea (including faba bean, lentil and vetch) symbioses are particularly sensitive to acid soils.

Where soils do support rhizobia, the communities are diverse and tend to become less effective at fixing nitrogen with time, when compared to commercial inoculant strains. The extent of ineffective symbioses formed can be modified by the host legume. Even so, symbioses between soil rhizobia and the host legume are commonly less than 50

per cent of the potential of symbiosis between the inoculant strain and host legume. It is not possible to predict the nitrogen fixing capacity of the rhizobia at a paddock level.

The good news is that inoculant strains, when applied at a high number, can compete with background soil rhizobia. This provides the opportunity to introduce effective strains in pulse crops and frequently renovated pasture systems.

Nodule occupancy by inoculant rhizobia declines with time in regenerating pastures. In these pastures there appear to be good prospects to develop 'symbiotically promiscuous' legumes that are better matched to the diverse communities of rhizobia that are now found in many soils.

4 RHIZOBIAL INOCULANTS – STRAINS AND QUALITY CONTROL

- Strains of rhizobia used in commercial inoculants must satisfy a number of criteria, including effectiveness at fixing nitrogen.

- Rhizobial inoculants are formulated and available in peat, clay or peat granules, liquids and as a freeze-dried powder.

- Inoculants are applied to the seed at sowing or directly to the soil in the vicinity of the seed at sowing.

- Rhizobial inoculants in Australia are subjected to independent quality testing by the Australian Inoculants Research Group (AIRG).

- Inoculants meeting the standards of the independent AIRG quality testing display the Green Tick Logo.

- The Green Tick Logo does not guarantee inoculant efficacy in the field, as this is influenced by a number of other factors.

- Testing of inoculants and preinoculated pasture legume seed at the point-of-sale indicate high quality of inoculants but problems with often very low numbers of rhizobia on preinoculated seed.

4.1 What are legume (rhizobial) inoculants?

Inoculants for legumes are products containing commercially prepared cultures of rhizobia protected in carriers that supply large numbers of viable rhizobia for the effective nodulation of legumes. The purpose of legume inoculation is to supply selected rhizobial strains in large numbers to the roots of the legumes soon after germination, optimising the chances of effective nodulation, symbiotic nitrogen fixation and plant and grain yield, while decreasing input costs.

Inoculants in Australia contain rhizobial strains that have been selected according to the following criteria established during many years of scientific research.

4.1.1 Effectiveness of rhizobia and their legume host range

There are thousands of strains of rhizobia that can nodulate and fix nitrogen with a particular legume host. However, the amount of nitrogen fixed can vary substantially, depending on the combination of plant host and rhizobia strain. Strains that are used in commercial inoculants are the most effective at fixing nitrogen with the range of legume species/cultivars in each of the inoculant groups. Strain testing with the target legumes is conducted first in glasshouse experiments and then in field trials across the range of soil types and environments where the legumes are grown commercially.

The result of using a highly effective rhizobial strains to fix nitrogen in subterranean clover plants grown in N-deficient medium is shown in Figure 4.1.

4.1.2 Genetic stability

The strain must maintain its symbiotic capacity (nodulation and nitrogen fixation performance) and other key traits during culture, manufacture and application. Strains are tested for genetic stability throughout the selection process and annually, once they are used as commercial inoculants.

FIGURE 4.1 Growth of subterranean clover in N-free medium inoculated with a highly effective rhizobial strain.

Host: T. subterraneum L. var WOODGENELLUP

Uninoculated — Inoculated with Rhizobium l. bv. trifolii
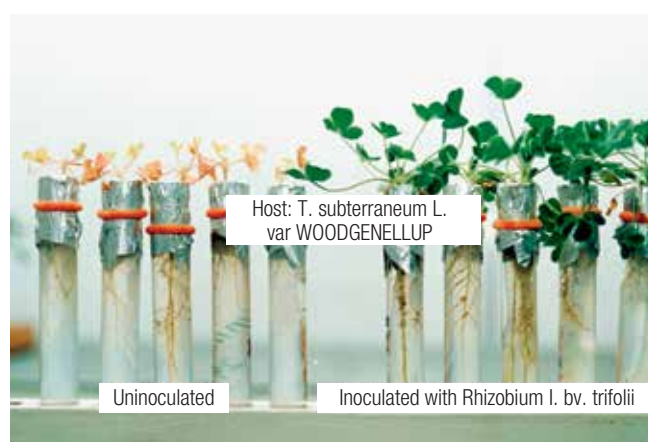
Strains of rhizobia are selected to ensure maximum nitrogen fixation. Important criteria are:

- Effectiveness
- Host range
- Field performance
- Soil persistence
- Genetic stability
- Manufacturability
- Inoculant survival

4.1.3 Potential for scale-up production as commercial inoculants

Rhizobial strains must be able to grow and survive in large numbers in commercial inoculant formulations (manufacturability). Inoculant companies test potential commercial strains for manufacturability in their production system and suitability for growth and survival in inoculant carriers prior to commercialisation.

4.1.4 Ability to survive during inoculant application

Strains vary in their ability to survive on seed. Seed inoculation is a convenient (and the most widely used) way to introduce rhizobia into the soil at sowing. Survival on seed needs to be high and is determined by the selection process. This is particularly important for pasture rhizobia destined for application to preinoculated seed.

4.1.5 Persistence in soil in absence of host – known as 'saprophytic competence'

This trait is more important for annual pastures than for pulse legumes or perennial pastures. Growers have an opportunity to re-inoculate pulse legumes when sowing annual crops. However, sowing and inoculation tends to be less frequent for annual legume pastures as plants are typically regenerated from soil seed banks. While persistence of perennial pasture roots allows continual colonisation and survival of inoculant strains, annual pasture legume-hosts are absent during the summer months and rhizobia must therefore persist in soil between growing seasons.

4.2 Inoculant formulations

There are several different commercial inoculant formulations available to growers to allow flexibility of application (Figure 4.2).

Formulations include peat, granular, liquid and freeze dried inoculants:

(i) **Peat inoculants** are the oldest and most common form of inoculant used in Australia. They are prepared by introducing selected rhizobial strains into gamma-irradiated (sterilised) finely milled peat. The final preparation has a relatively high moisture potential when compared with other solid formulations, which, if maintained, allows survival of rhizobia for up to 18 months.

(ii) **Granular pellets or chips** are made from either peat or clay.

(iii) **Freeze-dried powder**, where a rhizobial broth culture is concentrated as a powder in a glass vial after all the water has been removed. The powder is reconstituted later on-farm.

(iv) **Liquid inoculants** are suspensions of rhizobia in a protective liquid formulation.

4.3 Application of inoculants

Application of inoculants is covered extensively in Chapter 5. Peat, freeze-dried and liquid inoculants can be applied either to seed or directly to soil. Peat inoculants should either contain, or be mixed with, a sticker or an adhesive if they are to be applied to seed before sowing. The use of a sticker ensures that the rhizobia adhere to the seed and are evenly distributed into the paddock when the seed is sown. If peat, freeze-dried or liquid inoculants are applied directly to soil, they need to be suspended in clean potable water so they can be evenly distributed over the cropping area.

Seed inoculation can be done by growers or by commercial seed coaters. Seed coaters may inoculate freshly purchased seed with peat on request from growers for sowing within a few days (custom inoculation) or prior to sale of the seed (preinoculation). Many of the small seeded pasture species (lucerne and clover) in Australia are preinoculated (Figure 4.3) providing a convenient ready-to-sow product. Preinoculated seed is generally coated with a thick pellet containing several other plant growth enhancers. Descriptions of seed preinoculation processes and microbiological quality can be found in Gemell et al. (2005), Deaker et al. (2012) and Hartley et al. (2012).

4.4 Quality of inoculants

In Australia, during the 1940s and early 1950s, the area sown to legumes increased with the introduction of many new species, particularly pasture legumes, and this prompted a shift in the manufacture of inoculants from the public to the private sector.

Adoption of the US technology using peat as a carrier, and a lack of regulation of the quality of inoculants, eventually led to nodulation failures. In 1954, Professor Jim Vincent, an eminent microbiologist from the University of Sydney, asserted that poor-quality inoculants cost growers in lost production and would eventually discredit the practice of inoculation. He made basic recommendations for quality control and use of legume inoculants and established the first quality control laboratory as a joint venture between the University of Sydney and the NSW Department of Agriculture.

The quality-control and assurance of legume inoculants continues today within the Australian Inoculants Research Group (AIRG) under the auspices of the NSW Department of Primary Industries (DPI), based at Ourimbah. The 'National Code of Practice and Quality Trademark for Legume Microbial Inoculant Products used in Australian Crops and Pastures' can be accessed at the AIRG website (www.dpi.nsw.gov.au/research/centres/gosford/australian-inoculants-research-group).

4.5 How do we know if an inoculant is high-quality?

Since July 2010, rhizobial inoculants in Australia that have been

tested to meet strict quality standards display a registered trademark called the Green Tick Logo (Figure 4.4). The logo indicates that at the time of testing the product contained:

- the correct rhizobial strain for the target legume host;
- numbers of live rhizobia equal to or above a minimum standard; and
- zero or minimal numbers of other organisms (contaminants).

The Green Tick Logo indicates that an inoculant has been independently tested and satisfies Australian quality standards.

FIGURE 4.3 Preinoculated lucerne seed.

FIGURE 4.2 Commercial inoculant formulations available for inoculating crop and pasture legumes: A – moist peat; B – peat granules (left), bentonite clay (middle), attapulgite clay (right); C – liquid inoculants; D – freeze-dried inoculants.

The logo also indicates that labelling standards have been achieved. The label should display:

- the name of the target legume host;
- application method/s;
- storage conditions;
- expiry date/shelf life;
- guaranteed number of live rhizobia at the point of sale; and
- batch number.

Inoculants will only carry the logo if a representative sample of packets from the batch has been tested.

At the date of publication of this handbook, companies that are signatories to the 'National Code of Practice: Quality Trademark for Microbial Inoculant Products used in Australian Crops and Pastures', and producing and selling inoculants that carry the Green Tick Logo, are:

- BASF Agricultural Specialties Pty Ltd;
- New Edge Microbials Pty Ltd; and
- Novozymes Biologicals Australia Pty Ltd (see Appendix for contact details).

4.6 Who tests inoculant quality?

Inoculant manufacturers are responsible for ensuring their product is of high quality for consumers, and they conduct a number of tests in their own laboratories. The AIRG is responsible for independent quality assessment of legume inoculants in Australia. The group is funded through service agreements with the three inoculant manufacturers that are signatories to the Code of Practice and research projects with the Grains Research and Development Corporation (GRDC), the Rural Industries Research and Development Corporation (RIRDC) and the NSW DPI. The AIRG also has collaborative support from the research community through the University of Sydney and the National *Rhizobium* Program.

The AIRG is responsible for:

- maintaining, authenticating and issuing approved rhizobial strains for commercial release to the manufacturers who comply with the national Code of Practice incorporating the Green Tick logo;
- assessing the quality of inoculants at point of manufacture for compliance to the Code of Practice and at various points through the supply chain; and
- administering and promoting the Green Tick Logo trademark.

4.7 Numerical standards

In Australia, legume inoculants displaying the Green Tick Logo must contain no less than a minimum number of rhizobia that has been prescribed for each inoculant formulation for the shelf life of the product (Table 4.1).

These numerical standards for legume inoculants are based on scientific research that has defined the number of rhizobia required for adequate nodulation. Requirements for inoculants at an individual site will be affected to some extent by the climate and soil conditions at that site. The numerical standards were developed and are applied to ensure effective nodulation is likely to be achieved with each formulation.

FIGURE 4.4 Registered trademark for inoculants quality – the Green Tick Logo.

TABLE 4.1 Australian minimum standards for legume inoculants.

Product	Initial count after manufacture	Count throughout shelf life	Expiry (months)
Peat (CFU/g)	$\geq 1 \times 10^9$	$\geq 1 \times 10^8$	12*
Liquid (CFU/mL)	$\geq 5 \times 10^9$	$\geq 1 \times 10^9$	6
Granules (MPN/ha)	$\geq 1 \times 10^{10}$	$\geq 1 \times 10^{10}$	6
Freeze dried (CFU/vial)	$\geq 1 \times 10^{12}$	$\geq 5 \times 10^{11}$	6

CFU: culture forming units; MPN: most probable number.

Standards for inoculants applied to seed have been set to achieve particular numbers depending on seed size. For large seeded legumes (e.g. soybeans), the number is 100,000 rhizobia/seed; for medium seeds (e.g. lentils), 10,000 rhizobia/seed; for small seeds (e.g. subterranean clover and lucerne), 1,000 rhizobia/seed and very small seeds (e.g. white clover), 500 rhizobia/seed.

Numerical standards for CB376 for *Lotononis bainesii* are 2×10^8 rhizobia/g moist peat (2×10^7 rhizobia/g at expiry). Standard for liquids based on a three litre bottle used to treat one tonne of seed. Standard for freeze-dried based on vial used to treat one tonne of seed. (Information on standards from Australian Legume Inoculant Research Unit Annual Report 2007)

* Based on current data, 18 months expiry applies for groups E, F, G and N stored at 4ºC. Group G is applicable to strain WU425 only.

Research with peat inoculants has been more extensive than with other formulations and so there is more confidence in quality standards for peat. Standards for all inoculant formulations are under continual review and are adjusted as new data becomes available.

In addition, peat, liquid and freeze-dried inoculants should not contain a high number of other contaminating organisms. If contaminant organisms are present within the inoculant, they should be at least 10 to 100 times lower in number than the rhizobial strain.

Non-rhizobial contaminants and moisture content of peat inoculants are effective indicators of potential shelf life and are checked routinely. If a batch of inoculant is within one month of expiry, it may be given an extended expiry of six months, provided it passes all standards when retested by the AIRG.

Standards for preinoculated seed are the same as the standards for seed listed in the footnote in Table 4.1.

4.8 Does a high-quality inoculant guarantee efficacy in the field?

There are factors that may compromise field efficacy of an inoculant. While the quality tests ensure that inoculants contain high numbers of effective rhizobia at the time of testing, the quality of the inoculant can be affected by the way it is treated along the supply chain and how it is applied.

Rhizobia are living organisms susceptible to high temperatures. It is important that inoculants are always stored according to the manufacturer's recommendations because hot temperatures (>35°C) during transportation and storage kill the rhizobia, thereby reducing their numbers in the inoculant.

Rhizobia may be exposed to detrimental conditions during inoculant delivery to the crop. Desiccation on seed, and contact with incompatible chemicals (e.g. pesticides applied to seed, nutrient residues in spray tanks and acidic superphosphate fertiliser) are major factors that can affect survival of rhizobia during application (see Chapter 5).

The careful application of high-quality inoculants to legume crops increases the chances that nodulation, nitrogen fixation and yield will be optimised.

4.9 What is the quality of inoculants and preinoculated seeds in Australia?

Shelf life of inoculants is determined by measuring the survival of rhizobia in inoculant formulations over time in the distribution chain.

Between 2005 and 2010, the AIRG conducted 23 point-of-sale surveys of inoculant and preinoculated seed quality. The surveys covered 266 towns across the Australian grainbelt.

During this period 1556 legume inoculants for temperate and tropical legumes were tested for quality. In all surveys, three inoculant formulations were on sale to farmers, and purchased for testing in the following proportions:

- peat-based – 92 per cent;
- freeze-dried – 3 per cent; and
- granular – 5 per cent.

Each inoculant was assessed for quality and either passed or failed the standards. Pass rates between 2005 and 2010 ranged from 87 per cent to 94 per cent. There were 126 inoculant samples (eight per cent) that had numbers of rhizobia below the AIRG standard. Inoculants also failed if contamination with non-rhizobial organisms was too high.

Data obtained from monitoring survival of rhizobia on preinoculated seed has been alarming. The convenience of using pasture seed that has been preinoculated with rhizobia led to an increase in demand from growers, and the number of companies producing preinoculated seed has risen in recent years.

4.10 Quality of preinoculated seed (rhizobial numbers)

Point-of sale surveys preinoculated seed were conducted across 37 towns in the wheat/sheep belt, mainly in the eastern states. A total of 272 samples of seed of temperate and tropical legumes were obtained and tested. The majority

of samples were temperate legume pasture species. Despite many attempts by various seed coaters to improve the quality of preinoculated legume seed, numbers of rhizobia on seed collected from retail outlets has not improved since the quality was assessed in an earlier survey between 1999 and 2003 (Gemell et al. 2005). Generally survival of rhizobia on lucerne seed is better than survival on clovers.

The percentage of samples of each legume species passing minimum standards between 1999 and 2003 were as follows:

- lucerne – 73 per cent;
- subterranean clover – 32 per cent;
- white clover – 3 per cent;
- red clover – 4 per cent; and
- other species – 0 per cent.

Results from 2005:

- samples passed – 5 per cent;
- rhizobia detected – 60 per cent: and
- nil rhizobia – 40 per cent.

The number of rhizobia on preinoculated pasture seed products is highly variable and viability declines rapidly over time (Figure 4.5). Some of the samples meet the rhizobial numerical standards when less than 50-days-old (i.e. 50 days after inoculation) but virtually none of the older samples (i.e. >50 days) met the standards.

4.11 Non-rhizobial inoculants

Inoculants that contain potentially beneficial microorganisms other than rhizobia are also available in the market. These organisms do not produce root nodules on legumes but are marketed as enhancing plant growth in other ways.

There is scientific evidence that certain microorganisms can enhance plant growth through a range of mechanisms. Some organisms can increase root growth through the production of hormones or enzymes, theoretically improving nutrient uptake efficiency. Hormone-producing microorganisms have the potential to increase legume nodulation by rhizobia through increased root hair density

FIGURE 4.5 Survival of rhizobia on seed of different preinoculated pasture species over time. Data from the AIRG surveys of preinoculated seed, sourced at point-of-sale (resellers) during 2005-10.
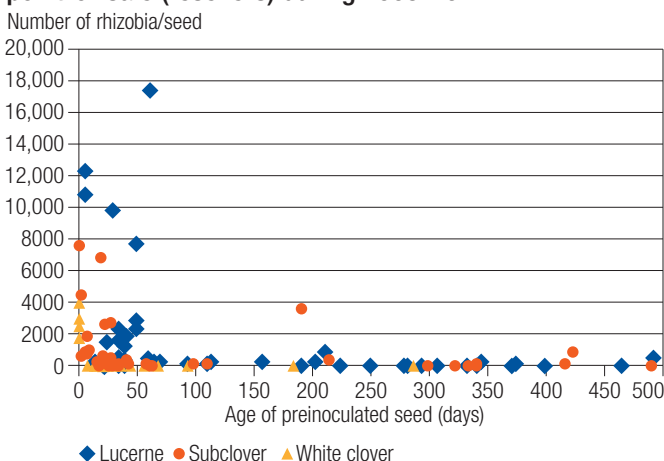
where nodulation is initiated. Another potentially beneficial microbially-mediated effect is the increased availability of nutrients by solubilisation of phosphorus and sulfur and chelation of iron.

Other microorganisms have been identified for their ability to protect plants against pests and diseases. This is either by direct antagonism of the pest or disease agent or by increasing plant resistance to attack.

While the evidence for beneficial effects on plants can be demonstrated in laboratory studies, results from field application are highly variable. Little is known about environmental conditions, specificity between selected microorganisms and plant host, or numerical requirements to achieve a beneficial effect. As a result, no standards have been set for these microbial inoculants and they are not subject to quality control. However, a system is being developed to extend the trademark system to allow product differentiation on the basis of confirming manufacturers' claims of microbial identity and quantity.

As the market for microbial inoculants is not regulated in Australia, products are not restricted from sale and consumers should be aware that quality and efficacy may be variable. In the meantime, research is continuing to find more about these inoculants and how their potential may be realised.

4.12 Concluding comments

The whole question of legume inoculants and their use starts with quality. If the quality is poor, then benefits from inoculation are highly unlikely. Successful production and use of legume inoculants is often associated with an effective, regulatory quality control (QC) program that primarily focuses on the quality of the rhizobial strains in the inoculants and their numbers as well as the numbers of contaminating microorganisms. The regulatory QC may be supported by appropriate legislation (e.g. Canada, Uruguay, France) or may be voluntary on the part of the inoculant manufacturers (e.g. Thailand, New Zealand, South Africa). In other countries, such as the US, regulatory control and independent testing has been considered unnecessary, with manufacturers conducting their own internal QC.

In Australia, we are fortunate that in the early 1950s Professor Jim Vincent had the presence of mind to recognise the harmful implications of poor-quality inoculants at the farm level and to set up an independent laboratory, jointly financed by the University of Sydney and NSW Department of Agriculture, to conduct quality assessment. Additionally, the laboratory acted as a resource to assist the industry to continually improve inoculants. Now, 60 years later, the system with its clearly-stated framework has survived essentially unchanged and has become the model that other countries follow.

We readily admit that the problems remain that have plagued the industry through those 60 years, such as genetic instability of inoculant strains, peat toxicities, poor survival of some strains in peat and, particularly, on preinoculated seed. Vigilance in detecting those problems through the ongoing testing program and diligence in addressing them has meant that Australian growers are now

generally supplied with high-quality product. The new Code of Practice incorporating the Green Tick Logo program should provide further support for the quest for quality inoculants.

5 INOCULATION IN PRACTICE

- Inoculation is relatively inexpensive and good insurance – always inoculate with AIRG-approved* inoculants.

- Match the correct inoculant group to each legume.

- Inoculants carry live root nodule bacteria (rhizobia), which die from exposure to sunlight, high temperatures, chemicals and freezing temperatures.

- Always use inoculants before their use-by-date has expired.

- Keep inoculants dry and cool, and reseal opened bags of inoculant. Use the resealed bags within a short time.

- Follow instructions on recommended rates of inoculation. Rates are either determined by the weight of seed (kilogram per tonne of seed) or by area (kilogram per hectare).

- Always sow freshly inoculated seed as soon as possible.

- When applying liquid or slurry inoculants, use clean, potable water and ensure the holding tank is free of toxic chemical residues.

- Do not add zinc or sodium molybdate to liquid or slurry inoculants.

- Check the product label or contact the manufacturer for compatibility of inoculants with fertilisers and seed dressings.

- Ensure inoculants remain cool in transport and do not leave inoculants or inoculated seed in the sun.

*AIRG is the Australian Inoculants Research Group, part of the NSW Department of Primary Industries.

5.1 Introduction

Inoculation is the application of root nodule bacteria (rhizobia) to a legume seed or soil in which the legume is sown. It is done to facilitate root nodulation. Improving the nodulation of a legume can increase symbiotic nitrogen fixation, crop biomass and grain yield and quality, and increase the amount of organic nitrogen contributed to the soil from legume shoot and root residues (Figures 5.1 and 5.2).

Some precautions need to be taken to ensure delivery of large numbers of rhizobia to the vicinity of the legume roots. Whichever inoculant is used, rhizobia are living organisms and their growth and survival can be reduced by coming into contact with chemicals and fertilisers, heat or freezing temperatures, sunlight, desiccation, and acidic (low pH) and highly alkaline (high pH) soil (see Chapter).

5.2 When is inoculation required?

When sowing legumes inoculation should always be considered due to the potential to increase nitrogen fixation and grain yield. The circumstances under which inoculation of specific legumes is required are covered in Chapter 7.

Important reasons to undertake inoculation include:

- the particular legume has not been grown in the paddock previously;
- it has been more than four years since that particular legume has been grown in the paddock;
- introduced newly selected strains with increased effectiveness and survival;
- the presence of acidic or highly alkaline soils in the paddock may limit survival of the rhizobia in the soil;
- the paddock is subjected to particularly hot, dry summers; and
- the legume has specific rhizobial requirements, e.g. lotus, biserrula, sulla.

FIGURE 5.1 Aerial biomass index image of chickpea plots 12 weeks after sowing, indicating plots inoculated with Rhizobium '+', and those that are uninoculated '-'. Blue is indicative of higher biomass, yellow of low biomass and red of bare earth.
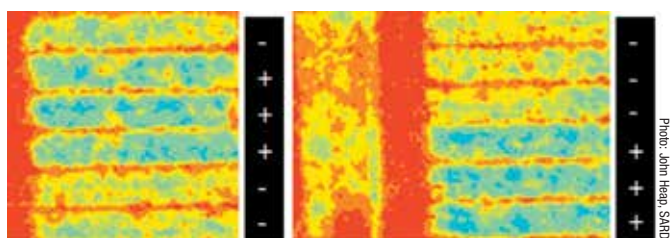
Photo: John Heap, SARDI

FIGURE 5.2 The clearly beneficial effects of inoculation on the growth of serradella. Plants inoculated with effective strains of rhizobia are green and well-grown. Plants inoculated with an ineffective strain are pale and unthrifty while the uninoculated plants have, to a large extent, died.
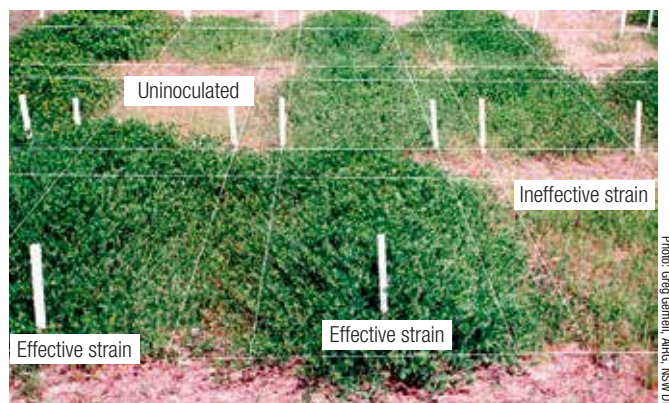
5.3 Which inoculant group should I use?

Crop and pasture legumes must be inoculated with the correct rhizobial strain for nodulation and nitrogen fixation. For example, chickpeas and field peas each require different inoculant rhizobia and will not nodulate unless the correct inoculant is used (see Table 5.1 and Chapter 7).

5.4 Which inoculant group do I need for a mixture of pasture species?

When using mixtures of different pasture legume species, each should be inoculated separately with the correct inoculant group. Once seed of each legume has been inoculated and dried off, the pasture species can be mixed together in the appropriate proportions for sowing.

5.5 What are the requirements for storing and handling inoculants?

For storage and transport of inoculants:

- always follow the manufacturer's instructions;

- keep inoculants in a cool, dry area (ideally below 10°C), except for a few inoculants for tropical/subtropical legumes, which should be stored at 20 to 25°C;

- do not freeze inoculants;

- minimise exposure to direct sunlight;

- store freeze-dried inoculants in the fridge, NOT in the freezer;

- use inoculants before their use-by-date.

- never expose inoculants to high temperatures, e.g. in a vehicle. Use an insulated box to keep them cool; and

- reseal inoculant packages after opening to reduce moisture loss and avoid contamination.

5.6 Can you use too much inoculant?

Inoculation of legumes at higher-than-recommended rates is not harmful to legume growth or production. Ensure blockages of equipment do not occur. Fewer problems result from liberal inoculation than from using inoculants at lower-than-recommended rates or not using inoculants

TABLE 5.1 Inoculant groups for some common legume species and the maximum amount of seed that should be treated by a 250 gram bag of inoculant.

Inoculant group	Common name of legume	Seed size	Maximum weight of seed treated by 250g inoculant
AL	Lucerne, strand medic, melilotus, disc medic	Small	25kg
AM	Burr medic, barrel medic, snail medic, sphere medic, murex medic	Medium	50kg
B	White clover, red clover, strawberry clover, alsike clover, berseem clover, ball clover, suckling clover, talish clover	Small	25kg
C	Subterranean clover, balansa clover, crimson clover, purple clover, arrowleaf clover, rose clover, gland clover, helmet clover, Persian clover, bladder clover	Small–medium	25–50kg
E	Field pea, vetch, narbon bean, lathyrus	Large	100kg
F	Faba bean, lentil	Medium–large	50–100kg
G	Lupin	Large	100kg
H	Soybean	Large	100kg
I	Cowpea, mungbean (green and black)	Large	100kg
J	Pigeon pea, lablab, horse gram	Large	100kg
N	Chickpea	Large	100kg
P	Peanut	Large	100kg
S	French and yellow serradella	Medium	50kg
Biserrula	Biserrula	Small	10kg
Sulla	Sulla	Medium	10kg

FIGURE 5.3 Rhizobial numbers on seed at sowing and their effect.

Plants nodulated (%) Grain yield (t/ha) narrow-leafed lupin

— Plants nodulated (%) — Grain yield (narrow-leafed lupin)

Number of rhizobia per seed

SOURCE: Roughley et al. 1993
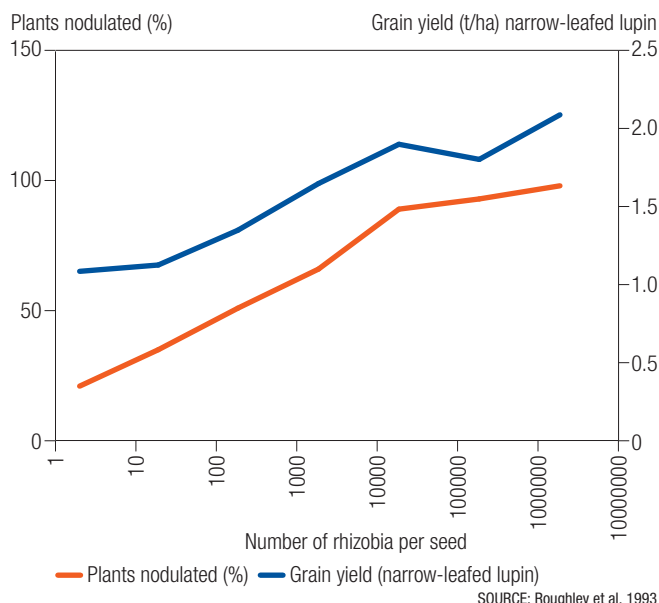
at all. Unnecessary inoculation represents a small cost to production, whereas poorly nodulated and N-deficient crops will cause a substantial reduction of production and profit.

5.7 How are numbers of inoculant rhizobia related to legume nodulation and yield?

Large numbers of rhizobia inoculated onto seed increase nodulation and grain yields (Figure 5.3). For pulses and grain legumes, inoculants usually contain enough rhizobia to deliver around 10^{10}–10^{11} (ten to one hundred billion) rhizobia per hectare (see Chapter 4). The recommendation for rhizobial numbers on seed at sowing when inoculated by peat slurry inoculants are 100,000 rhizobia per large seed (chickpeas, lupins) and 10,000 for smaller seeds (mungbeans, lentils). For preinoculated pasture legume seeds, the recommendations are 1000 rhizobia per medium-sized seed, such subterranean and lucerne, and 500 rhizobia per small seed, such as white clover.

5.8 Which formulation of legume inoculant should I use?

A range of different inoculant formulations are available to Australian legume growers (Table 5.2).

In selecting an inoculant formulation, consider the following characteristics:

- All inoculants are expected to work well when sown into moist soils, where rhizobial survival should be optimal.
- The cost of inoculants is influenced by such factors as the cost of production, the cost of freight and rate of application. Peat inoculants are considered both the highest quality and the least expensive option.
- Soil-applied inoculants (i.e. granular and liquids applied in-furrow) allow the separation of the inoculant from potentially harmful seed applications such as fungicides, insecticides and trace elements.

- Granular and in-furrow application of liquid inoculants have increased in popularity due to their ease-of-use. Granules are particularly attractive for large sowings of pasture legumes (i.e. more than one tonne of seed). Although the application of peat slurry to seed during busy seeding times is often viewed as inconvenient, it remains the most popular form of inoculation.
- Granular inoculants contain fewer rhizobia per gram than peat and need to be applied at higher rates and cost more per hectare.
- Liquid inoculants should be used immediately after dilution. Freeze-dried inoculant should be sown within five hours after application to seed. Peat slurry inoculant should be sown within 24 hours of application to seed. Granular inoculants can be stored for up to six months after manufacture.
- Current recommendations are that to ensure rhizobial survival, inoculated legume seed should not be sown into dry soil. In particular, freeze-dried and liquid inoculants should only be applied to moist seedbeds. Note that some manufacturers do recommend application into dry soil.
- Preinoculated pasture seed is seen as very convenient but varies in quality, with the number of rhizobia on seed at the point of purchase sometimes inadequate (see Chapter 4). Preinoculated seed coatings can add significant cost to pasture seed.

5.9 Peat inoculants

Peat inoculants are cost-effective and reliable, and the most commonly used formulation. These inoculants consist of finely ground peat with a single strain of rhizobia. The rhizobia are grown by the inoculant manufacturers to high concentrations in a nutrient broth in large fermenters, and then injected into packets containing sterilised peat. The rhizobia multiply further in numbers in the peat. Packets from selected batches are independently tested by the Australian Inoculants Research Group (AIRG), and only batches that reach the stringent standards carry the Green Tick Logo (see Chapter 4). Each packet has a use-by-date, which should be adhered to.

TABLE 5.2 Inoculant formulations available to Australian growers.

Inoculant formulation	Composition
Peat	High organic matter soil, milled and irradiated, with rhizobia added in a nutrient suspension
Freeze dried	Concentrated pure cells of rhizobia following extraction of water under vacuum
Granular	Clay or peat granules impregnated with rhizobia
Liquid	Suspension of rhizobia in a protective nutrient solution
Preinoculated seed	Seed coated with polymers and peat inoculant

FIGURE 5.4 Peat inoculant is easily seen on faba beans (top left) and peas (lower left) when compared with uninoculated seeds (right).

Peat inoculants are best applied as a slurry on the seed but can be mixed with water and injected into a moist seedbed at sowing. Simply sprinkling the peat into the seed box is not recommended as this results in poor contact between the rhizobia and the seed and may lead to patchy and inconsistent nodulation.

5.9.1 Preparation, water quality and application of peat slurries to seed

The inoculant is mixed with clean water and sometimes an adhesive to form a slurry. Adhesive solutions are used to improve the contact of inoculant with seed and to protect the rhizobia from desiccation. Most peat inoculants for grain legumes already include an adhesive in the peat and only water is required to create the slurry. In contrast, peat inoculants for pasture legumes usually do not contain adhesive and the peat slurry is made using an adhesive solution prepared separately.

The use of rainwater or preferably drinking (potable) water is recommended for the preparation of all slurries.

It is important that the pH of the water is checked and is between 5.5 and 7.0 or rapid death of the rhizobia will probably result. It is critical to avoid toxic chemicals and residues particularly if the water is sourced from bore water or a storage tank. The water must not contain high levels of dissolved salts, spray rig washings containing pesticides or detergents, or swimming pool water that may be chlorinated.

5.9.2 Preparation of adhesive solution for pasture legumes

Adhesive solutions or 'stickers' such as Seedstik™ are often used where the seed is to be lime pelleted.
To prepare one litre of Seedstik™ adhesive solution:

- for a solution of 20 per cent, sprinkle 200 grams of the granulated powder into 200 millilitres of hot (~80°C) water, stirring vigorously until the powder is dispersed;

- slowly add 800mL of cold water while still stirring vigorously, until an even gel is produced;

- sticker is best prepared the day before inoculation. Sticker should be used within three days; and

- periodically stir the solution until fully dissolved. Cool the solution to less than 30°C before use. Thoroughly stir the solution prior to use. Combine peat inoculant and sticker together for immediate application to seed.

Less concentrated adhesive solutions (refer to the manufacturer's instructions) may be used when seed is not lime pelleted. Many other adhesives have been used to apply rhizobia to seed, however, not all adhesives are compatible or protective of rhizobia (Deaker et al. 2004; Deaker et al. 2007; Hartley et al. 2012). It is important that adhesives be used that are recommended for use with legume inoculants.

5.9.3 Application of the slurry to seed

The slurry is mixed with the seeds using a concrete mixer, shovelling on a cement floor, or by using a rotary coater, on-the-go applicator or auger to provide even coverage of the seed (Figure 5.4). Slurry inoculant can be applied to the seed during various pre-seeding transfers including augering of seed from a silo to truck, or truck to seeder. Care must be taken to avoid crushing or cracking the seedcoat. Slurry must be applied in a calibrated flow to ensure consistent distribution across the seed lot.

Inoculated seed should be sown as soon as possible, ideally on the same day as inoculation. For grain legume inoculants already containing adhesive, a 2.5kg packet when mixed with water will provide sufficient rhizobia for 1000kg of a larger seeded grain legume e.g. lupin or 500kg of a medium size grain legume e.g. lentils (see manufacturer's instructions on packet label for exact amounts of seed and water).

5.9.4 Field inoculation

Peat is made up into a slurry as per manufacturer directions in a clean drum and mixed well (Figure 5.5A). The slurry is ideally pumped rather than poured from the container (Figure 5.5B) into the path of seed going up the slow moving, flighted auger (Figure 5.5C). Inoculated seed is augered into the grain/grouper bins and transported to the planter/airseeder in the paddock (Figure 5.5D). Freeze-dried inoculum can be applied to seed in the same way as peat slurry and as per the manufacturer's instruction. Inoculant rates on seed are given on inoculant packets and should be applied to the correct weight of seed. Volumes needed may vary according to pumping rates and auger speeds. If seed is transferred with a tabulator or conveyer auger, a mixing ladder will be needed to enhance inoculant distribution on the seed.

5.9.5 Lime pelleting of pasture legumes

Pasture seed is often coated with fine lime immediately after the application of the peat slurry to help dry the seed and to prevent clumping (Figures 5.6 and 5.7). Liming also protects rhizobia against acid soils and acidic fertilisers, such as superphosphate.

Lime pelleting may improve survival of the rhizobia when delays between inoculation and sowing are unavoidable. It also reduces the clumping of seed from the slurry mix and forms a seed pellet favourable for easy flow in the sowing process. However, lime pelleting is not required when sowing podded seed such as serradella or soft-seeded sulla as the seed pod absorbs the slurry and does not affect flowability.

Grain legumes are not lime pelleted. Similarly, tropical pastures legumes (except *Leucaena leucocephala*) should not be lime pelleted because it has been reported to kill the applied rhizobia. Most temperate pasture legume seeds, i.e. those grown in the southern and western grain regions,

FIGURE 5.5 Peat inoculant made into a slurry in a drum (A); slurry pumped out of the container (B); slurry pumped from the container into the path of the seed going up the auger (C); and inoculated seed is augered into the bins and transported to the planter/airseeder (D).

should be lime pelleted using fine lime (calcium carbonate) following inoculation with the peat slurry and adhesive. Slaked, hydrated lime and builder's lime are too alkaline and will kill the rhizobia and should not be used. Keep in mind that the pellet can increase the weight of the seed substantially, so that sowing rates may need to be adjusted.

To lime pellet pasture seed:
- pour the mixture of peat slurry and sticker over the seed and mix in a rotating drum (concrete mixer) until seeds are evenly coated;
- immediately add the appropriate amount of very fine lime (such as Seed Cote™, Microfine® or Omyacarb®) in one step to the rotating seed, and roll for one to three minutes; and
- allow pelleted seed to dry in a cool place out of direct sunlight.

PLEASE NOTE: Preparation of a small trial batch is always recommended, particularly if the process is being undertaken for the first time.

Good quality pelleted seed is:
- evenly coated with the lime (see Figure 5.8); and
- firm enough when dry to withstand a light rolling between the fingers, without the lime flaking off.

Poor quality pelleted seed is:
- powdery, with soft pellets indicating too much lime or uneven mixing, or both;
- pasty with the seed surface showing, the result of too much adhesive. This may be rectified by adding more lime;
- clumped together, the result of too much adhesive or inadequate mixing prior to adding lime; or
- hard, glossy or smooth resulting from too little lime, or too much mixing after adding the lime.

5.9.6 Using peat inoculants for liquid injection

Inoculum, suspended in potable water, is injected into the seed furrow in a band. Peat is mixed into dilute slurry or placed into a porous bag (calico bag or fine muslin, cheesecloth or nylon stocking) before adding to the tractor-mounted water tank. Peat inoculants are finely milled products and readily disperse in water. Despite this, the use of a fine filter, such as a stocking, is encouraged to ensure that any extraneous material does not block the liquid injection system. The liquid inoculum is made by mixing the required amount of peat inoculant, for a specific amount of seed, into water. For example, if one large (1.2 kg) packet of peat inoculates 500kg of seed then at a seeding rate of 100kg/ha the liquid (300–500L) should be injected over 5ha.

FIGURE 5.7 Subterranean clover uninoculated (left) and inoculated and lime pelleted (right).

FIGURE 5.8 Three different batches of lime pelleted clover seed inoculated with a group C slurry mix. The seeds on the left display insufficient lime or uneven mixing, the seeds on the right (clumpy) show too much sticker. The seeds in the centre indicate an even amount of mixing and adequate lime addition.

For more details on applying liquid inoculants see Sections 5.11 and 5.12.

5.10 Freeze-dried inoculants

Inoculants containing freeze-dried rhizobia are available as powders in 30g glass vials (Figure 5.9). They become active when the powder is reconstituted with liquid. The product comes with a protective polymer in a separate bottle, which assists survival of the rhizobia. A vial will treat between 25 and 500kg of seed, depending on the legume species. These products allow for liquid injection of inoculants into the seeding furrow or seed can be coated immediately prior to sowing. Treated seeds need to be sown into moist soil within five hours of application. Contact with pesticides and fungicides must be avoided. Do not freeze this product.

5.10.1 How do I apply freeze-dried inoculant?

Remove cap and rubber bung from the glass vial, add potable water, replace bung and shake until all powder is dissolved. For liquid injection into the seeding furrow, add the vial of inoculant solution to 2L of cool water containing the protective polymer, supplied by manufacturer of the freeze-dried product. Add this solution to the spray tank and deliver 50 to 100L of clean water per hectare into the furrow during sowing. It is important to ensure that the protective agent is added to the tank mix, prior to the addition of the freeze-dried rhizobia.

To coat seed, add dissolved solution from the vial into 2.5L of water (containing protective polymer). Apply to the seed until evenly coated and allow to dry before sowing.

5.11 Liquid inoculants

Liquid inoculants should only be used where the seedbed is moist. Liquid injection of inoculant into furrows is increasing in practice, due to the relative ease of applying liquid inoculants to broad acre crops. It is very important that the tanks on spray rigs and seeders be thoroughly clean of residues, which can be toxic to rhizobia. The concentrated inoculant should be diluted with good-quality, clean water of neutral pH before application. Diluted inoculant should be delivered to the sowing furrows at rates of 50 to 100L/ha. Inject liquid inoculant immediately or within six hours.

FIGURE 5.9 Concentrated rhizobia in a freeze-dried formulation which can be applied to legume seed or to injected into the soil at sowing.

DO NOT MIX PEAT, FREEZE DRIED AND LIQUID INOCULANTS WITH:

- chemicals containing high levels of zinc, copper or mercury;
- fertilisers and seed dressings containing sodium molybdate, zinc, manganese and molybdenum;
- fungicides such as Sumisclex® or Rovral®
- insecticides containing endosulfan, dimethoate, omethoate, or carbofuran.

5.12 Applying inoculants by water injection

Water injection methods can use peat, freeze-dried or liquid forms of inoculum. The inoculants are diluted with water in tanks mounted on tractors (Figure 5.10) and applied through spray lines attached behind each planting tyne/boot (Figure 5.11). Agitators and in-line filters may be necessary, particularly for peat-based inoculum. Rates of inoculum need to be calculated for planting rates (kilograms of seed per hectare) and water volumes able to be carried. Typically application rates are 50 to 100L/ha.

5.13 Granular inoculants

Granular inoculants can simplify the delivery of rhizobia to the legume. For most granular inoculants, a third seeding box is required as mixing with seed or fertiliser is not recommended. The technology is an alternative to the standard peat slurry on seed and can provide greater flexibility and practical solutions in sowing operations. The physical separation of rhizobia from the seed also allows insecticides and fungicides to be applied to the seed, which may otherwise kill the rhizobia.

5.13.1 Types of granules

Granular inoculants can be manufactured from prilled peat, clay (bentonite or attapulgite) or a mixture of peat and clay and vary in appearance and characteristics such as particle size and uniformity of particle size (Figure 5.12). Granules should be stored in a dry, cool area away from direct sunlight. Clay-based granules can be stored for up to six months after manufacture without refrigeration.

Peat-based granules should be sown with the seed into moist soil. Clay-based granules have been promoted as being more reliable when dry sown. However, it is important to note that dry sowing may reduce nodulation and that the outcome may vary with soil moisture, soil temperature and the time between inoculation and crop emergence.

GRANULAR INOCULANTS

- Granules should be drilled into the furrow with the seed to ensure rhizobia are placed in close proximity to the emerging legume root.

- Preferably granules should be applied from a third box separated from seed and fertiliser.

- Granules can be added to the seed box; however, differences in particle size may lead to settling and uneven delivery of inoculant and seed.

FIGURE 5.11 Spray lines attached behind each planting tyne/boot dispense inoculants by water injection.

A common feature of granular inoculants is that they have fewer rhizobia per gram than the peats used for slurry inoculation. They must be applied at higher rates to achieve similar levels of nodulation. Granules are typically applied at 5 to 10kg/ha when sowing on 18cm row spacings, depending on manufacturer, the strain and number of rhizobia per gram of product. Lower rates of attapulgite and peat granules can be used with wide row spacings according to manufacturers' guidelines e.g. if row spacings are doubled, the application of inoculant can be halved (Table 5.3). However, bentonite clay granules are recommended to be sown at a rate of 8 to 10kg/ha no matter what row spacing is used at sowing. When sowing mixtures of pasture legumes, the full rate of granular inoculant per hectare for each pasture inoculant group must be used.

Granular products differ in their ability to be mixed with seed or fertiliser, and manufacturers' recommendations should always be followed. In general, excessive auguring should be avoided to ensure that the particle size is maintained and to minimise dust. Granules are best distributed through a third sowing box, rather than mixed with seed because differences in granule and seed size may result in separation or settling and uneven distribution of both granules and seed.

Contact of granular inoculants with moisture during seeding operations should be avoided and they should not be stored in the seeder boxes overnight because some products can absorb moisture, stick together and cause blockages in seeding equipment.

5.14 Preinoculated and custom-inoculated seed

Some seed companies sell pasture seeds that contain rhizobia as part of a specialised seed coating process. The coating may include insecticides, fungicides and micronutrients. This has provided more flexibility with problems such as sowing delays. It is advisable to sow as soon as possible after the seed coating treatment.

The main use of preinoculated seed is for pasture species, particularly lucerne and annual medics, because the rhizobia for these species survive well in this form.

FIGURE 5.12 Examples of attapulgite clay granules (left), peat granules (middle) and bentonite clay granules (right) used to deliver rhizobia to grain and pasture legumes.

PREINOCULATED SEED

If purchasing preinoculated seed for clovers, serradella, biserrula and sulla, ensure the seed has been freshly coated, as rhizobial numbers can reduce significantly within days for these species.

Testing of preinoculated seed samples collected from retail outlets has indicated that many samples did not meet the AIRG standard for numbers of rhizobia on the seed (see Chapter 4).

5.15 Are there compatibility issues between seed-applied inoculants and fertilisers, chemicals and pesticides?

As rhizobia are living organisms, it is very important that inoculants are kept away from toxic substances that will reduce their viability, such as fertilisers, fungicides, insecticides and herbicides. Inoculated seed should not come in direct contact with fertiliser because it will kill the rhizobia through desiccation and exposure to acidity. Certain pesticides can also have an impact on rhizobial survival and nodulation.

There are three major factors to be considered:

- **Are the chemicals acidic in solution?** Most rhizobia are sensitive to solutions with pH values below 5.0 or above 7.5.

TABLE 5.3 The influence of row spacing on application rates for the three different types of granular inoculants.

Row spacing (cm)	Attapulgite clay granule rate (kg/ha)	Peat granule rate (kg/ha)	Bentonite clay granule rate (kg/ha)
18	6.0	5.6	8–10
20	5.3	4.9	8–10
23	4.6	4.4	8–10
25	4.2	3.9	8–10
28	3.8	3.6	8–10
31	3.5	3.3	8–10
33	3.2	3.0	8–10
36	3.0	2.8	8–10
38	2.8	2.6	8–10

- **Do the preparations contain toxic chemicals?** Metals such as mercury, copper and zinc are harmful. Effects of other active ingredients may be difficult to predict.
- **Is there prolonged direct contact between the substance and inoculated seed?** Direct contact between the inoculated seed and other substances should be avoided at all times. If contact is made, and for only a short period the effect may be reduced.

5.15.1 Fertiliser compatibility

Superphosphate and related products are acidic and toxic to rhizobia when in direct contact, and contact between seed and fertiliser should be avoided even if the seed has been lime pelleted.

Inoculated seed should not be sown or be in contact with any fertiliser except lime, dolomite or gypsum. If contact cannot be avoided, lime pellet the seed first and do not store it mixed in with the fertiliser — sow immediately.

5.15.2 Adding molybdenum at inoculation

Low molybdenum (Mo) in the soil can cause a reduction in the nodulation and nitrogen fixation of a legume crop, particularly in soils with a low pH (<6.0). Adding Mo to seed is more cost-effective and ensures even distribution of Mo in the paddock. However, sodium molybdate is toxic to rhizobia and should not be applied to inoculated seed. Use either molybdenum trioxide (66 per cent Mo) or ammonium molybdate (54 per cent Mo) for seed application.

When sowing pasture legumes in molybdenum-deficient soils, 50g of Mo is required per hectare, equivalent to that supplied in 250kg/ha 0.02% Mo superphosphate. Then, every four to five years, 25g of Mo per hectare should be applied as a maintenance dressing (e.g. 125kg/ha of 0.02% Mo superphosphate). In some areas, responses to larger quantities of molybdenum have occurred. Check local recommendations.

5.15.3 Fungicide compatibility

Seed-applied fungicides are marketed specifically for the purpose of killing or inhibiting the growth of disease-causing fungi and are considered preventative. Seed-applied fungicides (sometimes called pickles) can reduce the survival of rhizobia on seed. Table 5.4 indicates the compatibility of rhizobia with various seed-applied fungicides. Note that rhizobial survival is dependent on the period of time that the inoculant is in contact with the seed-applied fungicides prior to sowing. Recent tests have shown that Metalaxyl and Metalaxyl-M, when applied to inoculated seed, contribute to a reduction in the number of rhizobia, and therefore should be used strictly in accordance with the manufacturer's instructions.

5.15.4 Insecticide compatibility

- Bendiocarb and permethrin, used to protect seed from ants, are safe (although limited trials indicate that there may be some reduction in nodulation).
- Imidacloprid is safe to use with rhizobia provided treated seed is sown into moist soil within one day of treatment for subterranean clover and murex medic, or within six

days of treatment for other species such as white clover, serradella, lucerne and barrel medic.
- Dimethoate can harm rhizobia.
- Follow label instructions carefully.

5.15.5 Herbicide compatibility

Rhizobia are relatively tolerant of herbicide concentrations recommended for field use. Because of the differences in susceptibility between the host and their rhizobia, it is difficult to make accurate assessments of the general impact of herbicides and additives on all legumes and rhizobia, and the specific impacts on plant growth, nodulation and nitrogen fixation. However, recommendations have been made that rhizobia are killed by the herbicides MCPA and 2,4-D. Recently it has also become evident that application of residual sulfonylurea-based chemicals are affecting the production of pasture legumes. Research is ongoing to clarify our understanding of these interactions.

5.16 Dry sowing of inoculated legume seed

Dry sowing of inoculated seed is not recommended where the legume is being sown in the paddock for the first time or where soil conditions are hostile to survival of the rhizobia. In paddocks with frequent use of the same legume and where effective nodulation was recently observed, the risk of nodulation failure resulting from dry sowing is greatly reduced.

5.17 Formulations of inoculants containing co-inoculants

With co-inoculants, an additional microorganism is applied with the rhizobia. A range of co-inoculants have recently been introduced to the market. Some have had the extra microorganism added during manufacture of the peat or granular inoculant, but sometimes the extra microorganism is supplied separately. These organisms include strains of

Bacillus subtilis and *Penicillium bilaii*, added in addition to a rhizobial inoculant. The mode of action varies according to the particular microbe co-inoculated with the rhizobia; these co-inoculants are marketed as increasing root growth, nodulation, phosphorus uptake, or reduce the incidence of pathogens affecting root growth. Advice from the individual manufacturer should be sought.

5.18 Concluding comments

This chapter has highlighted the **dos** and **don'ts** when inoculating legume seed to achieve effective root nodulation. Rhizobia are living organisms and their survival can be severely reduced when this is not kept in mind. When handling inoculants remember that many things are toxic to rhizobia such as direct contact with chemicals and fertilisers, high or freezing temperatures, sunlight, desiccation, and acidic (low pH) and highly alkaline (high pH) soil.

Legumes must be inoculated with the correct rhizobia strain (inoculant group) for maximum benefit. In Australia, the inoculant rhizobia are currently available in different carriers: peat, freeze-dried powders, granules and as preinoculated seed. The shelf life of these products varies from several weeks in the case of some preinoculated seeds to three years for the freeze-dried powder. The cost of inoculation can vary depending on the product. Peat is the cheapest form of inoculant to purchase but there are additional application costs in time and labour to consider. The more expensive options can be easier to use and offer flexibility at sowing.

Although inoculation of legumes can be perceived as a difficult exercise, by following some simple instructions and precautions you can ensure delivery of large numbers of the commercial inoculum to the target legume roots. Successful inoculation should improve nodulation, resulting in increased symbiotic nitrogen fixation and yield of the legume, and ultimately produce more yield and higher grain quality in the following non-leguminous crops.

TABLE 5.4 Compatibility of different rhizobia groups with seed-applied fungicides and insecticides. Information sourced from commercial product information guides (BASF and Novozymes)

Inoculant group / crop	Fungicide type	Planting window of inoculated seed
E – pea, vetch	P-Pickel T	6 hours
	Gaucho® 600 FL	4 hours
F – faba bean, lentil	Gaucho® 600 FL	24 hours
	P-Pickel T	24 hours
	Thiram	Compatibility not known
G – lupin	Rovral	6 hours
	Thiram	24 hours
H – soybean	not compatible with seed dressings	
N – chickpea	P-Pickel T	6 hours
	Thiram	6 hours
	Apron® XL 350 6 hours	6 hours
	Gaucho® 600 FL	6 hours
P – peanut	not compatible with seed dressings	

Well-nodulated faba bean growing near Moree in northern New South Wales. In this region, farmers often apply the peat-based inoculant in liquid slurry form to the faba bean seed during various pre-seeding transfers, including augering of seed from silo to truck or truck to seeder.

High-yielding inoculated soybean ready for harvest, grown in rotation with sugarcane in north-coastal New South Wales. Growers inoculate soybeans using mainly peat and liquid inoculant formulations and are looking to capture the benefits of the soybean by reducing amounts of fertiliser N applied to the subsequent cane.

PHOTOS: DAVID HERRIDGE

6 LEGUME NITROGEN FIXATION AND ROTATIONAL BENEFITS

- Legume–rhizobia symbioses fix approximately 2.7 million tonnes nitrogen (N) annually in Australian agricultural systems, with a nominal value of about $4 billion.

- At the paddock scale legumes fix, on average, about 110 kilograms of N per hectare annually. The range is large, from close to zero to more than 400kg N/ha.

- The amount of nitrogen fixed increases as potential legume dry matter yield (biomass) increases, but is reduced by high levels of soil nitrate.

- The actual amount of nitrogen fixed in any one paddock varies with the species of legume, site and season and the applied agronomic management.

- The fixed nitrogen is used by the legume itself for growth.

- The legume residues left in the soil after the grain is harvested or the grazed/cut pasture legume phase is terminated represent, upon decomposition, a potent source of plant-available nitrogen for subsequent cereal and oilseed crops.

- Cereals grown after legumes generally out-yield cereals grown after non-leguminous crops. The extra yield is mostly due to the higher levels of soil nitrate following the legumes but will also include other factors such as a disease-break effect.

- Depending on the circumstances, the economic benefits of including legumes in crop production systems can be substantial.

6.1 Introduction

Grain and pasture legumes are valued components of Australian agricultural production systems. More than a century ago J.L. Thompson (1895) summarised their worth in rotations as contributing to: more economical use of manures; more economical use of nutrients in the soil; improved distribution of labour on the farm; improved weed control; improved soil conditions through the benefits of deep-rooted and air feeding crops; improved productivity of following cereal crops; improved management of plant pathogens and insects; improved management of livestock; and spread of economic risk.

Nothing much has changed. Growers still grow legumes as rotation crops because it helps to spread risk and manage weeds, pests and diseases in the production system. A number of the pulses (food legumes) are also valuable crops in their own right, attracting high prices for good-quality grain. Arguably, the major enduring value of legumes relates to their ability to form a mutually beneficial (symbiotic) association with rhizobia, a soil bacterium.

This symbiotic association starts when rhizobia infect the roots of the legume and form nodules. In the nodules, the rhizobia convert gaseous atmospheric nitrogen (N_2) into ammonia (NH_3), which is then largely used by the legume for growth. In return, the legume provides the rhizobia with nutrients, energy and habitat.

The principal beneficiary of nitrogen (N) fixation is the legume itself. It is self-sufficient in N, it can grow in essentially any soil without inputs of fertiliser N. The amount of N fixed is influenced by the type of legume, its health and yield, soil nitrate levels and a range of environmental factors. The legume also produces N-rich residues that remain in the soil after the crop is harvested (Figure 6.1). The mineral

FIGURE 6.1 Cycling of N through the legume phase of a rotation to the following cereal crop.
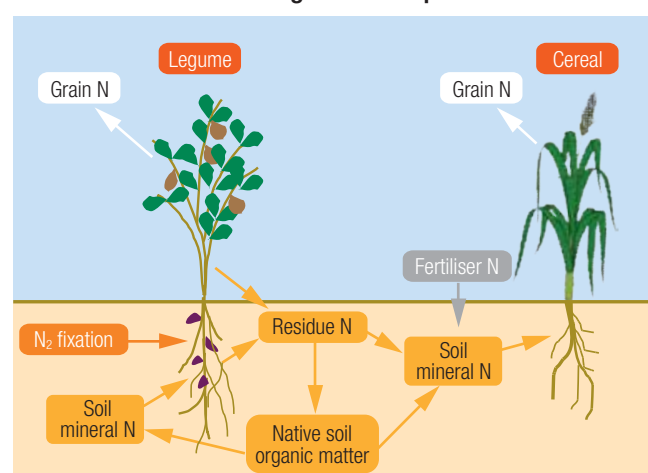
TABLE 6.1 Annual contribution of symbiotically (legume) fixed nitrogen.		
	Globally	Australia
Amount N fixed (million tonnes)	40	2.7
N fertiliser equivalent (million tonnes)	50	3.4
Economic value (billion dollars)	63	4.3

The incorporation of legumes into rotations helps reduce reliance on high-cost fertiliser N.

N released from these residues as they decompose is taken up by the following cereal (or oilseed) crop in the rotation. Legumes have a role in supplying nitrogen to the farming system following their harvest.

This chapter examines legume N fixation within global and Australian contexts, the drivers of legume N fixation and how they might be managed and, finally, the benefits of legumes and legume N in our agricultural systems.

6.2 Legume nitrogen fixation – globally and on Australian farms

Agricultural legumes fix a lot of N. Globally, there are 185 million hectares of crop legumes and more than 100 million hectares of pasture and fodder legumes and they fix about 40 million tonnes of N every year (Herridge et al. 2008). This represents a significant saving of fertiliser N that would otherwise need to be applied and has substantial positive economic and environmental consequences.

6.2.1 Economic consequences

Almost all the fixed N is available for use by the growing legume. If we compare this to an approximate 80 per cent conversion of fertiliser N into plant N, then the 40 million tonnes of biologically fixed N has a fertiliser-N equivalence of 50 million tonnes. This represents more than 50 per cent of current global inputs of nitrogenous fertilisers. The nominal annual value of this fixed N is about $60 billion, assuming a cost of fertiliser N of $1.25/kg.

The situation for Australian agriculture is equally impressive. The 23 million hectares of legume-based pastures are estimated to fix about 2.5 million tonnes of N every year. Nitrogen fixation by the crop legumes is estimated at approximately 0.2 million tonnes annually. Using the same assumptions above, the economic value of the N fixed

\by legumes in Australia's agricultural systems is close to $4 billion annually.

Table 6.1 summarises the economic contributions of nitrogen fixation by legumes in agriculture.

6.2.2 Environmental consequences

Legume nitrogen fixation is basically a solar driven process. Plants use solar energy to convert atmospheric carbon dioxide (CO_2) to carbohydrates. Some carbohydrates are transferred to the nodules, where they are used by rhizobia as an energy source.

By way of contrast, industrial N fixation, which is used to produce nitrogenous fertilisers, requires high temperatures and pressures and the expenditure of large amounts of fossil fuels. The transport and application of the N fertilisers are also energy demanding and nitrous oxide, a potent greenhouse gas, is often emitted from soils following application of nitrogenous fertiliser. All these processes result in large amounts of greenhouse gas emissions. Current estimates suggest that 10 tonnes CO_2 equivalents are emitted per tonne of N fertiliser used.

While biological nitrogen fixation will not replace the need for N fertilisers in agriculture, legume-based rotations can significantly reduce the amounts used.

6.3 Comparing nitrogen fixation by the different crop and pasture legumes

Not all legumes have the same capacity for nitrogen fixation. There are inherent differences among the commonly grown legumes. External factors, such as how much water they receive from rainfall and irrigation, also impact N fixation.

6.3.1 How much nitrogen do crop legumes fix?

Table 6.2 lists the major crop legumes grown by Australian growers. The numbers in the table are averages, derived from many studies. They provide an overview of crop legume N fixation only – the values are not representative of all paddocks sown to these crops. The amounts of N fixed by individual crops will reflect environmental and management effects.

The second column shows the percentage of crop N derived from nitrogen fixation (per cent of N fixed) for each of those crops. Clearly, navy beans have a weak capacity for N

TABLE 6.2 Estimates of the amounts of N fixed annually by crop legumes in Australia.						
Legume	% N fixed	Shoot dry matter (t/ha)	Shoot N (kg/ ha)	Root N (kg/ha)	Total crop N (kg/ ha)	Total N fixed[1] (kg/ha)
Lupin	75	5.0	125	51	176	130
Pea	66	4.8	115	47	162	105
Faba bean	65	4.3	122	50	172	110
Lentil	60	2.6	68	28	96	58
Soybean	48	10.8	250	123	373	180
Chickpea	41	5.0	85	85	170	70
Peanut	36	6.8	190	78	268	95
Mungbean	31	3.5	77	32	109	34
Navy bean	20	4.2	105	43	148	30

1 Total N fixed = per cent N fixed x total crop N; Data sourced primarily from Unkovich et al. 2010.

fixation, fixing only 20 per cent of its requirements for N. At the other end of the scale are faba beans and lupin, which both have strong capacities for N fixation.

For all crops, the remaining N requirements have to be supplied from soil and/or fertiliser sources.

> The total amount of N fixed by a legume is determined by its nitrogen fixation capacity and dry matter production.

The percentage of legume N derived from nitrogen fixation is only part of the story. The total amount of N fixed per hectare is also strongly influenced by the size of the crop (i.e. the more biomass the crop produces, the more it potentially fixes).

Crops such as soybeans, faba beans and peanuts often produce large amounts of biomass because they tend to be irrigated or are grown in high-rainfall areas. Other crops such as mungbeans and lentils are low-yielding crops often grown under water-limited conditions. Both root and shoot N contribute to the total amount of N fixed by a crop. Root N, listed in the fifth column of Table 6.2, is substantial for all crops, and in particular for chickpeas and soybeans.

The more N that is fixed by the legume, the greater the inputs of N-rich residues into the cropping system. In this context, the N contained in and associated with the roots is very important. These N inputs are the basis for the legume effect on the improvement of soil-N fertility and yields of subsequent crops. When all of these factors are taken into account, soybeans, lupin, and faba beans fix the most N on an area basis. The low estimate for navy beans reflects its low efficiency of N fixation coupled with the fact that all commercial crops are fertilised with N.

6.3.2 How much N do pasture legumes fix?

All pasture legumes have a relatively strong capacity for N fixation, as shown in Table 6.3. As with the crop legumes, a major factor affecting the amounts of N fixed by the different pasture legumes is their production of biomass. The annual clovers, for example, typically produce twice the biomass as the annual medics and fix nearly twice as much N.

The majority of legume-based pastures in Australia are dominated by subterranean clover and the annual medics and, to some degree, lucerne. Therefore, the overall value for nitrogen fixation by pasture legumes across the whole of the country would likely be approximately 110 to 120kg N/ ha annually.

6.3.3 How much N will legumes fix in my paddock?

The N fixation data for crop and pasture legumes in Tables 6.2 and 6.3 were derived from very large amounts of data across a range of sites. As stated above, these values are intended to provide a broad picture of the average amounts of N fixed by the major crop and pasture legumes in Australian agriculture.

The actual amounts of N fixed by legumes in specific paddocks will vary enormously with site, season, and

management by the grower. In the next section, we look at some of the management effects on legume nitrogen fixation.

6.4 How does crop and soil management affect legume nitrogen fixation?

The amount of N fixed by legumes essentially depends on how well the legume grows and the level of nitrate in the soil. The lower the soil nitrate and the greater the biomass produced, the greater the amount of N fixed.

In the Australian environment, legume growth is most strongly determined by the amount of water that the crop or pasture can access. Management practices can be optimised to maximise water use and provide the legume with ideal, stress-free growing conditions, including low soil nitrate.

6.5 Soil nitrate suppresses legume nitrogen fixation

Soil nitrate is a potent inhibitor of legume nodulation and nitrogen fixation. At low soil nitrate (i.e. less than 50kg N/ ha in the top metre or so of soil), the legume reliance on nitrogen fixation (% N fixed) is generally high. As soil nitrate increases, legume nodulation and nitrogen fixation become more and more suppressed. Eventually, at very high soil

TABLE 6.3 Estimates of the amount of N fixed annually by the pasture legumes in Australia.

Legume	%N fixed	Shoot DM (t/ha)	Total crop N (kg/ha)	Total N fixed (kg/ha)
Annual clovers	60	5.8	234	140
Subterranean clover	81	2.8	150	120
Annual medics	74	2.6	110	80
Perennial clovers	72	4.0	180	130
Lucerne	60	4.4	298	180

Values for annual shoot dry matter (DM) production are taken from Unkovich et al. 2010, and are aggregated from 240 individual values. Note that vetch is not included.

FIGURE 6.2 Impact of soil nitrate on chickpea nitrogen fixation in northern NSW.

Chickpea N_2 fixation (kg N/ha)
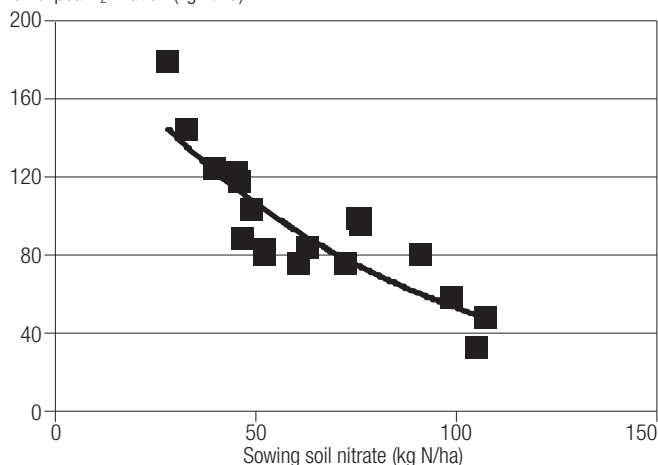
SOURCE: Unpublished data of WL Felton, H Marcellos, DF Herridge, GD Schwenke and MB Peoples

nitrate (more than 200kg N/ha), nodulation and nitrogen fixation will be close to zero. Figure 6.2 illustrates that the impact of soil nitrate on chickpea crops in northern NSW, where nitrate levels greater than 40kg N/ha had a suppressive effect on nitrogen fixation.

The actual amount of soil nitrate that will inhibit legume nodulation and nitrogen fixation in a specific paddock will vary with the legume species and environmental conditions. Nitrogen fixation of faba beans, for example, is far less prone to the suppressive effects of soil nitrate, compared with crops such as chickpeas and field peas.

Aggressive cultivation, heavy use of nitrogenous fertilisers and long pre-crop fallows all increase soil nitrate levels.

> Low soil nitrate leads to greater N_2 fixation activity.

6.6 What are the best management practices to improve legume growth and nitrogen fixation?

Apart from inoculating the legume seed with the appropriate rhizobia (see Chapter 5), optimising basic agronomy (best management practice) is the key to legume productivity and therefore N fixation. This means maintaining a good cover of

TABLE 6.4 Effects of tillage on soil water and nitrate at sowing, and on chickpea growth, grain yield and nitrogen fixation.

	No till	Cultivated
Sowing soil water (mm)	144	109
Sowing soil nitrate (kg N/ha)	71	86
Shoot dry matter (t/ha)	5.4	4.7
Grain yield (t/ha)	2.01	1.83
% N fixed	55	44
Crop N fixed (kg/ha)	107	75

Data are the means of 21 site/years of experiments
SOURCE: Unpublished data of WL Felton, H Marcellos, DF Herridge, GD Schwenke and MB Peoples
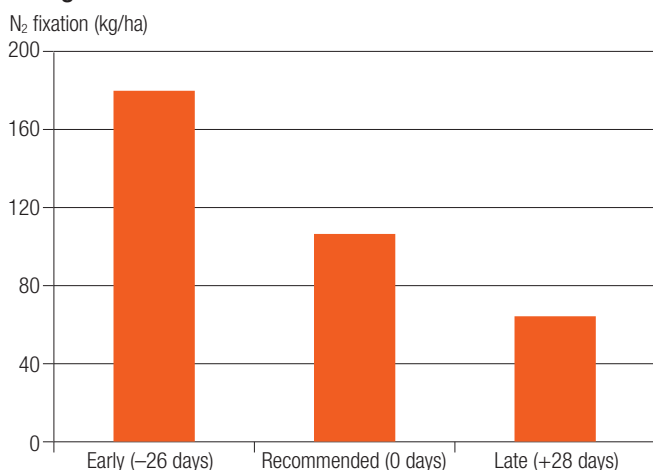
stubble on the soil surface in the pre-crop fallow, sowing on time and establishing the appropriate plant density. It also means optimising nutrient inputs, reducing acidity with lime, and managing weeds, disease and insects.

6.6.1 Tillage practices
One management option for cropping that has gained popularity in recent years is no-tillage. No-tillage may lead to increased soil water and decreased soil nitrate accumulation during the pre-crop fallow and in-crop.

Studies in northern NSW show a positive effect of no-tillage on productivity and nitrogen fixation of chickpeas (Table 6.4). No-till plots had more soil water at sowing and less nitrate-N than the cultivated soils. As a result, chickpea biomass, grain yields and nitrogen fixation increased.

However, under no-tillage non-legume crops (e.g. cereals, oilseeds) additional fertiliser N may be required to supplement the reduced soil nitrate.

6.6.2 Sowing practices
The N fixation potential of legumes may be maximised by sowing on time and at the appropriate density.

Sowing on time takes full advantage of growing-season rainfall and temperatures. Studies with field peas in Victoria and southern NSW showed that N fixation increased from 64kg N/ha to 180kg N/ha by planting earlier (Figure 6.3).

The use of narrow row spacing and/or high plant density can improve N fixation. Increasing lucerne density from 5 to 40 plants/m² more than doubled crop biomass and nitrogen fixation in lucerne-based pastures in south-eastern Australia (Figure 6.4). Scientists in northern NSW also found that N fixation of faba beans and chickpeas increased with higher plant densities (Schwenke et al. 1998).

6.6.3 General soil conditions
Soil acidity and phosphorus (P) deficiency are common constraints to legume N fixation. Soils that are very acidic or very alkaline may result in reduced N fixation.

FIGURE 6.3 The early-sown pea had the highest rates of nitrogen fixation.

N₂ fixation (kg/ha)

SOURCE: O'Connor et al. 1993

FIGURE 6.4 Increasing legume density increases nitrogen fixation.

N₂ fixation (kg/ha)

Lucerne density (plants/m²)
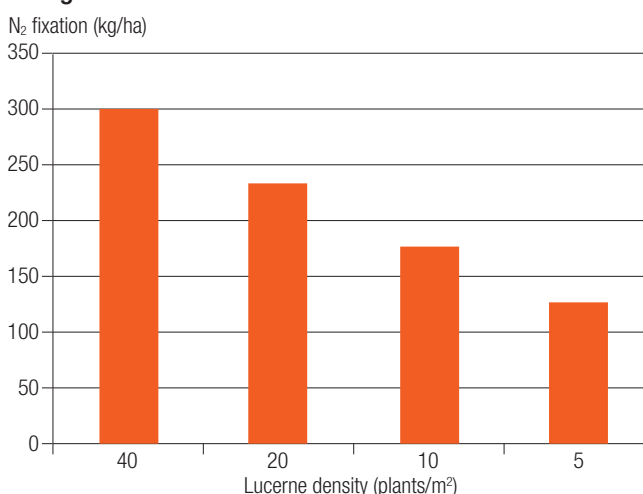
SOURCE: Peoples et al. 1998

In a three-year study of subterranean clover pastures in south-eastern Australia, applying lime and P fertiliser increased total yields and N fixation. The two amendments together were more effective than either one alone, resulting in average N fixation increases of 100 per cent (Figure 6.5).

Increasing soil pH with lime application decreases the availability of aluminium and manganese. At elevated concentrations, both of these metals are toxic to legume roots and rhizobia (Peoples et al. 1995).

Not all plant species react similarly. Different species, and even different cultivars, of legumes may have different tolerances to soil conditions. A legume that fixes a lot of N under one set of conditions may not perform as well under another set of conditions.

For example, a study in southern Australia showed that lupin fixed approximately 80 per cent more N than field peas in acidic soils. However, in alkaline soils the field peas fixed more N. The results were due to changes in both crop biomass and N fixation rates (Evans et al. 1989).

Other soil constraints to N fixation include salinity, sodicity, and nutrient toxicities and deficiencies. Such constraints must be addressed if potential legume biomass production is to be realised.

FIGURE 6.5 **Optimising plant nutrition increases nitrogen fixation.**
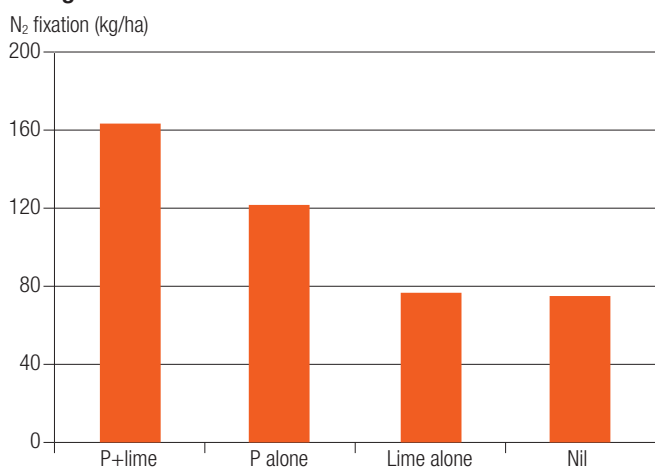
N$_2$ fixation (kg/ha)

SOURCE: Peoples et al. 1998

Research has also established that nitrogen-fixing legumes may have additional nutritional requirements, compared with plants that do not fix N. For example, nodulated legumes have higher requirements for calcium, boron and molybdenum (O'Hara et al. 1988).

6.7 What are the nitrogen and rotational benefits of crop legumes?

As previously stated, N fixation provides 'free' N to the legume eliminating the need for additional inputs of fertiliser N. This is only the first part of the story. Incorporating legumes into a cropping system also provides rotational benefits. Rotational benefits include an N benefit and a biological benefit. Both factors often lead to significantly increased yields of subsequent crops.

A substantial body of research examining the rotational benefits of N-fixing crop legumes and legume pasture leys in Australia's wheat production systems has now been published. These include, among others, the Sustainable Agriculture Through Wheat and Good Legumes (SATWAGL) experiments at Wagga Wagga, NSW (Heenan and Chan 1992) and the Tarlee, SA, pasture and pulse rotations (Schultz 1995).

6.7.1 What is the N benefit?

The N benefit of a legume phase of a rotation comprises the mineral N conserved in the soil during growth of the legume and the addition of N-rich residues following legume harvest.

Since legumes fix a percentage of their required N, they use less of the available soil N. The residual soil N that is not used is normally carried over to the subsequent cropping year.

Legumes also produce N-rich residues, which decompose in the soil after the pulse crop is harvested or pasture senesces. The mineral N released during decomposition is then available to be taken up by the following crop (see Figure 6.1, page 41).

6.7.2 How do the N-rich legume residues contribute to the N benefit?

To determine how much N-rich legume residues contribute to the N benefit, we need to examine crop, residue and soil N values for the two years of a legume-cereal rotation. Table 6.5 shows factors affecting the N balances of chickpea/wheat and wheat/wheat rotations in northern NSW.

In the first year of the sequence, all crops were grown in a soil with moderate nitrate at sowing (67kg N/ha). Chickpeas fixed 135kg N/ha and produced far more residue-N than both wheat crops. The chickpea residues were also N-enriched (lower carbon (C):N ratio) compared to the wheat residues.

The low C:N ratio of the chickpea residues means that mineral N (ammonium and nitrate) was released into the soil during microbial decomposition, resulting in net mineralisation.

C:N ratio of plant residues	The ratio of carbon to nitrogen in the plant residues. The carbon content of plant materials is fairly constant at about 40 per cent but the N content varies considerably, from about 0.4 per cent to 3.0 per cent. The C:N ratio thus varies accordingly from 100:1 to 13:1.
Net mineralisation and immobilisation	The decomposition of residues by the soil microbes will either result in the release of mineral N into the soil (net mineralisation) or tie up mineral N (immobilisation). Net mineralisation is associated with residues with C:N ratios of less than 30 and immobilisation with those with C:N ratios of more than 30.

In order to decompose higher C:N wheat residues, microbes must use soil mineral N. The use of this N results in net N immobilisation of the mineral N into microbial biomass within the soil.

Thus, we can estimate that the chickpea residues released 16kg mineral N/ha into the soil during the six-to-seven-month summer fallow, compared to 21 to 22kg N/ha immobilised by the wheat residues during the same period.

In Table 6.5, at crop sowing time in Year 2 at the end of the summer fallow, nitrate in the soil following chickpeas was much higher than following wheat crops. As a result, grain yields and grain N were higher after chickpeas.

The data clearly shows the key role the residues have in determining how much plant-available N will be in the soil at the time of sowing the next crop. Both the amount and the concentration of N in those residues (described by the C:N ratios in the example) are critical.

Legumes produce residues with a higher N concentration compared to cereals.

6.7.3 What is the biological benefit?
The biological benefit is largely related to the break-crop effect of the legume phase on soil and stubble-borne diseases of cereals.

The benefit depends on the nature of the disease. Diseases with a broad host range, such as *Rhizoctonia*

TABLE 6.5 Explaining the N and yield benefits of a chickpea-wheat rotation compared with unfertilised or N-fertilised wheat-only sequences.

	Chickpea/ wheat (0 N)	Wheat (0 N)/ wheat (0 N)	Wheat (100kg/ ha N)/ wheat (0 N)
Year 1 (chickpea or wheat)	Chickpea	Wheat	Wheat
Sowing soil nitrate (kg N/ha, 1.2m depth)	67	67	67
Fertiliser N applied (kg N/ha)	0	0	100
Grain yield (t/ha)	2.3	2.3	3.2
Total crop N (kg /ha)	205	55	115
Crop N fixed (kg /ha)	135	0	0
Residue N (kg/ha)	133	20	55
Residue C:N	25:1	50:1	44:1
Estimated mineralisation (+) or immobilisation (−) (kg N/ha)	+16	−22	−21
Year 2 (wheat only)	Wheat	Wheat	Wheat
Sowing soil nitrate (kg N/ha, 1.2m depth)	102	53	74
Wheat grain yield (t/ha)	2.8	1.7	1.8
Wheat grain N (kg/ha)	55	30	33

TABLE 6.6 Biological break benefit.

Rotation	Crown rot incidence (%)	Yield (t/ha)
Wheat/wheat	20–27	2.7
Chickpea/wheat	15	3.0

Source: Kirkegaard et al., 2004

solani, are not effectively controlled by legume rotations. However, the increased available soil N can enhance plant health and help to minimize the impact of the disease.

Host-specific diseases such as take-all (*Gaeumannomyces graminis*) and crown rot (*Fusarium* spp.) can usually be managed using legumes (and crops such as canola) as a break crop. Crop legumes are generally more effective than pasture legumes because the latter tend to be part of a mixed legume-grass sward with the grasses acting as disease carriers, except where pasture leys are managed to remove the grass component.

For example, a SA study showed that seminal root infection in wheat by take-all was three per cent following a legume, compared to eight per cent following wheat (King 1984).

Similarly, the data from northern NSW in Table 6.6 shows substantially less crown rot in wheat after chickpeas than following wheat. There were associated yield increases as all crops were well-fertilised with N, so the increased yield after chickpea was not related to an N benefit from the chickpea.

In general, the non-N biological benefit of legumes to grain yield in the following crop may range from negligible to more than 2t/ha.

6.7.4 How does a legume break the cereal pathogen cycle?
The numbers of the pathogen in the soil decrease when the host (cereal crop) is not present. A study of different sites in south-eastern Australia showed lower numbers of the crown-rot fungi in soils after legume-based rotations than continuous cereals (Evans et al. 2010).

Similarly, cereal cyst nematode (*Heterodera avenae* Woll.) populations in SA and Victoria decreased to almost undetectable levels following two years of peas or fallow. In comparison, numbers after two years of resistant wheat were four eggs per gram of soil, and 15 eggs per gram of soil after susceptible wheat (Table 6.7).

6.7.5 What are the yield benefits of crop legumes in rotation?
Pulses and other legumes are usually grown in rotation with cereals. The benefits to the system are measured in terms of increased soil total and plant-available (nitrate) N, and grain N and yield of the subsequent cereal crop, all relative to a cereal/cereal sequence.

Studies on different cropping systems in different regions of Australia have typically found that cereals grown after crop legumes commonly yield an additional 0.5 to 1.5 t/ha grain compared with cereals grown after cereals without fertiliser N.

To generate equivalent yields in the cereal-cereal

TABLE 6.7 Population changes of cereal cyst nematode under different rotational regimes.

	Nematode eggs/gram soil		
	Initial	1984	1985
Wheat (Resistant)	40	9	4
Wheat (Susceptible)	33	19	15
Field pea	43	8	0.1
Fallow	38	6	0.3

Source: Fisher and Hancock, 1991

sequence, 40 to 100kg fertiliser N/ha would need to be applied.

For example, 167 experiments were conducted in WA between 1974 and 2007 to examine the rotational benefit of the narrow-leafed lupin and field peas on subsequent wheat crops (Seymour et al. 2012). Over all experiments, the rotational benefit of lupin was 0.6 tonnes of wheat grain/ha and for pea was 0.45t/ha. For both, the benefit was a combination of extra N plus disease-break (principally take-all).

It is worth noting that the benefits were more substantial in the high-rainfall areas that produced higher-yielding lupin crops, and with the more recent trials (Table 6.8). The larger benefits during the 1990s were likely related to improved agronomy (weed management etc.) of both the lupin and wheat crops. Significant benefits (0.4t wheat grain/ha) persisted into a second wheat crop.

Rotation trials at different sites in Victoria and NSW show consistent increases in wheat grain yield following both faba bean and lupin crops (Figure 6.6). The yield benefit of the legume rotation was equivalent to fertilising with 80kg N/ha.

Rotation trials in the grain-growing regions of northern NSW and southern Queensland showed that both yield and grain protein of wheat increased substantially following chickpea, compared to the wheat/wheat sequence. A summary of the results is presented in Table 6.9.

The yield benefit of chickpea was equivalent to fertilising with 75 to 150 kg N/ha. The major factor in the increased wheat yields was soil nitrate. In NSW, there was, on average, an additional 35kg nitrate-N/ha in the 1.2m profile after chickpeas compared with the continuous wheat.

TABLE 6.8 Effects of size of the lupin crop (lupin grain yield) and year of study on rotational benefits of the narrow-leafed lupin on wheat grains yields in WA.

Lupin grain yield / years of experiments	Increase in wheat grain yield following lupin (t/ha)
0.5–1.0t/ha	0.5
1.0–1.5t/ha	0.7
> 1.5t/ha	0.9
1974–80	0.4
1981–90	0.5
1991–97	1.0

Cereals grown after crop legumes commonly out yield cereals grown after cereals.

Comparable benefits were established in the grainbelts of southern NSW and Victoria, where wheat yield and grain protein were greater in legume/wheat rotations than in wheat-wheat rotations (Figure 6.7).

In a number of cases, yield increases were more than 200 per cent. On average, wheat after lupin yielded an additional 0.9 t grain/ha and wheat after pea yielded an additional 0.7 t/ha, compared with wheat after wheat.

Again, increased plant available N was the main factor governing yield increases. Plant available N increased by 54 per cent following lupin and 61 per cent following peas.

6.7.6 How does the inclusion of a crop legume in the rotation impact on the overall profitability of the system?

Crop legume/cereal rotations often show improved gross margins compared with the cereal/cereal sequences. When the gross margins for the crop sequences in Table 6.5 were calculated, the chickpea/wheat rotations were far more profitable (Table 6.10).

After Year 1, there was not a lot of difference between the gross margins of chickpea and the N-fertilised wheat. In Year 2 however, wheat after chickpea had gross margins more than double those of the wheat/wheat sequences. The least profitable sequence was the unfertilised wheat followed by unfertilised wheat. Over the two years of the sequences, the chickpea/wheat rotation had a gross margin that was 50 to 90 per cent greater than those of the continuous wheats.

Legume-wheat rotations can be twice as profitable as wheat-wheat rotations.

FIGURE 6.6 Rotation crop impact on wheat grain yield at three Victorian Department of Primary Industries' sites.

Wheat grain yield (t/ha)

Rutherglen, Vic | Mininera, Vic | Culcairn, NSW

SOURCE: Victoria DPI, unpublished data
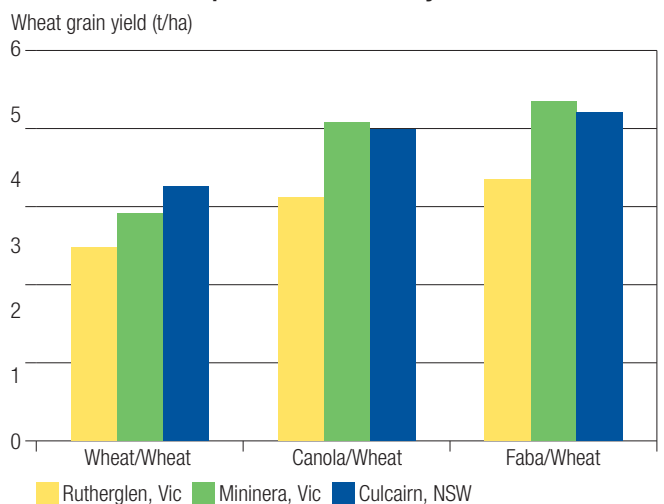
TABLE 6.9 Benefits of chickpeas on yield and grain protein of the following wheat crop.

Sites / rotations	No fertiliser N		+ fertiliser N (75–150kg/ha)	
	Yield (t/ha)	% protein	Yield (t/ha)	% protein
Wheat after wheat	2.1	11.2	2.7	13.2
Wheat after chickpea	2.8	12.2	2.9	13.8

Data sourced from Lucy et al. 2005, representing the summary of a decade of rotations in the northern grainbelt of NSW.

6.7.7 How long does the rotational benefit last?

The rotational benefits of crop legumes for following cereal crops last for one to two seasons, depending on particular circumstances. A study of six sites in northern NSW showed an average yield benefit following chickpea of 46 per cent (3.2t/ha for wheat after chickpea versus 2.2 t/ha for wheat after wheat; Marcellos et al. 1993). For five of the six sites in this study, there were no effects of the chickpeas on yields of a second wheat crop. In WA, on the other hand, the benefit of the narrow-leafed lupin lasted into a second wheat crop, likely through disease-break effects (Seymour et al. 2012).

6.8 What are the benefits of pasture legume rotations?

Pasture legumes provide high-quality feed for grazing animals. Therefore a major benefit of pasture legumes is enhanced productivity of the pasture, which flows through to animal production.

Pasture legume leys also benefit soil N and soil structure. These benefits can be derived from single or multi-year pasture leys. When the pasture is moved into crop production, these benefits enhance productivity of subsequent cereal crops grown on the same land.

Research at Tamworth in northern NSW clearly illustrated the benefit of legume-based pasture leys on soil total N. The well-managed, intensively grazed lucerne pasture on a black

FIGURE 6.7 Average percentage increase in wheat yields and grain proteins for wheat following either lupin or peas, relative to wheat following wheat. Values are averages from 18 experiments.

% increase relative to wheat/wheat

SOURCE: Evans et al. 1991
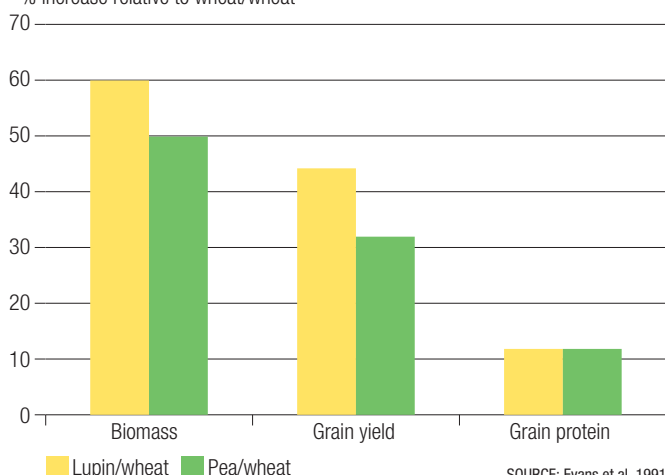
TABLE 6.10 Simple gross margin analysis of the N and yield benefits of a chickpea-wheat rotation compared with unfertilised or N-fertilised wheat-only sequences

	Chickpea/wheat (0 N)	Wheat (0 N)/ wheat (0 N)	Wheat (100 kg/ ha N)/wheat (0 N)
Year 1	Chickpea	Wheat	Wheat
Grain yield (t/ha)	2.3	2.3	3.2
Grain ($)[1]	920	575	800
Cost of production ($)[2]	465	270	400
Gross margin ($)	455	305	400
Year 2 (wheat only)	Wheat	Wheat	Wheat
Grain yield (t/ha)	2.8	1.7	1.8
Grain ($)	700	425	450
Cost of production ($)	270	270	270
Gross margin ($)	430	155	180
2-year gross margin ($)	885	460	580

Yields taken from Table 6.5 and are the means of no-tillage and cultivated treatments at two sites in northern NSW (source: unpublished data of WL Felton, H Marcellos, DF Herridge and GD Schwenke).
1 Chickpea at $400/t; wheat at $250/t; 2 NSW DPI figures

earth added about 140kg N/ha per year. Higher levels of soil total N were maintained during more than nine years of following wheat cropping (Figure 6.8).

> Legume-pasture leys increase soil N and enhance productivity of subsequent crops.

Comparable benefits were found on a red earth soil, where the lucerne pasture added about 110kg N/ha per year.

Additional studies in the Tamworth region showed the positive impact of pasture legume leys on nitrate-N and subsequent wheat yields (Table 6.11).

Grazed pasture leys accumulated 290 to 854kg of biomass-N per hectare during three years of growth. Following the pasture phase, up to 215kg of nitrate-N/ha became available for crop growth. By comparison, nitrate levels were 15kg/ha in the adjacent continuous wheat plots.

Increased grain yields and protein in subsequent wheat crops reflected the substantial inputs of legume N into the soil. The benefits of the pasture leys were still apparent after three years of wheat crops, particularly for lucerne pastures.

The long-term benefits resulted in savings on N fertiliser inputs, as shown in Table 6.12.

Single–year pasture leys are also excellent for increasing soil nitrate and enhancing wheat production. Research on one-year lucerne and annual medic leys at Warra in southern Queensland demonstrated that soil nitrate following the legume ley increased by as much as 180 per cent compared to that following wheat (Weston et al. 2002).

In those trials, the higher soil-water use by lucerne meant that the additional soil nitrate following lucerne did not translate into higher yields of the following wheat crops, but the extra nitrate meant far higher grain protein (13.1 per cent) than for continuous wheat (9.7 per cent).

Pasture legumes typically provide greater soil N increases than crop legumes. This difference is related to greater biomass return to the system, longer growth periods, and

TABLE 6.11 Summary of data from pasture ley rotation experiments at NSW Department of Primary Industries, Tamworth.

Previous crop / pasture ley	Years duration	Shoot biomass dry matter (t/ha)	Shoot biomass N (kg/ha)	Nitrate-N at sowing[1] (kg/ha)	Wheat grain yield[2] (t/ha)	Wheat grain protein[2] (%)
Lucerne	3	24.7	854	215	2.9	12.7
Clover	3	12.7	425	150	2.8	10.4
Annual medic	3	10.8	290	110	2.2	9.5
Wheat	1	3.3	37	15	1.1	9.6

Data sourced from Holford and Crocker 1997 and Holford et al. 1998. Data are the means of six replicates and averaged over two soil types (black and red).
1 Nitrate-N levels to 1.2m at sowing in the first year after the pasture ley or after continuous wheat
2 Averaged over three years

TABLE 6.12 Savings in fertiliser N (kg/ha) from the three-year legume pasture leys at NSW Department of Primary Industries, Tamworth.

Previous crop/pasture	Wheat crop 1	Wheat crop 2	Wheat crop 3	Average 3 wheat crops
Lucerne	45*	120	65	80
Clover	>100	60	45	70
Annual medic	70	30	25	45

Data from long-term rotation experiments on black and red soils during 1988–93.
* low because of the soil drying effect of the lucerne ley.

greater nitrogen fixation efficiency.

An additional benefit of pasture legumes is the impact the extra organic N can have on soil structure. Figure 6.9 clearly shows the positive effect of pasture leys on aggregate stability of a red-earth soil in the Victorian grainbelt. Aggregate stability declined once wheat cropping recommenced.

The effect of organic N on soil structure varies with the type of clay and the clay content of the soil (Russell 1987). With vertosols (black earths high in clay content), there is little relationship between soil organic matter and structure.

On the other hand, loss of organic matter can have serious negative effects on structure of soils of less than 30 per cent clay (e.g. red-brown earths), or with high proportions of sand and silt (e.g. sands, sandy loams).

Much of the agriculture in Australia's southern and western grainbelts was built around sequences of pasture leys and cereals. As agricultural land used for cropping continues to lose organic matter and structural integrity, the role of pasture leys in restoring organic fertility and productivity may need to be expanded.

Legume pasture leys have a positive impact on soil structure as well as soil fertility.

6.9 Concluding comments

Legumes have been used as a source of food ever since humankind first tilled the soil many thousands of years ago.

From very early times, legumes were recognised as 'soil improvers'. The farmers of ancient Mesopotamia grew peas and beans in their agricultural systems because they realised

FIGURE 6.8 Build-up of soil total N under a well-managed, intensively grazed lucerne pasture on a black earth at Tamworth and the subsequent run-down during wheat cropping. Soil N levels under the wheat monoculture are shown also.

Soil total N (%, 0-15cm)
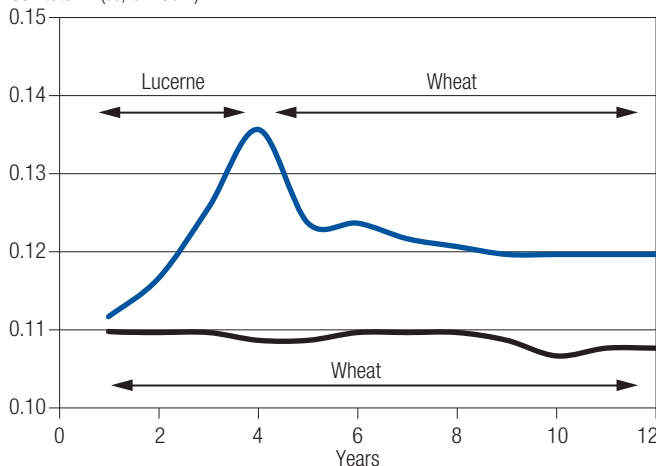
A 0.01 per cent increment in soil N to 15cm depth is equivalent to 180kg N/ha.

SOURCE: Holford 1981

FIGURE 6.9 Positive effects of pasture leys on aggregate stability of a red earth at Rutherglen, Victoria.

Water stable aggregates (% of cont. wheat)
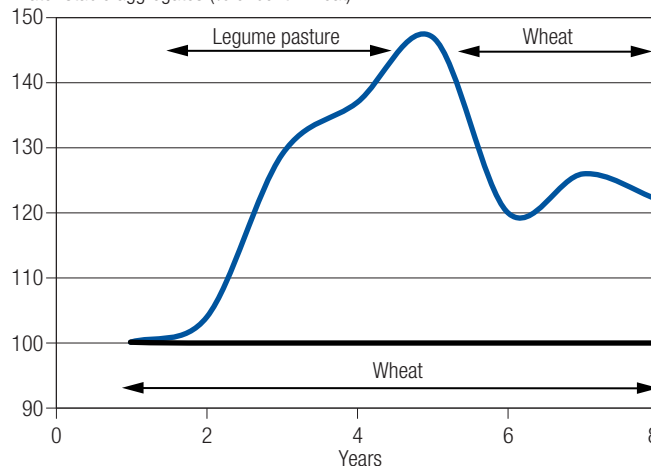
SOURCE: Reeves 1991

that cereals, their mainstay crops, were healthier and higher yielding when grown after a legume break-crop.

Nothing much has changed. Growers still grow legumes as rotation crops because of the N benefits and because it helps them to spread risk and manage weeds, pests and diseases in the production system, and improve soil health.

In this chapter, we have tried to flesh out the nature of legume nitrogen fixation and the rotational benefits of legumes by summarising some of the more recent research data on the topics. We have also provided examples of how legume nitrogen fixation and yields might be optimised through crop and pasture management.

Optimising legume yields within any system can only be achieved through best management practice in agronomy where production is not constrained by soil deficiencies, poor agronomy, insects, disease, weeds or nutrients. Once this is achieved, further yield gains may be made through using elite, high-yielding varieties that are well-adapted to the location.

Nodulation must also be optimised, either through well-conducted inoculation or by growing the legume in soils that are known to contain high numbers of effective, compatible rhizobia. Previous chapters in this handbook examined the mechanisms of the rhizobia-legume symbiosis, and explored management decisions regarding when and how to inoculate.

7 LEGUME INOCULATION FACT SHEETS

In this Chapter, we present the full list of rhizobial strains that are available to be used by Australian farmers, followed by a series of Fact Sheets for inoculating the more widely-grown legumes.

7.1 List of rhizobial strains used in Australian inoculants

Inoculant group	Rhizobial strain	Legume common name	Legume botanical name
AL	RRI128	Lucerne or alfalfa	*Medicago sativa*
		Strand medic	*Medicago littoralis*
		Melilotus	*Melilotus albus*
		Disc medic	*Medicago tornata*
AM	WSM1115	Barrel medic	*Medicago truncatula*
		Burr medic	*Medicago polymorpha*
		Snail medic	*Medicago scutellata*
		Sphere medic	*Medicago sphaerocarpus*
		Gama medic	*Medicago rugosa*
		Murex	*Medicago murex*
B	TA1	White clover	*Trifolium repens*
		Red clover	*Trifolium pratense*
		Strawberry clover	*Trifolium fragiferum*
		Alsike clover	*Trifolium hybridum*
		Talish clover	*Trifolium tumens*
		Berseem, Egyptian clover	*Trifolium alexandrinum*
		Cluster or ball clover	*Trifolium glomeratum*
		Suckling clover	*Trifolium dubium*
C	WSM1325	Subterranean clover	*Trifolium subterraneum*
		Balansa clover	*Trifolium michelianum*
		Bladder clover	*Trifolium spumosum*
		Crimson clover	*Trifolium incarnatum*
		Purple clover	*Trifolium purpureum*
		Arrowleaf clover	*Trifolium vesiculosum*
		Rose clover	*Trifolium hirtum*
		Gland clover	*Trifolium glanuliferum*
		Helmet clover	*Trifolium clypeatum*
		Persian or shaftal clover	*Trifolium resupinatum*
D	CC829	Lotus	*Lotus pedunculatus*
E	SU303 or WSM1455	Pea, field pea	*Pisum sativum*
		Tares or common vetch	*Vicia sativa*
		Woolly pod vetch	*Vicia daisycarpa*
		Grass pea	*Lathyrus sativus*

		Bitter vetch	*Vicia ervilia*
		Narbon bean	*Vicia narbonensis*
		Lathyrus	*Lathyrus cicera*
F	WSM1455	Faba, tick or broad bean	*Vicia faba*
		Lentil	*Lens culinaris*
G	WU425 or WSM471	Narrow-leaf lupin	*Lupinus angustifolius*
		Mediterranean white lupin	*Lupinus albus*
		Yellow lupin	*Lupinus luteus*
		Sandplain lupin	*Lupinus cosentinii*
H	CB1809	Soybean	*Glycine max*
I	CB1015	Cowpea	*Vigna unguiculata*
		Mungbean (green gram)	*Vigna radiata*
		Mungbean (black gram)	*Vigna mungo*
J	CB1024	Pigeon pea	*Cajanus cajan*
		Lablab, hyacinth bean	*Lablab pupureus*
		Horse gram, biflorus	*Macrotyloma uniflorum*
		Perennial horse gram	*Macrotyloma axillare*
L	CB376	Lotononis	*Lotononis bainesii*
M	CB756	Velvet bean, banana bean	*Mucuna deeringiana*
		Siratro	*Macroptilium atropurpureum*
		Phasey bean	*Macroptilium lathyroides*
		Puero, tropical kudzu	*Pueraria phaseoloides*
		Calopo	*Calopogonium mucunoides*
		Glycine	*Neontonia wightii*
		Butterfly pea	*Clitoria ternatea*
N	CC1192	Chickpea (desi and kabuli)	*Cicer arietinum*
P	NC92	Peanut or groundnut	*Arachis hypogaea*
S	WSM471 or WU425	Yellow serradella	*Ornithopus compressus*
		Slender serradella	*Ornithopus pinnatus*
		Pink serradella	*Ornithopus sativus*
		Hybrid serradella	*Ornithopus compressus X sativus*
		Birdsfoot	*Ornithopus perpusillus*
SPECIAL	CB82	Fine stem stylo	*Stylosanthes guianensis* var. *intermedia*
		Stylo	*Stylosanthes guianensis* var. *guianensis*
		Townsville stylo	*Stylosanthes humilis*
		Shrubby stylo	*Stylosanthes viscosa*

	CB1923	Centro	*Centrosema pubescens*
		Centurion	*Centrosema pascuorum*
	CIAT3101	Pinto peanut	*Arachis pintoi*
	CB627	Desmodium	*Desmodium intortum*
	CB3126	Desmanthus	*Desmanthus virgatus*
	CB3060	Leucaena	*Leucaena leucocephala*
	CB1650	Caribbean stylo (verano)	*Stylosanthes hamata*
	CC1502	Tree lucerne or tagasaste	*Chamaecytisus palmensis*
	CB2312	Bargoo jointvetch	*Aeschynomene falcata*
	WSM1592	Sulla	*Hedysarum coronarium*
	CC283b	Caucasian clover, kura clover	*Trifolium ambiguum*
	CB3035	Guar or cluster bean	*Cyamopsis tetragonoloba*
	SU277	Fenugreek	*Trigonella foenum-graecum*
	CB3481	Caatinga stylo	*Stylosanthes seabrana*
	SU343	Lotus	*Lotus corniculatus*
	WSM1497	Biserrula	*Biserrula pelecinus*
	CB3171	Calliandra	*Calliandra* spp.
	CC1099	Sainfoin	*Onobrychis viciifolia*
	CC511	French or common bean	*Phaseolus vulgaris*
		Lima bean, butter bean	*Phaseolus lunatus*
		Scarlet runner bean fire bean	*Phaseolus coccineus*
	CB1717	Burgundy bean	*Macroptilium bracteatum*
	5G1B	Adzuki bean	*Vigna angularis*
	CB2312	Jointvetch	*Aeschynomene americana*
	CB3090	Gliricidia	*Gliricidia* spp.

Pasture legumes
- Annual clovers (group C)
- Annual medics (group AM)
- Biserrula (Special biserrula)
- Lotus (group D and special lotus)
- Lucerne, strand and disc medic (group AL)
- Perennial clovers (group B)
- Serradella (groups G and S; see serradella with lupin above)
- Sulla (special sulla)

The fact sheets are arranged in the following order:

Grain legumes (pulses and oilseed legumes)
- Chickpea (group N)
- Field pea, vetch (group E) and faba bean, lentil (group F)
- Lupin and serradella (groups G and S)
- Peanut (group P)
- Mungbean and cowpea (group I)
- Soybean (group H)

7.2 CHICKPEA
inoculation fact sheet

Chickpea	Strain: CC1192 (Group N)
Cicer arietinum	*Mesorhizobium ciceri*

Legume use and rhizobia distribution

Chickpea plantings have been steadily increasing over the past decade to an annual total of more than 500,000 hectares throughout Australia. About 90 per cent of these areas are in New South Wales and Queensland. Chickpea rhizobia are generally present in soils where chickpea has been recently grown, although numbers can vary substantially with soil type and environment.

Inoculation method

Peat inoculants applied to the seed remains the most commonly used method of inoculation for chickpea. Some inoculant is also applied as granular and freeze-dried formulations. Seed can be coated with either the peat or freeze-dried inoculant formulations as slurries just prior to planting or during transfer (augering). Alternatively, peat or freeze-dried inoculum can be applied in-furrow when planting using a water-injection system. Granular inoculum can be dispensed into the seed row with the seed at planting.

Key considerations

Where chickpea has not been grown before, inoculation is required to achieve good nodulation. Even where background populations of rhizobia are present, inoculation may be worthwhile because the background rhizobia are often not as effective at fixing nitrogen.

Nodulation

Nodules are indeterminate and often multi-lobed (see Figure 7.1).

For chickpeas, 10 to 30 nodules per plant is satisfactory after about eight weeks of plant growth.

Likelihood of response to inoculation for sown chickpea	
HIGH	• Chickpeas not previously grown.
MODERATE	• Previous chickpea crop was grown more than four years ago (recommended pulse rotation); OR • legume nodulation or growth below expectation.
LOW	• Recent history of well nodulated chickpea crop in past two years.

FIGURE 7.1 Roots of deep-sown chickpea plants showing multi-lobed nodules particularly around the crown (i.e. site of inoculation).

7.3 FIELD PEA, VETCH, FABA BEAN and LENTIL inoculation fact sheet

Field pea and vetch	Strain: SU303 (group E)
Pisum sativum *Vicia* species	*Rhizobium leguminosarum* bv. *viciae*
Fababean, broad bean and lentil	Strain: WSM1455 (group F)
Vicia faba *Lens culinaris*	*Rhizobium leguminosarum* bv. *viciae*

Legume use and rhizobia distribution

The same species of rhizobia can nodulate legumes in inoculant groups E and F. The rhizobia have been widely distributed following decades of, particularly, pea and vetch cultivation. Present combined sowings of pea, faba bean and lentil are about 600,000 hectares per year. Spread and survival of the rhizobia has also been assisted by vetch, which is broadly naturalised and also sown as a forage/green manure crop.

Although the rhizobia have been widely distributed, their moderate sensitivity to soil acidity means they sometimes occur at levels below what is needed for optimal nodulation.

Inoculation method

Inoculation usually occurs by pouring or spraying a slurry of peat inoculant over seed during transfer (augering) prior to sowing. Peat, granule and freeze-dried inoculant formulations can also be used.

Key considerations

Two inoculant strains are provided for these legumes to optimise nitrogen fixation potential of the different legume hosts. For this reason only group F should be used on faba beans and lentils. Group E (SU303) is preferred for field peas, but group F (WSM1455) can be used in its place as it is only marginally less effective.

Rhizobia for these legumes are moderately sensitive to soil acidity. Their number may be sub-optimal or absent where soil pH is less than 6.0, even where there has been a recent history of legume host. About 20 per cent of soils in South Australia and Victoria and 60 per cent of soils in Western Australia contain insufficient rhizobia to maximise pea nodulation.

For up to 30 per cent of soils, effectiveness of the rhizobia with field pea ranges from 50 to 70 per cent of the commercial inoculant strain and therefore many field pea crops may benefit from inoculation. Grossly ineffective symbioses are rarely observed.

Crops such as faba beans that have potential to produce a lot of dry matter have a higher demand for nitrogen and therefore may be more responsive to inoculation than field pea.

Nodulation

More than 100 pink nodules per plant are often observed after eight to 10 weeks plant growth in loam-clay soils. For lighter textured soils 20 nodules per plant is deemed satisfactory (see Figure 7.2).

Likelihood of response to inoculation for sown pea, faba bean, lentil and vetch	
HIGH	• Soils with pH(CaCl$_2$) below 6.0 and high summer soil temperatures (>35°C for 40 days); OR • legume host (pea, faba bean, lentil, vetch) not previously grown.
MODERATE	• No legume host (pea, faba bean, lentil, vetch) in previous four years (recommended pulse rotation); OR • Prior host crop not inoculated or lacked good nodulation.
LOW	• Loam or clay soils with neutral or alkaline pH and a recent history of host crop with good nodulation.

FIGURE 7.2 **Well-nodulated roots of field pea (left) and faba bean (right).**

7.4 LUPIN and SERRADELLA inoculation fact sheet

Lupin	Strain: WU425 or WSM471 (group G)
Lupinus species Narrow-leafed, white, yellow and sand-plain	*Bradyrhizobium* spp. *lupinus*
Serradella	Strain: WSM471 or WU425 (group S)
Ornithopus species Yellow, pink, hybrid, slender and birdsfoot	

Legume use and rhizobia distribution

Legumes in the groups G and S inoculation groups are nodulated by the same species of rhizobia (i.e. *Bradyrhizobium* spp). Commercial plantings of serradella began in the 1950s while significant plantings of lupin commenced in the 1970s. Both legumes are adapted to acidic to neutral sandy soils and are therefore widely grown in WA where they have been sown on several million hectares. The rhizobia tend to be persistent where the legume has been grown, but remain confined to those areas because, unlike the clovers and medics, an array of legume species that host the rhizobia have not dispersed and naturalised in Australian soils.

Inoculation method

Lupin is usually inoculated by pouring or spraying a slurry of peat inoculant over seed during transfers (augering) prior to sowing. Peat, granular and freeze-dried inoculant formulations are also used.

Inoculation of serradella is mostly done with the application of a slurry of peat. Where podded serradella is being inoculated, adjustments to liquid volumes are required to ensure even distribution and survival of inoculant and the manufacturer's instructions should be carefully followed (see Chapter 5). Granular inoculant in furrow can also be used. Lime pelleting has been shown to be advantageous to rhizobial survival and serradella nodulation in eastern Australia, even though serradella rhizobia are naturally acid tolerant. Lime pelleting of serradella is recommended in all states except WA.

Key considerations

Since late 2006, two inoculant groups are available and can be used for both lupin and serradella. They are group G, containing strain WU425, or group S, containing strain WSM471.

Rhizobia for these legumes are highly tolerant of soil acidity but some instances of inadequate number in soil after fours years legume absence have been recorded. Top-up inoculation may be worthwhile where the host crop has been absent four or more years.

As these legumes are often grown on very sandy soils that are acutely deficient in available nitrogen, nodulation failure can result in total-crop or pasture failure. Where there is no previous history of lupin or serradella, inoculation is essential.

Nodulation

For serradella more than 20 pink nodules per plant is satisfactory after eight to 10 weeks plant growth. For lupin, nodules can be difficult to count, but the collar region (top of root system) is typically covered by nodule material in well nodulated plants (see Figure 7.3).

Likelihood of response to inoculation for sown lupin and serradella	
HIGH	• Lupin or serradella not previously grown in paddock.
MODERATE	• No legume host in past four years. • Previous host crop not inoculated and legume growth or nodulation below expectation.
LOW	• Sowing in the north and central regions of the Western Australian wheat/sheep belt; OR • recent history (past four years) of vigorous lupin/serradella growth and good nodulation.

FIGURE 7.3 **Examples of well-nodulated serradella (left) and lupin (right).**

7.5 PEANUT
inoculation fact sheet

Peanut (or groundnut)	Strain: NC92 (group P)
Arachis hypogaea	*Bradyrhizobium* spp.

Legume use and rhizobia distribution

Australian growers produce about 40,000 tonnes of peanuts annually from about 15,000 hectares. More than 90 per cent of these are grown in Queensland with a few growers also in northern NSW and northern WA. One third of production is rain-fed and two thirds is irrigated, with respective average yields of 2 and 5t/ha.

Inoculation method

Water injection of peat or freeze-dried inoculum is recommended to eliminate damage to the seed from applying a slurry coating. Alternatively, granular inoculum can be dispensed with the seed at planting.

Key considerations

Inoculation every season is recommended to maximise yields as native or 'background' rhizobia compete strongly with the inoculated strain for root infection but are not as effective at fixing nitrogen.

Nodulation

Peanuts can form many nodules (i.e. more than 100/plant). It is virtually impossible to state the number of nodules per plant after eight to 10 weeks of plant growth that might be considered satisfactory (See Figure 7.4).

Likelihood of response to inoculation for sown peanut	
HIGH	• Peanut not previously grown.
MODERATE	• Most other situations due to likely presence of poorly effective rhizobia.
LOW	• Recent and/or intensive cultivation of peanut

FIGURE 7.4 Photo of well-nodulated peanut.

7.6 MUNGBEAN and COWPEA inoculation fact sheet

Mungbean	Strain: CB1015 (group I)
Vigna radiata, V. mungo	*Bradyrhizobium* spp.
Cowpea	
Vigna unguiculata	

Legume use and rhizobia distribution
Mungbeans are the more widely grown legume in this inoculant group with the majority being grown in southern and central Queensland and northern NSW.

Inoculation method
Peat inoculants applied to the seed remain the most commonly used method of inoculation for this legume. Inoculant is also available in granular and freeze-dried forms. Seed can be coated with either the peat or freeze-dried inoculant formulations as slurries just prior to planting, commonly by applying to the seeds during transfers (augering). Alternatively, peat or freeze-dried inoculum can be applied in-furrow when planting using a water-injection system or granular inoculum can be dispensed with the seed at planting.

Key considerations
Soil nitrate may depress nodulation and nitrogen fixation of mungbean, even at relatively low mineral nitrogen supply.

Nodulation
For mungbean and cowpea, more than 20 nodules per plant is satisfactory after eight to 10 weeks of plant growth (see Figure 7.5).

Likelihood of response to inoculation for sown mungbean and cowpea	
HIGH	• No previous mungbean, cowpea or other related *Vigna* species.
MODERATE	• Most other situations due to likely presence of poorly effective rhizobia.
LOW	• Recent and/or intensive cultivation of mungbean or cowpea.

FIGURE 7.5 Well-nodulated mungbean from field.

7.7 SOYBEAN
inoculation fact sheet

Soybean	Strain: CB1809 (Group H)
Glycine max	*Bradyrhizobium japonicum*

Legume use and rhizobia distribution

Soybean is grown in areas of adequate-to-high summer rainfall or where irrigation is possible. This includes a wide area from northern Queensland, along the coastal sugar belt and in central Queensland, to the Darling Downs, into the NSW coastal hinterland and to inland cropping regions of southern NSW and Victoria. They are also grown in the northern irrigation areas of WA.

Inoculation method

Peat inoculants applied to the seed remain the most commonly used method of inoculation for this legume. Inoculant is also available in granular and freeze-dried forms. Seed can be coated with either the peat or freeze-dried inoculant formulations as slurries just prior to planting, and are commonly applied to the seeds during transfer (augering). Alternatively, peat or freeze-dried inoculum can be applied in-furrow when planting using a water-injection system or granular inoculum can be dispensed with the seed at planting.

Key considerations

When grown with irrigation or under high-rainfall conditions, soybeans can produce considerable shoot biomass (seven to eight tonnes per hectare) and grain yield (four tonnes per hectare) and fix as much as 300 to 400kg N/ha. Soybean is specific in its requirement for rhizobia. Soybean will not nodulate with the same range of naturalised soil rhizobia as mungbean or cowpea. Therefore, good agronomy and good inoculation practice are necessary to achieve yield and nitrogen fixation potentials.

Nodulation

For soybeans more than 20 nodules per plant is satisfactory after eight to 10 weeks of plant growth (see Figure 7.6).

Likelihood of response to inoculation for sown soybean	
HIGH	• No previous soybean crop. Highly alkaline or highly acidic soils.
MODERATE	• Soybean cultivated in paddock more than three to five years ago.
LOW	• Recent and/or intensive cultivation of soybean.

FIGURE 7.6 **Well-nodulated soybean roots dug from soil when plants were mid-flowering.**

7.8 ANNUAL CLOVERS
inoculation fact sheet

Annual Clovers	Strain: WSM1325 (group C)
Trifolium species	*Rhizobium leguminosarum* bv. *trifolii*
Subterranean, balansa, Persian, arrowleaf, rose, gland, crimson, purple, bladder, cupped and helmet	

Legume use and rhizobia distribution

Subterranean clover is the most widely sown legume in this group. It is sown on about 300,000 hectares annually and occurs on more than 10 million hectares of neutral to acid soils in southern Australia. Many non-sown clover species that have naturalised extensively have assisted the widespread proliferation of clover nodulating rhizobia.

Inoculation method

Inoculation is mostly done with the application of a slurry of peat followed by pelleting with fine lime or other suitable product. Large sowings of bladder clover in WA and NSW has resulted in granular inoculants being used.

The availability of preinoculated seed has increased. However, survival of the rhizobia is often poor and therefore freshly inoculated (coated) seed is preferred.

Granule and freeze-dried inoculant formulations are available.

Key considerations

The majority of Australian soils with a history of growing annual or perennial clovers contain clover nodulating rhizobia. Effectiveness of the naturalised soil rhizobia with subclover is often sub-optimal, averaging 50 per cent of the commercial inoculant strain. Inoculation will help overcome sub-optimal symbioses in short-term pastures.

Some annual clover species, notably gland, bladder and arrowleaf clovers are less compatible with naturalised soil rhizobia and inoculation is considered essential to ensure adequate establishment.

Clover symbioses are reasonably tolerant of low soil pH, but ideally soil pH should be greater than 5.5. Background soil rhizobia should not be relied upon in very low pH soils, even where good nodulation is observed in the pasture before renovation. Disruption of background rhizobia from soil micro-sites during pasture renovation may result in their death with the site becoming responsive to inoculation.

Nodulation

50–100 pink nodules per plant after eight week's growth indicates good nodulation of subclover (see Figure 7.7).

Likelihood of response to inoculation for sown annual clovers	
HIGH	• Gland, bladder and arrowleaf clovers should always be inoculated. • All annual clovers where there is no history of clover having grown. • Soils with pH (CaCl$_2$) below 5.0 and where there is tillage at pasture renovation.
MODERATE	• No clover host in past four years and soil pH below 5.5. • Clover present, but growth or nodulation below expectation. May be associated with development of sub-optimal populations of soil rhizobia. High numbers of rhizobia on sown seed will compete with soil rhizobia at sowing but potency will diminish after several seasons.
LOW	• Soils with neutral or alkaline pH and a recent history of good clover growth and nodulation.

FIGURE 7.7 **Well-nodulated subterranean clover. Plant grown in greenhouse (left) and plant from field (right).**

7.9 ANNUAL MEDICS
inoculation fact sheet

Annual Medics	Strain: WSM1115 (group AM)
Medicago species (except strand and disc) Barrel, burr, snail, murex, sphere and gama	*Sinorhizobium medicae*

Legume use and rhizobia distribution
The diverse medic species in this inoculation group are grown in the medium-to-low-rainfall cropping regions where soils are neutral to alkaline and not subject to waterlogging. They have been grown extensively since the1930s and therefore their rhizobia are also widely distributed.

Inoculation method
Inoculation is mostly done with the application of a slurry of peat followed by pelleting with fine lime or other suitable product. Granule and freeze-dried inoculant formulations are available.

Key considerations
The majority of Australian soils that are neutral or alkaline in pH and have a history of growing annual medic (both sown and naturalised species) will contain medic-nodulating rhizobia.

Effectiveness of the naturalised soil rhizobia is often sub-optimal, averaging 50 per cent of the commercial inoculant strain. Inoculation will help overcome sub-optimal symbioses in short-term pastures.

Mildly acidic soils (pH 5.0 to 6.0) where the more acid tolerant species, namely burr, murex and sphere medic are grown, often contain insufficient rhizobia for good nodulation at establishment.

The group AL inoculant should not be used as a substitute because the inoculant strain (RRI128) is less effective at fixing nitrogen with some medic species in this group.

Nodulation
10-20 pink nodules per plant after eight week's growth indicates good nodulation of annual medics (see Figure 7.8).

Likelihood of response to inoculation for sown annual medics	
HIGH	• Burr, sphere and murex medic sown on soils with pH ($CaCl_2$) below 6.0; OR • no presence or history of sown or naturalised medic.
MODERATE	• Medic present, but growth or nodulation below expectation. May be associated with development of sub-optimal populations of rhizobia. Mean effectiveness of soil rhizobia with burr medic estimated to be 30 per cent. High numbers of rhizobia on sown seed will compete with soil rhizobia at sowing but potency will diminish after several seasons.
LOW	• Loam or clay soils with neutral or alkaline pH and a recent history of vigorous medic growth and good nodulation

FIGURE 7.8 **Well-nodulated medic plants grown in greenhouse (left) and field (right).**

7.10 BISERRULA inoculation fact sheet

Biserrula (special)	Strain: WSM1497
Biserrula pelecinus	*Mesorhizobium ciceri* bv. *biserrulae*

Legume use and rhizobia distribution

A relatively new annual pasture legume with the first cultivar Casbah registered in 2001. It is presently grown on about 100,000 hectares, mainly in mixed-farming areas. Approximately 90 per cent of plantings occur in WA.

Inoculation method

The two common methods of inoculation are peat-slurry lime pelleted seed or seed sown with granular inoculant. Increased inoculation rates (above recommended rates) of one 250g packet of inoculant for 10kg seed are recommended.

Key considerations

Because biserrula and its rhizobia are relatively new to Australian agriculture it is essential to inoculate if the legume has not been recently grown in the paddock. Biserrula and their associated rhizobia are very specific. The plant does not nodulate with the rhizobia associated with other indigenous or cultivated legumes.

The inoculant strain WSM1497 persists in low pH soils based on observations of good nodulation on regenerating plants five years after introduction of the inoculant strain.

Nodulation

At least five large (>5mm) and 10 small nodules per plant after eight week's growth indicates good nodulation of biserrula (see Figure 7.9).

FIGURE 7.9 Well-nodulated biserrula.

Likelihood of response to inoculation for sown biserrula	
HIGH	• Biserrula host not been previously grown.
MODERATE	• No biserrula in past four years; OR • last host crop not inoculated or lacked 'good' nodulation near top of root system.
LOW	• Loam or clay soils with neutral or alkaline pH and a recent history (past two years) of host crop with good nodulation.

7.11 LOTUS
inoculation fact sheet

Lotus (group D)	Strain: CC829
	Bradyrhizobium sp.
	Strain: SU343 (Special)
Lotus pedunculatus (syn uliginosus) Lotus corniculatus	Mesorhizobium loti.

Legume use and rhizobia distribution
The use of these perennial pasture legumes is largely restricted to permanent pastures in the medium-to-high-rainfall districts of eastern Australia and their rhizobia will be similarly restricted in their distribution. Although there are some naturalised species of lotus, they occur in low numbers and are unlikely to maintain rhizobia in sufficient number to negate the need for inoculation.

Inoculation method
Inoculation is mostly done with the application of a slurry of peat followed by pelleting with fine lime or other suitable product. One packet of peat inoculant (250g) will inoculate 10kg seed. Freeze-dried products are available.

Key considerations
A different inoculant strain is provided for each species of lotus, recognising that they have different rhizobial needs. Lotus pedunculatus is particularly specific in its rhizobial need. The two inoculant strains should not be interchanged. The rhizobia have moderate tolerance of soil acidity.

Nodulation
Expected nodulation after eight to10 weeks is considered to be more than 30 pink nodules per plant. (see Figure 7.10).

FIGURE 7.10 **Example of well-nodulated lotus plant.**

Likelihood of response to inoculation for sown lotus	
HIGH	• Lotus not previously grown.
MODERATE	• No lotus in past four years; OR • prior lotus pasture not inoculated or lacked good* nodulation near top of root system.
LOW	• Loam soils with neutral pH and a recent history (past two years) of lotus with good nodulation.

*Good nodulation of lotus at eight weeks after planting is considered to be more than 30 pink nodules

7.12 LUCERNE, MELILOTUS (albus), STRAND and DISC MEDICS inoculation fact sheet

Lucerne, Melilotus (albus)	Strain: RRI128 (group AL)
Strand and disc medic	
Medicago sativa, Medicago littoralis Medicago tornata Melilotus albus	*Sinorhizobium meliloti*

Legume use and rhizobia distribution

About 300,000 hectares of lucerne are sown annually, with stands persisting on three to five million hectares. It is most widely grown in NSW and least grown in WA, where summer rainfall is scarce.

By comparison the area sown annually to strand and disc medic is less than 20,000 hectares. However, established pastures of strand medic persist over wide areas of SA's Eyre Peninsula and the Mallee region bordering SA and Victoria. Medic sowings are generally aimed at renovation of pastures in these areas, which support large populations of rhizobia which are able to nodulate both medic and lucerne.

Lucerne is also often sown in permanent pasture areas where sown and naturalised medics do not commonly occur. Soils in these areas are unlikely to support suitable rhizobia for lucerne.

Inoculation method

Peat, granule and freeze-dried inoculant formulations are available. Most seed sold through retail outlets is preinoculated.

Key considerations

Inoculation is always recommended for lucerne because establishment of good plant density at sowing is critical to long-term production and cannot be recovered if compromised nodulation leads to poor establishment.

Most lucerne seed is sold preinoculated. Seed should not be used where the period since inoculation exceeds six months, even if it has been stored under cool dry conditions. Seed that exceeds this expiry period should be re-inoculated.

The lucerne and medic symbioses are very sensitive to low pH. Coating the inoculated seed with fine lime is advisable to provide protection from acidic fertilisers and aid establishment in acid soils.

Where soil pH is less than 6.0, soils will often contain no suitable rhizobia and will be highly responsive to inoculation.

The group AM inoculant should not be used as a substitute for AL because the inoculant AM strain (WSM1115) is less effective at fixing nitrogen with lucerne, strand and disc medic.

Nodulation

Young lucerne plants should have at least five pink nodules per plant at eight to 10 weeks after sowing. 10 to 15 nodules are ideal at this time.

For mature lucerne plants where tap root development has occurred, nodules may be restricted to the finer lateral roots and to a depth of 30cm in the soil. Nodules on mature

FIGURE 7.11 Well-nodulated lucerne grown in (A) greenhouse and (B) field; and (C) strand medic.

lucerne are therefore easily detached and difficult to find.

The strand medics are sometimes referred to as 'shy nodulators' due to the low number of nodules commonly observed on their roots. This is a characteristic of the plant and so the presence of five nodules at eight to 10 weeks after sowing is regarded as satisfactory.

Nodules tend to rapidly develop lobed or coral type structures (see Figure 7.11).

Likelihood of response to inoculation for sown lucerne, strand & disc medic	
HIGH	• Lucerne should always be inoculated at sowing. • Soils with pH (CaCl$_2$) below 6.0. • No history or presence of sown or naturalised medic.
MODERATE	• Medic present, but growth or nodulation below expectation. Maybe associated with development of sub-optimal populations of medic rhizobia in the soil. High number of rhizobia on sown seed will compete with soil rhizobia at sowing but potency will diminish after several seasons.
LOW	• Loam or clay soils with neutral to alkaline pH and a recent history of vigorous medic growth and good nodulation.

7.13 PERENNIAL CLOVERS
inoculation fact sheet

Perennial clovers	Strain: TA1 (group B)
	Strain: CC283b (Caucasian clover only)
Trifolium species White, strawberry, red, talish, alsike and caucasian	*Rhizobium leguminosarum* bv. *trifolii*

Legume use and rhizobia distribution

White clover is the most widely sown legume in this group. It is grown on more than five million hectares, generally in high-rainfall (>700mm) coastal areas and cooler tableland districts or elsewhere where irrigation is available. Many sown and non-sown clover species that have naturalised in the areas where perennial clovers are grown have assisted the widespread proliferation of clover nodulating rhizobia.

Inoculation method

Peat and freeze-dried inoculant formulations are available. Most seed sold through retail outlets is preinoculated.

Key considerations

The majority of Australian soils with a history of growing annual or perennial clovers contain clover nodulating rhizobia, but their effectiveness is often sub-optimal. Inoculation will help overcome sub-optimal symbioses and can be important to ensure that the early growth of smaller seeded perennial legumes is vigorous.

Clover symbioses are reasonably tolerant of low soil pH, but ideally soil pH should be greater than 5.5. Background soil rhizobia should not be relied upon in very low pH soils, even where good nodulation is observed in the pasture before renovation. Disruption of background rhizobia from soil micro-sites during pasture renovation may result in their death, resulting in the site becoming responsive to inoculation.

Most perennial clover seed is sold preinoculated. Survival time of rhizobia strain TA1 on seed is less than for other rhizobia. Seed should not be used where the period since inoculation exceeds two weeks, even if it has been stored under cool dry conditions. Seed that exceeds this expiry period should be re-inoculated. Freshly inoculated seed is preferred.

Seed size of many perennial clovers is small and inoculation rate needs to be adjusted accordingly. For white clover the standard 250g packet of peat inoculant is recommended for the inoculation of 25kg of seed.

The group C inoculant (WSM1325) for annual clovers should not be used as a substitute for the group B inoculant (TA1). Nitrogen fixation by the perennial clovers is significantly better with strain TA1.

Nodulation

Young clover plants should have at least 10 pink nodules per plant at eight to 10 weeks after sowing (see Figure 7.12).

Likelihood of response to inoculation for sown perennial clovers	
HIGH	• Caucasian clover should always be inoculated. • All perennial clovers where there is no history of clover having grown. • Soils with pH (CaCl$_2$) below 5.0 and where there is tillage at pasture renovation.
MODERATE	• No clover host in past four years and soil pH below 5.5. • Clover present, but growth or nodulation below expectation. May be associated with development of sub-optimal populations of soil rhizobia. High numbers of rhizobia on sown seed will compete with soil rhizobia at sowing but potency will diminish after several seasons.
LOW	• Soils with neutral or alkaline pH and a recent history of good clover growth and nodulation.

FIGURE 7.12 Well-nodulated white clover showing an abundance of nodules on the tap root and close to the crown of the plants.

7.14 SULLA
inoculation fact sheet

Sulla (special)	Strain: WSM1592
Hedysarum coronarium	*Rhizobium sullae*

Legume use and rhizobia distribution

Sulla is comparatively new to Australian agriculture, having only been sown on about 10,000 hectares annually since 2007. It is suited to moderate-to-high-rainfall zones (400 to 1000mm) and soils with pH ($CaCl_2$) in the range 5.5 to 8.0, but prefers alkaline soils. It is essential to inoculate sulla as their associated rhizobia are very specific and the species rarely nodulates with background rhizobia in the soil.

Inoculation method

Inoculation is mostly done with the application of a slurry of peat followed by pelleting with fine lime or other suitable product. Seed sold through retail outlets may be preinoculated.

Key considerations

Sulla tends to be a 'shy' nodulator and young seedlings quickly develop nitrogen deficiency symptoms where nodulation is inadequate. Higher rates of inoculation can be used to ensure adequate nodulation. One packet of peat inoculant (250g) should be used to inoculate 10kg seed. In preinoculated seed, the rhizobia have a very short shelf life and so seed is best sown as soon as possible after inoculation.

Nodulation

For sulla, four large (>5 mm) nodules per plant is satisfactory after eight to 10 weeks of plant growth (see Figure 7.13).

FIGURE 7.13 **Well-nodulated sulla plant.**

Likelihood of response to inoculation for sown sulla	
HIGH	• Sulla not previously grown; OR • soils with pH ($CaCl_2$) below 6.0.
MODERATE	• No sulla in past four years; OR • growth or nodulation of previous crop below expectation.
LOW	• Loam or clay soils with neutral or alkaline pH and a recent history (past two years) of sulla with good* nodulation.

* Good nodulation of sulla at eight weeks after planting is considered to be more than four large (>5mm) pink nodules.

APPENDIX: LEGUME INOCULANT MANUFACTURERS IN AUSTRALIA

Company: BASF Agricultural Specialties Pty Ltd, Australia and New Zealand
Address: 1205 Old Pacific Hwy, Somersby, NSW, 2250
Phone: 1800 803 440   02 4340 9410
Fax: 02 9475 0956
Email: info@basf.com
Web: www.basf.com.au

Company: New Edge Microbials Pty Ltd
Address: 951 Garland Avenue, Albury, NSW, 2640
Phone : 02 6025 0044
Fax: 02 6040 0237
Email: newedge@microbials.com.au
Web: www.microbials.com.au

Company: Novozymes Biologicals Australia Pty Ltd
Address: Lot 1, Bush's Lane, Bendigo, Victoria, 3550
Phone: 03 5443 6331
Fax: 03 5441 6611
Email: rgv@novozymes.com (Rob Velthuis, General Manager)
Web: www.bioag.novozymes.com

Company: ALOSCA Technologies Pty. Ltd.
Address: Unit 1/ 50 Atwell Street, Landsdale, WA, 6065
Phone: 08 6305 0123
Fax: 08 6305 0112
Email: cpoole@alosca.com.au (Chris Poole)
Web: www.alosca.com.au

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Review

Environmental impacts of dredging and other sediment disturbances on corals: A review

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ABSTRACT

A review of published literature on the sensitivity of corals to turbidity and sedimentation is presented, with an emphasis on the effects of dredging. The risks and severity of impact from dredging (and other sediment disturbances) on corals are primarily related to the intensity, duration and frequency of exposure to increased turbidity and sedimentation. The sensitivity of a coral reef to dredging impacts and its ability to recover depend on the antecedent ecological conditions of the reef, its resilience and the ambient conditions normally experienced. Effects of sediment stress have so far been investigated in 89 coral species (~10% of all known reef-building corals). Results of these investigations have provided a generic understanding of tolerance levels, response mechanisms, adaptations and threshold levels of corals to the effects of natural and anthropogenic sediment disturbances. Coral polyps undergo stress from high suspended-sediment concentrations and the subsequent effects on light attenuation which affect their algal symbionts. Minimum light requirements of corals range from <1% to as much as 60% of surface irradiance. Reported tolerance limits of coral reef systems for chronic suspended-sediment concentrations range from <10 mg L^{-1} in pristine offshore reef areas to >100 mg L^{-1} in marginal nearshore reefs. Some individual coral species can tolerate short-term exposure (days) to suspended-sediment concentrations as high as 1000 mg L^{-1} while others show mortality after exposure (weeks) to concentrations as low as 30 mg L^{-1}. The duration that corals can survive high turbidities ranges from several days (sensitive species) to at least 5–6 weeks (tolerant species). Increased sedimentation can cause smothering and burial of coral polyps, shading, tissue necrosis and population explosions of bacteria in coral mucus. Fine sediments tend to have greater effects on corals than coarse sediments. Turbidity and sedimentation also reduce the recruitment, survival and settlement of coral larvae. Maximum sedimentation rates that can be tolerated by different corals range from <10 mg cm^{-2} d^{-1} to >400 mg cm^{-2} d^{-1}. The durations that corals can survive high sedimentation rates range from <24 h for sensitive species to a few weeks (>4 weeks of high sedimentation or >14 days complete burial) for very tolerant species. Hypotheses to explain substantial differences in sensitivity between different coral species include the growth form of coral colonies and the size of the coral polyp or calyx. The validity of these hypotheses was tested on the basis of 77 published studies on the effects of turbidity and sedimentation on 89 coral species. The results of this analysis reveal a significant relationship of coral sensitivity to turbidity and sedimentation with growth form, but not with calyx size. Some of the variation in sensitivities reported in the literature may have been caused by differences in the type and particle size of sediments applied in experiments. The ability of many corals (in varying degrees) to actively reject sediment through polyp inflation, mucus production, ciliary and tentacular action (at considerable energetic cost), as well as intraspecific morphological variation and the mobility of free-living mushroom corals, further contribute to the observed differences. Given the wide range of sensitivity levels among coral species and in baseline water quality conditions among reefs, meaningful criteria to limit the extent and turbidity of dredging plumes and their effects on corals will always require site-specific evaluations, taking into account the species assemblage present at the site and the natural variability of local background turbidity and sedimentation.

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1. Introduction

Coastal construction, land reclamation, beach nourishment and port construction, all of which involve dredging, are increasingly required to meet the growing economic and societal demands in the coastal zone worldwide. In tropical regions, many shorelines are not only home to people but also to coral reefs, one of the most biodiverse ecosystems on earth (Hoeksema, 2007). World-wide, ~3 billion people depend more or less directly on coral reefs for a significant part of their livelihood, obtaining their protein needs or other essential commodities (Bryant et al., 1998). Even if not necessarily sustaining human life in many wealthier regions of the world, the economic value of the realised tourism potential of coral reefs can be enormous. For example, three southern Florida counties (Miami-Dade, Broward and Palm Beach) derive ~6 billion dollars annually from reef-oriented tourism and fisheries (Johns et al., 2001). Clearly, coral reefs are a biologically as well as economically valuable resource worth protecting. Unfortunately, coastal construction and dredging is frequently unavoidable in their immediate vicinity (Salvat, 1987).

The excavation, transportation and disposal of soft-bottom material may lead to various adverse impacts on the marine environment, especially when carried out near sensitive habitats such as coral reefs (PIANC, 2010) or seagrass beds (Erftemeijer and Lewis, 2006). Physical removal of substratum and associated biota from the seabed, and burial due to subsequent deposition of material are the most likely direct effects of dredging and reclamation projects (Newell et al., 1998; Thrush and Dayton, 2002). Dredging activities often disturb sediments reducing visibility and smothering reef organisms (Dodge and Vaisnys, 1977; Bak, 1978; Sheppard, 1980; Fortes, 2001). Coastal engineers and conservation officials need to balance the needs of a healthy economy, of which construction and dredging are often an integral part, with those of a healthy environment. Managing these potentially conflicting priorities can at times be a formidable challenge, particularly where coral reefs are concerned (Smith et al., 2007).

In many cases, dredging operations have contributed to the loss of coral reef habitats, either directly due to the removal or burial of reefs, or indirectly as a consequence of lethal or sublethal stress to corals caused by elevated turbidity and sedimentation. Dredging activities potentially affect not only the site itself, but also surrounding areas, through a large number of impact vectors (e.g. turbid plumes, sedimentation, resuspension, release of contaminants, and bathymetric changes) (Wolanski and Gibbs, 1992). Effects can be immediate or develop over a longer time frame and they may be temporary or permanent in nature. Some coral species appear to be more sensitive than others to increases in turbidity or sedimentation that are commonly associated with dredging operations. Their responses to such increases may depend on typical local conditions and vary between seasons. In general, the impact from dredging on corals and coral reef ecosystems is complex and far from fully understood. Yet there is an extensive body of experience to learn from. This experience lies with dredging contractors, in assessment reports, in monitoring data and in scientific literature derived from field-based and laboratory studies.

In this review we examine the environmental impacts of dredging on corals. We outline the type and level of dredging operations near coral reefs; provide an overview of the general impacts of sediment disturbances on corals; examine the current state of knowledge regarding sensitivity among and within coral species, tolerance limits and critical thresholds; and, finally, discuss mitigating factors and the potential for recovery. Where appropriate, these findings are illustrated with case studies. The focus of this review is limited to the effects of dredging on corals. The nomenclature of the coral species discussed in this review has been updated according to the most recent taxonomic revisions. The effects of dredging on other reef-associated organisms were not considered, except those depending on corals as specific host organisms. A similar analysis for seagrasses can be found in Erftemeijer and Lewis (2006). Information sources for the review included peer-reviewed scientific literature, "grey" literature in the form of environmental impact assessments, consultancy and technical reports, and additional information obtained from members of Working Group 15 of the Environmental Commission of the World Association for Waterborne Transport Infrastructure (PIANC, 2010). While the emphasis of this review is on the impacts of dredging, the findings and implications presented here are equally applicable to other sediment disturbances as sources of elevated turbidity or sedimentation (rivers, natural resuspension, flood plumes, bottom-trawling, etc.).

2. Dredging near coral reefs

An overview of 35 selected case studies of documented dredging operations in, near or around coral reef areas is presented in Table 1, which provides an indication of the scale and type of impact that dredging operations can have on corals and coral reefs. Undoubtedly, there are many more cases of coral damage associated with dredging operations worldwide, some of which are reported in confidential documents or in local languages, to which access is limited. Many of the historical dredging operations and port developments near coral reefs have never been documented and effects on corals were rarely quantified. The actual scale of dredging damage to coral reefs worldwide can therefore be assumed to be much greater than the available literature may suggest. Not all dredging projects near coral reefs lead to mortality of corals and not all observed changes in coral health in the immediate vicinity of dredging sites are necessarily the result of dredging-induced turbidity. Indeed, distinguishing the effects of anthropogenic disturbances from natural dynamics in the marine environment can be a challenge and calls for an appropriate monitoring design (Underwood, 2000; Stoddart et al., 2005). Nevertheless, the cumulative effects of dredging, filling and other coastal construction activities in coral reef environments have collectively resulted in major adverse ecological impacts on many reefs (Maragos, 1993).

Coral reefs are generally recognised as biogenic structures, but it is rarely appreciated that over half of the material in most coral reef complexes is actually made up of sediments (Hubbard et al., 1990; Dudley, 2003). Over 90% of the sediments on most coral reefs consist of carbonate (aragonite, high-magnesium calcite and calcite) produced by the growth and subsequent destruction of reef organisms as well as pre-existing reef rock through physical, chemical and biological erosion processes. Only on nearshore fringing reefs do silicate mineral grains from weathered and eroded igneous or metamorphic rocks (terrigenous sediments) constitute a significant part of the sedimentary material (Dudley, 2003). With time, the skeletons of primary and secondary reef organisms and loose sediments may be changed into a firm sedimentary rock (reef rock) and eventually into a dense solid limestone through consolidation of reef material, binding, cementation and diagenesis (Hubbard et al., 1990; Dudley, 2003). Levels of sedimentation in coral reef environments can vary substantially over spatial and temporal scales, often by several orders of magnitude within kilometres and weeks (Wolanski et al., 2005). Sedimentation is usually highest on inshore reefs and sheltered, wave-protected parts of reef systems, and decreases with distance from shore and with increasing exposure to wave energy (Wolanski et al., 2005).

Due to their geotechnical nature, limestone and coral materials tend to break when dredged and/or transported hydraulically

Table 1
Selected case studies of dredging operations near coral reefs and their impacts.

Country	Location	Year	Activity/purpose	Scale of impact/damage	References
Arabian Gulf	Various countries & locations	1990s–2008	Various mega-reclamations, coastline modifications and associated dredging	Widespread loss and degradation of productive coastal habitats incl. large stretches of coral reef	Sheppard et al. (2010)
Australia	Mud Island, Moreton Bay	1940s–1991	Coral dredging for cement manufacture	Loss of corals, development of shingle ridges that have restricted tidal flushing impacting adjacent mangroves	Allingham and Neil (1995)
Australia	Magnetic Island	1972	Dredging	Reduction in herbivores and reef dwellers	Marszalek (1981)
Australia	Cleveland Bay and Magnetic Island, Queensland	1970s	Capital & maintenance dredging at Ross River mouth and disposal at various dump sites in Cleveland Bay (peak in the early – mid 1970s)	Extensive burial of seagrass and coral habitats and impacts on mangroves (in combination with cyclones)	Pringle (1989)
Australia	Nelly Bay	2000–04	Capital dredging (35,000 m³) for marina	18 ha Construction area; no detectable impact immediately outside construction area	Chin and Marshall (2003) and Koloi et al. (2005)
Australia	Dampier, DPA & HI	2003–04	Capital dredging for port construction/ expansion total dredged volume 4.1 million m³	one site 80% loss within 1 km from dredging site, no discernable change due to dredging at other sites	Blakeway (2005) and Stoddart and Stoddart (2005)
Australia	Hay Point	2006	Capital dredging for port construction/ expansion total dredged volume 9 million m³	2–5% Loss of coral cover at 2 islands up to 6 km away from dredging site	Smith et al. (2007)
Australia	Dampier, HI	2006–07	Capital dredging for port expansion total dredged volume 3.4 million m³	<10% Gross coral mortality at impact sites	Hanley (2011)
Australia	Cape Lambert A	2007–08	Capital dredging for port construction/ expansion total volume 2.5 million m³ in <5 months	<3% Net coral mortality at impact sites	Hanley (2011)
Australia	Mermaid Sound, Pluto	2007–10	Capital dredging for port construction/ expansion total dredged volume 14 million m³	<6% Reduction in coral cover (Zone A) due to thermal bleaching; <5% net coral mortality in Zone B; <10% coral bleaching at monitoring sites in Zone C	Hanley (2011)
Bahrain	Fasht Al-Adham (east coast)	1985–92	Dredging and industrial development	Loss of at least 22 hectares of coral reef and degradation of a further 8 ha due to increased turbidity and sedimentation	Zainal et al. (1993)
France	Guadeloupe	1979	Dredging	Unbalanced fish community – disappearance of 20 out of 29 spp.	Galzin (1982)
French Polynesia	Tahura lagoon, Moorea	1981	18 ha dredged	Destruction of corals, reduced species composition, changes in invertebrate fauna favouring gastropods instead of crustaceans, disruption of stability of reef & lagoon ecosystems	Naim (1981)
French Polynesia	Tiahiti (36 sites)	1959–1983	Dredging by hydraulic shore & bucket dredgers total volume 2.5–3.0 × 10⁶	Dredge & fill destroyed 43% of fringing reefs in Papette and 75% in FAAA region; hard bottoms colonized by turf algae after dredging; fish populations reduced	Gabrie et al. (1985)
Hong Kong	Ninepin Islands	1991–93	Trailer dredging of up to ∼400 million m³ at 20 borrow areas	Build-up of fine sediment in shallow water; 40% reduction in live coral in 3 months; sign. increase in % *Acropora* colonies damaged	Hodgson (1994)
Indonesia	Turtle Island, Bali	1997	Dredging & reclamation (20 million m³)	No detectable impacts at 1 km from work area; used an adaptive monitoring & mgt. approach	Driscoll et al. (1997)
Kiribati	Fanning Island	1971	Dredging	Live coral cover reduced from 62% to 31% over time	Roy and Smith (1971)
Malaysia	Bintulu	2005	Dredging at borrow areas (4 million m³)	No detectable impacts at nearest reef ∼2 km from borrow area	Doorn-Groen (2007)
Micronesia	Truk Atoll, Eastern Caroline Islands	1981	Dredging (2 million cubic yards)	Fish abundance and diversity significantly reduced	Amesbury (1981)
Netherlands Antilles	Piscadera Bay, Curacao	1972	Dredging	*Porites astreoides* (plating form) died as result of inability to reject sediment; calcification rates of *Madracis mirabilis* and *Agaricia agaricites* decreased by ∼33% over a 4-week period	Bak (1978)
Netherlands Antilles	Bonaire	1980–83	Dredging and large coastal resort development	Significant coral mortality due to sedimentation and excavation for channel & breakwater construction	van 't Hof (1983)
Thailand	Phuket	1981	Tin dredging; 11.6 km² dredged with 3 tin dredgers (200,000 yd³/month)	Reefs adjacent to dredging severely damaged by sedimentation (4% coral cover compared to 26–34% in non-impacted areas)	Chansang et al. (1981)
Thailand	Phuket	1986–87	Dredging of 1.3 million m³ by hydraulic dredgers (9-months dredging & disposal operation)	30% Reduction in coral cover and a decline in species diversity for up to 1 year; maximum conc. 286 mg/l; rapid recovery in 22 months	Brown et al. (1990)
Singapore	coastline	1970s–90s	Coastal reclamation and dredging along almost the entire shoreline of Singapore	Loss of approx. 60% of Singapore's coral reefs; remaining reefs subjected to sediment impact	Hilton and Manning (1995) and Chou (2006)
Singapore	Southwest Islands	2006	Dredging and reclamation (9 million m³)	No detectable impacts 300 m outside direct impact area; used adaptive monitoring & management approach	Doorn-Groen (2007)

Table 1 (*continued*)

Country	Location	Year	Activity/purpose	Scale of impact/damage	References
UK	Diego Garcia, Chagos	1980	Dredging	Coral diversity unaffected by dredging	Sheppard, 1980
UK	Castle Harbor, Bermuda	1941–1943	Extensive dredging and filling for construction of Kindley Airfield (US navy base)	Mass coral mortality due to dredging in harbor area major shift in nearby reef community structure towards more tolerant coral species	Dodge and Vaisnys (1977) and Flood et al. (2005)
USA	Johnston Atoll	1966	Dredging (440 ha)	Reduction of living corals by up to 40%; reduction in reef fish abundance & development of blue-green on dead coral	Brock et al. (1965)
USA	Kaneohe Bay, Hawaii	1974	Dredging	Up to 30% of corals died & overgrown with algae	Banner (1974)
USA	Johnston Atoll	1976	Airfield construction activities	40% Reduction in coral cover due to siltation from airfield construction activities	Amerson and Shelton (1976)
USA	Miami Beach, Florida	1977	Large-scale dredging operations	1 cm sediment cover on nearby reef surface in <2 h; partial mortality & paling of affected corals; up to 32% of corals exhibiting signs of stress; small colonies displayed tissue mortality	Marszalek (1981)
USA	Southeast Florida	1995	Dredge & fill (350,000 m³) for beach widening	Burial & loss of 5 ha of nearshore hard-bottom habitat; 30× drop in fish density, 10× drop in fish diversity	Lindeman and Snyder (1999)
USA	Florida	1985–2004	26 Projects involving filling and dredging for beach nourishment and port development	217 Acres of reef impacted by cumulative effects	PBS&J, (2008)
USA	Florida	2005–06	Dredging for Broward County beach nourishment (10.9 km of beach with 1.5×10^6 m³ of sand)	Increased sedimentation during construction, no effects on %cover; minor to moderate coral stress; rapid post-dredging recovery	Fisher et al. (2008)
USA	Key West (Florida)	2006	Key West harbour dredging project	No significant effects on % live coral cover; some paling & bleaching	CSA (2007)

(Schlapak and Herbich, 1978; Maharaj, 2001). From the freshly broken surface, very fine silt and colloidal material can be released into the water, creating milky white "clouds". These fine sediment clouds are difficult to control, as they can remain in suspension for prolonged periods and thus spread over large areas under the action of currents, wind and waves. It is therefore imperative to minimise the need for dredging coral material and to exercise great care and accuracy when dredging in coral reef environments. Some excellent guidelines on best management practices for dredging and port construction near coral reefs were published recently (PBS&J, 2008; PIANC, 2010). In the case of contaminated sediment, dredging may also lead to deleterious effects on water quality and reef-associated biota by the release of contaminants (Brown and Holley, 1982; Lay and Zsolnay, 1989; Esslemont et al., 2004). Dredgers and port engineers possess a wide range of tools to reduce their impact on the environment either by design or by choice of low-impact building methods (Bray, 2008). Various environmental regulatory agency permitting processes are intended to give engineers the information required to maintain any given project's impacts within the legally required, or otherwise agreed-upon, limits. Given the potential for adverse effects of dredging on sensitive marine habitats such as coral reefs, the management and monitoring of those activities that elevate turbidity and sediment-loading is critical. In practice, however, this has proved difficult as the development of water quality threshold values, upon which management responses are based, are subject to a large number of physical and biological parameters that are spatially and temporally specific (Sofonia and Unsworth, 2010).

It should be noted here that many coral reef environments demonstrate substantial natural variability in background turbidity due to resuspension as a result of metocean conditions such as tides, wind, waves, storms, cyclones, tsunamis and river floods, which in some areas can increase the suspended-sediment concentrations to levels similar to those occurring during dredging (Harmelin-Vivien, 1994; Schoellhamer, 2002; Anthony et al., 2004; Larcombe and Carter, 2004; Orpin et al., 2004; Storlazzi et al., 2004; Ogston et al., 2004; Kutser et al., 2007; Jouon et al., 2008).

It is almost impossible to predict levels and patterns of increased turbidity and sedimentation during dredging operations without

sophisticated numerical modelling of site-specific hydrodynamic and sediment transport processes (Winterwerp, 2002; Hardy et al., 2004; Aarninkhof and Luijendijk, 2010). Total suspended sediment (TSS) concentrations experienced at a given distance from a dredging operation may vary by up to two orders of magnitude depending on the scale of the operation, the techniques used, background water quality conditions and the nature of the substrate that is dredged (or disposed of). Kettle et al. (2001) recorded suspended-sediment concentrations of >150 mg L^{-1} to be laterally confined to within about 100 m of a dredger in Cleveland Bay (Townsville, Australia). Plumes exceeding 20 mg L^{-1} extended for up to about a kilometre from the actual dredging or placement operation (Kettle et al., 2001). Thomas et al. (2003) reported a general regime of suspended-sediment concentrations >25 mg L^{-1} (90% of the time) for several months during dredging operations over fringing coral reefs at Lihir island (Papua New Guinea) with regular (short-term) peak increases above 1000 and 500 mg L^{-1} (in severe and transitional impact zones) in an area that normally experience background TSS concentrations of <5 mg L^{-1}. In contrast, Stoddart and Anstee (2005) recorded suspended-sediment concentrations above 10 mg L^{-1} for 42% of monitoring days at impacted coral reef sites (within 1 km of dredging locations, occasionally peaking to ~60 mg L^{-1}) during dredging operations in Mermaid Sound (Dampier, Western Australia) against a low background level of ~4 mg L^{-1} at reference sites.

A poor understanding of responses of corals to sediment disturbances can result in inappropriate management of dredging projects that may lead to preventable coral mortality or unnecessarily high costs from down-time and delays in dredging operations. There are many examples of dredging operations near coral reefs where inadequate management has contributed to significant damage to reefs and mortality of corals (Table 1). Conversely, exaggerated (over-conservative) thresholds used for predicting levels of coral mortality from dredging can lead to unrealistically high levels of predicted coral mortality over large areas of presumed impact. A review of ten recent (large) capital dredging projects near coral reefs in the Pilbara region (Western Australia) described how conditions governing environmental controls and monitoring requirements have become increasingly comprehensive, prescriptive and onerous since 2003 (Hanley, 2011). However, in none of these case

studies was there evidence of any breach (non-compliance) of the permitted levels of impacts on corals. In fact, observed mortality of corals in these projects typically was far below predictions and could in many cases be attributed to other factors not related to dredging (e.g. cyclonic events and thermal bleaching). The review warned about the consequences of such routine overestimation of dredging impacts to corals, including the misinformation of the public, unrealistically large offset packages and unnecessarily large monitoring and baseline programs to areas well outside the real range of impacts (Hanley, 2011). These examples from Western Australia, along with the various case studies summarised in Table 1, clearly demonstrate the need for strengthening capacity in predicting and managing impacts of dredging through thorough literature reviews, a critical evaluation of past dredging projects near corals, and targeted experimental research (Lavery and McMahon, 2009).

The main effects of dredging and port construction on corals—besides direct physical removal, damage or burial—include temporarily increased turbidity and enhanced sedimentation. In order to understand how corals are affected by enhanced turbidity and sedimentation, it is important to first gain some basic understanding on how corals function.

3. The impacts of sediment disturbance on corals

With the exception of free-living species, corals—once settled—are sessile organisms (Hoeksema, 1988, 1993; Hubmann et al., 2002; Hoeksema and de Voogd, 2012). As they cannot move away from unfavourable conditions, growth-form and physiological changes regulate their interactions with the environment. Much of the success of reef-building corals relies on symbiotic, unicellular algae called zooxanthellae, which live as symbionts inside the coral tissue (primarily the gastrodermis) and produce the majority of the coral's energy requirements through photosynthesis. Because of this symbiosis, most corals require light to survive (Achituv and Dubinsky, 1990). The major problems arising from turbidity and sedimentation derived from coastal construction and dredging are related to the shading caused by decreases in ambient light and sediment cover on the coral's surface, as well as problems for the feeding apparatus under a sediment blanket and energetic costs associated with mucus production, sediment clearance and impaired feeding. Suspended sediments, especially when fine-grained, decrease the quality and quantity of incident light levels, resulting in a decline in photosynthetic productivity of zooxanthellae (Falkowski et al., 1990; Richmond, 1993). Non-photosynthetic corals are an exception to this but while they may not suffer from light reduction, they can be impacted by high loads of suspended sediment through clogging and smothering. Many corals are primarily light-traps and thus their growth form is not necessarily optimised for sediment-shedding. As a result, certain morphologies are prone to collect more sediment from the water column than the coral is able to clear (Hubbard and Pocock, 1972; Bak and Elgershuizen, 1976; Dodge and Vaisnys, 1977; Rogers, 1983; Stafford-Smith, 1993; Sanders and Baron-Szabo, 2005). Turbidity reduces ambient photosynthetically active radiation (PAR) and leads to a decrease in zooxanthellae productivity which can result in starvation. Sediment settling on coral tissue causes additional shading and smothering, and in this way contributes to a further decrease of the photosynthetic activity by zooxanthellae and can even lead to coral bleaching (Glynn, 1996; Brown, 1997).

High turbidity and sedimentation rates may depress coral growth and survival due to attenuation of light available to symbiotic zooxanthellae and redirection of energy expenditures for clearance of settling sediments. Thus, the potential effects of sediment input not only include direct mortality, but also involve
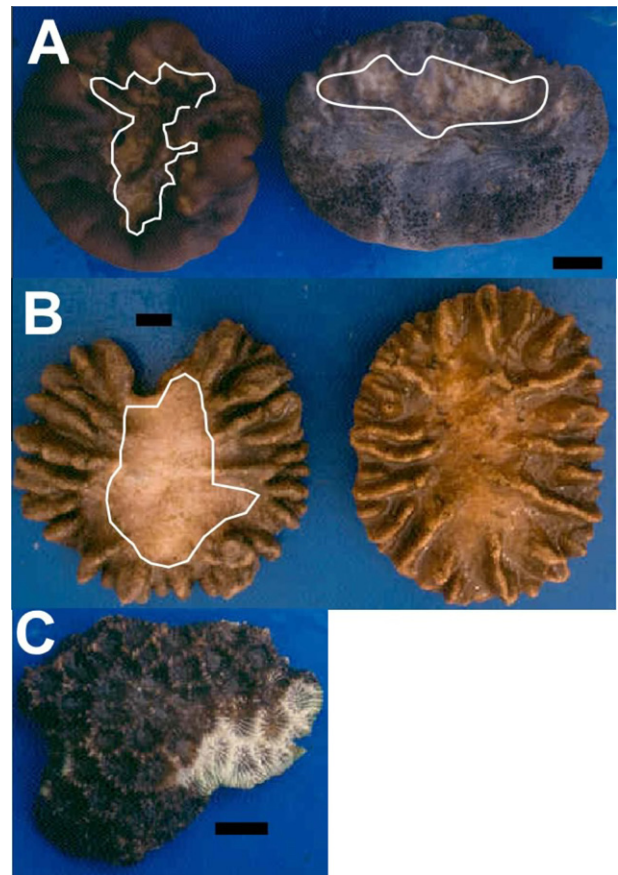
Fig. 1. Sublethal effects of sedimentation on corals. (A) After two weeks of intense sedimentation, large tissue necroses appeared on these *Lobophytum depressum* (left) and *Lobophytum patulum* (right). (B) At the same time *Sinularia dura* colonies were bleached where the sand had rested on them (left experimental animal, right control animal). (C) tissue necrosis on *Favites pentagona* after four weeks of sedimentation.

sublethal effects such as reduced growth, lower calcification rates and reduced productivity, bleaching, increased susceptibility to diseases, physical damage to coral tissue and reef structures (breaking, abrasion), and reduced regeneration from tissue damage (Fig. 1). Sediment disturbance can also affect coral recruitment and have impacts on other (non-coral) reef-dwelling organisms. As pointed out by Johannes (1975), selective mortality of corals results in the migration or death of other fauna, suggesting that the environmental tolerances of the associated reef community are unlikely to exceed those of the component corals. As the stress level caused by enhanced turbidity and sedimentation increases, the response of corals shifts from photo-physiological effects, changes in polyp activity and mucus production at the level of individual coral polyps, to colour changes, bleaching and partial tissue necrosis of coral colonies (Meesters et al., 1992; Stafford-Smith, 1993; Riegl, 1995; Riegl and Branch, 1995; Fabricius, 2005). Ultimately, severe and long-lasting stress from sustained sediment disturbances may result in wide-spread coral mortality, changes in community structure and major decreases in density, diversity and coral cover of entire reef systems (Table 2; adapted from Gilmour et al., 2006).

The risk and severity of impacts from dredging on corals is directly related to the intensity, duration and frequency of exposure to increased turbidity and sedimentation (Newcombe and MacDonald, 1991; McArthur et al., 2002). Very high sediment stress levels over relatively short periods may well result in sublethal and/or lethal effects on corals, while long-lasting chronic exposure

Table 2
Schematic cause-effect pathway for the response of corals and coral communities to sedimentation and turbidity. Level of stress increasing from top to bottom (adapted from Gilmour et al., 2006).

	Sedimentation	Turbidity
Stress		
Photophysiological stress	• Reduced photosynthetic efficiency of zooxanthellae and auto-trophic nutrition to coral	• Reduced photosynthetic efficiency of zooxanthellae and autotrophic nutrition to coral; switch to heterotrophic feeding, ingestion of sediment particles
Changes in polyp activity	• Extrusion of mesenterial filaments following severe stress • Increased ciliary or polyp activity, and tissue expansion in some species, to remove sediment	• Increased ciliary or polyp activity to feed
Mucus production	• Increased mucus production or sheeting to remove sediment	• Evidence of mucus production
Severe stress		
Sediment accumulation	• Accumulation of sediment on tissue of susceptible growth forms due to failure of mechanisms of rejection	
Change in coral colour	• Change in coral colour arising from changes in the density of zooxanthellae and photosynthetic pigments • Paling of coral due to partial bleaching	• Change in coral colour arising from changes in the density of zooxanthellae and photosynthetic pigments • Darkening of coral in response to reduced light due to photoacclimation
Bleaching	• Considerable whitening of corals due to the expulsion of a large proportion of zooxanthellae from the colony	• Not known
Partial mortality	• Injury to coral tissue, loss of polyps and partial mortality of the colony • Decrease in (live) coral cover	• Injury to coral tissue, loss of polyps and partial mortality of the colony • Decrease in (live) coral cover
Mortality	• Mortality of small-sized colonies and partial mortality of large corals • Mortality of susceptible species and size classes. • Decreased density, diversity and coral cover • Changes in community structure • Wide-spread mortality of corals • Major decreases in density, diversity and coral cover • Dramatic changes in community structure, and shifts towards the dominance of non-coral species, such as sponges and algae	• Mortality of susceptible species and size classes • Decreased density, diversity and coral cover • Changes in community structure • Wide-spread mortality of corals • Major decreases in density, diversity and coral cover • Dramatic changes in community structure, and shifts towards the dominance of non-coral species, such as sponges and algae
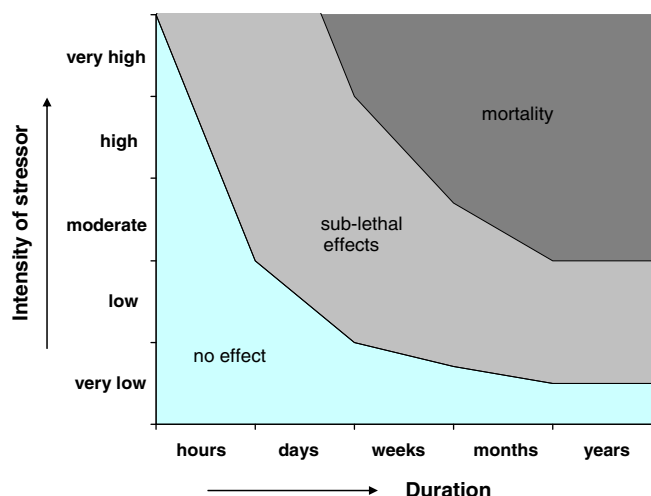
Fig. 2. Conceptual relationship between the intensity and duration of a stress event and the risk of sublethal and lethal effects on corals. This graph shows the general relationship between the magnitude of an increase in turbidity or sedimentation above background levels (vertical axis), how long it lasts (horizontal axis) and the onset of (sub)lethal effects on corals. Actual thresholds will vary by location based on typical ambient conditions, sediment properties (e.g. grain-size) and the sensitivity of the coral species.

to moderate levels of sediment stress may induce similar effects (Fig. 2). Repetitive stress events could result in deleterious effects much sooner if corals have not been allowed sufficient time to recover between consecutive disturbances (McArthur et al., 2002). Excessive sedimentation from land runoff and dredging events superimposed on other stresses from natural processes and anthropogenic activities can cause substantial impacts on coral health and dramatic declines in live coral cover (Field et al.,

2000). It should be noted, however, that a number of studies have demonstrated the occurrence of coral reefs (often with high live coral cover) in areas of high and fluctuating turbidity and sedimentation, for example from the inner shelf of the Great Barrier Reef (Mapstone et al., 1989; Hopley et al., 1993; Larcombe et al., 1995; Anthony and Larcombe, 2000). Tolerance of corals to increased turbidity and sedimentation may vary seasonally and geographically, similar to what has been demonstrated for thermal thresholds (Weeks et al., 2008).

In this section we provide a brief overview of the main impacts of sediment disturbance on corals by first examining turbidity (light for photosynthesis), then sedimentation (feeding and respiration), then effects on sexual recruitment (larval survival and settlement) and, finally, the impact of associated nutrients and contaminants.

3.1. Turbidity and light for photosynthesis

Turbidity and light availability in the marine environment are measured and expressed in a number of different ways. Common measures for turbidity include concentration of total suspended solids (TSS, in milligrams per litre), suspended-sediment concentration (SSC, in milligrams per litre), nephelometric turbidity units (NTU), Secchi disc readings (in centimetres), and attenuation coefficient (k_d). Conversion factors between these different measures are site-specific, depending on various local factors, including particle-size distribution, contribution of phytoplankton and organic content (Gray et al., 2000; Thackston and Palermo, 2000). Light availability is generally measured directly in micromole photons per square metre per day, or expressed as a relative measure (minimum light requirement) in percentage of surface irradiance (% SI). Photosynthetically active radiation (PAR) is most commonly taken as being between 400 and 700 nm, which corresponds approximately to visible light (Kirk, 1977). At any depth, the

underwater light field is highly variable and exactly how much light reaches any particular habitat will depend on factors such as orientation of the sun, the weather, shading, reflection, and refraction (Weinberg, 1976; Falkowski et al., 1990). The amount of light an organism will be exposed to is also contingent upon its vertical angle and compass direction (Weinberg, 1976; Falkowski et al., 1990; Dunne and Brown, 2001).

Light reduction is probably the most important of all sediment-related effects on corals. Light decreases exponentially with depth due to a process of attenuation (extinction), i.e. the absorption and scatter of light by water molecules, particulate solids, and dissolved matter (Weinberg, 1976; Falkowski et al., 1990). Maximal growth and development of reef corals usually occurs down to 30% to 40% of subsurface irradiance (SI) and rarely is any significant reef formation found below 10% SI (Achituv and Dubinsky, 1990). Photosynthetic carbon fixation by zooxanthellae in *Montastrea annularis* (a species with one of the widest depth distributions) was found to decrease by more than 93% between 0.5 and 50 m depth (Battey and Porter, 1988). Available light was found to be the primary factor responsible for monthly variations in growth of three hermatypic coral species in Curaçao (Bak, 1974). Shading by large *Acropora hyacinthus* table corals (causing light levels to fall exponentially to ~1% of outside values as a light meter was moved under the table) was found to significantly reduce "understorey" coral density, cover and diversity beneath the table corals compared with adjacent unshaded areas (Stimson, 1985). Shading of a 20 m² area of San Cristobal Reef off south-western Puerto Rico for five weeks altered community structure, decreased net reef productivity and caused bleaching and death of several hard coral species (Rogers, 1979).

As a response to lower light levels, most mesophotic reef corals often exhibit flat, plate-like morphologies to maximise light capture and may also utilise different symbionts (Bongaerts et al., 2010, 2011). Such plate-like morphology, however, more easily traps sediment, and although this increased susceptibility to sedimentation is normally not problematic due to the relatively lower rates of sedimentation on the deeper reef, increased sediment levels can result in large-scale mortality among mesophotic corals (Bak et al., 2005; Bongaerts et al., 2010).

Even in clear tropical waters, light intensity is reduced by 60% to 80% in the top 10 m of water (Kinzie, 1973) but attenuation increases in turbid waters (Kirk, 1977). Concordantly, the total energy available for the life processes of autotrophs is also reduced (Thurman, 1994), affecting coral distribution (Roy and Smith, 1971; Jaubert and Vasseur, 1974; Titlyanov and Latypov, 1991) as well as photosynthesis and respiration (Rogers, 1979; Telesnicki and Goldberg, 1995). Decreases in algal productivity causes a drop in the nutrition, growth, reproduction, calcification rate and depth distribution of corals. In some coral species, this drop in productivity can eventually result in the coral starving (Richmond, 1993). In Singapore, chronic levels of sedimentation over the last 30–40 years has resulted in underwater visibility being reduced from 10 m recorded in the early1960s to a contemporary average of 2 m (Chou, 1996). Chuang (1977) found only 10% of surface light reached down to 8 m depth, 5% to 10 m depth and 0.35% to 16 m depth at two sampling stations, whereas Todd et al. (2004a) found <0.6% surface PAR reaching 8.9 m at one of their "best" sampling sites. There is very little coral cover around Singapore beyond 8 m depth. Wave-driven resuspension of bottom sediments in shallow areas and/or tidal currents transporting material off corals may also be important, preventing direct negative effects of sedimentation on reefs in such marginal environments (Chou, 1988; Bak and Meesters, 2000).

Results of field studies on coral distributions have indicated a negative correlation between suspended sediment loads and hard coral abundance (Rice and Hunter, 1992). Coral communities are generally better developed, are more diverse and have greater coral cover and rates of coral growth the lower the sediment load (Rogers, 1990; Fabricius, 2005). Long-term exposure to elevated levels of suspended sediment can cause reduced coral growth and reduced reef development (Rice and Hunter, 1992), although recent studies from nearshore reefs in the Great Barrier Reef would argue against this, where there is evidence of spatially relevant and temporally persistent reef-building having occurred over millennial timescales (Larcombe et al., 1995; Anthony and Larcombe, 2000).

Monitoring data from the west coast of Barbados indicated a 20% reduction in the annual growth rate of *Montastraea annularis* in response to a 28% increase in average long-term background suspended-sediment levels (Hawker and Connell, 1989). Coral cover and diversity are greatly reduced near sources of terrigenous sediment input and runoff (e.g. rivers) and tend to increase with distance from the river mouth (Acevedo et al., 1989; Hoeksema, 1990; van Katwijk et al., 1993; Kleypas, 1996; Woolfe and Larcombe, 1999; Nugues and Roberts, 2003; Fabricius, 2005; Dikou and Van Woesik, 2006a; Cleary et al., 2006, 2008; Golbuu et al., 2008; Hennige et al., 2010; van der Meij et al., 2010). In the geological record, increased turbidity has been implicated as a major factor in the demise of several coral reefs in the western Atlantic (Adey et al., 1977; Lighty et al., 1978; Macintyre, 1988; Achituv and Dubinsky, 1990; Kleypas, 1996). At larger spatial scales, however, increased terrigenous sediment supply due to human impacts on catchments may not necessarily lead to increased turbidity or sedimentation at reefs further offshore and corals can indeed survive well in some turbid environments (Larcombe and Woolfe, 1999; Perry and Larcombe, 2003; Perry, 2005; Perry and Smithers, 2010).

There is some indication that elevated turbidity can reduce thermal bleaching damage to reefs, suggesting a photo-protective effect during thermal anomalies making shallow-water corals in turbid waters less susceptible to bleaching than those in clear waters (Phongsuwan, 1998; Piniak and Storlazzi, 2008) but this requires further study.

3.2. Sedimentation: feeding and respiration

Sedimentation and burial in the marine environment are measured and expressed in a number of different ways. Sedimentation (sometimes also called "siltation" or "deposition") is usually expressed as a rate (in mg cm^{-2} d^{-1}) or in thickness (mm) of the sediment layer (instantaneous, or accumulating over time). Water turbidity and sedimentation correlate only in part because increased turbidity does not necessarily lead to increased sediment deposition (Larcombe and Woolfe, 1999). A range of methods is available for field measurements of sediment accumulation or sediment elevation change in underwater environments, all of which have merits and shortcomings (Thomas and Ridd, 2004). Despite their widespread use in this setting, sediment traps do not provide quantitative information about "net" sedimentation on coral surfaces (Storlazzi et al., 2011). Sediment traps can, however, yield useful information about the relative magnitude of sediment dynamics in different areas, as long as trap deployment standards are used for trap height, trap-mouth diameter, height of trap mouth above the substrate and spacing between traps (Jordan et al., 2010; Storlazzi et al., 2011).

Sedimentation on coral reefs may cause smothering of coral polyps (Fig. 3; Fabricius and Wolanski, 2000), inhibiting photosynthetic production and increasing respiration as well as creating a diffusion barrier. In a study by Abdel-Salam and Porter (1988), daytime photosynthesis in corals exposed to sediments decreased, while at night-time respiration increased. Stafford-Smith (1993) measured a drop in photosynthesis to respiration (P:R) ratios for

Fig. 3. Partial coverage of corals with sediment transported by plume and currents from nearby dredging works (Photo courtesy: Tony Ayling).

smothered corals. Corals will attempt to clean themselves of this sediment by a combination of ciliary action and the production and sloughing off of mucus sheets. This, however, is expensive in energy and can lead to exhaustion of mucus-producing cells (Peters and Pilson, 1985; Riegl and Bloomer, 1995; Riegl and Branch, 1995). At the individual (colony) level, energy diverted to clearing the colony surface of sediment can lead to growth inhibition and a reduction in other metabolic processes (Dodge and Vaisnys, 1977; Rogers, 1983; Edmunds and Davies, 1989). At the population level, increased sedimentation may inhibit sexual population recruitment, cause changes in the relative abundance of species, decrease live coral cover and reduce the abundance and diversity of corals and other reef fauna, including fish (Brock et al., 1965; Amesbury, 1981; Rogers, 1990; Gilmour, 1999; Bray and Clark, 2004). It may also, however, cause increased rates of asexual reproduction in free-living corals that show partial mortality (Gilmour, 2002, 2004).

Furthermore, cover by sediment interferes with the coral's feeding apparatus, by causing polyps to retract and tentacular action to cease. Sufficient sediment overburden may make it completely impossible for corals to expand their polyps and thus can inhibit the coral compensating for its losses in autotrophic food production by heterotrophic activity. While some corals are able to ingest sediment particles in turbid conditions and derive some nutritional value from them (Rosenfeld et al., 1999; Anthony et al., 2007) or even build up higher lipid energy reserves (Anthony, 2006), most corals cease activity when confronted with heavy sediment loads.

Corals can withstand a certain amount of settling sediment, as this occurs naturally (Rogers, 1977, 1990; Perry and Smithers, 2010). Many species have the ability to remove sediment from their tissues, either passively (through their growth form) or actively (by polyp inflation or mucus production, for example). Sediment rejection is a function of morphology, orientation, growth habit and behaviour of the coral and the amount and type of sediment (Bak and Elgershuizen, 1976). Corals growing in areas where they typically experience strong currents or relatively high wave energy generally have no need for effective (active) sediment rejection mechanisms, as the turbulence of the water assists in the passive cleaning of any sediment that may have accumulated on the coral tissue (Riegl et al., 1996; Hubmann et al., 2002; Sorauf and Harries, 2010). Many branching corals appear very effective in passive rejection of sediment because of their colony morphology, but they may suffer from reduced light levels. Massive and plating coral colonies, on the other hand, though usually more tolerant of turbid conditions, are more likely to retain sediment because of their shape and a lack of sediment rejection capabilities and thus tend to have a relatively low tolerance to sedimentation (Brown and Howard, 1985).

Various species of free-living mushroom corals that live on reef flats and slopes can occur on a range of substrata, whereas those that live deeper on the sandy reef bases usually live on sediment (Hoeksema and Moka, 1989; Hoeksema, 1990, 1991b). As juveniles, mushroom corals live attached and only after a detachment process

do they become free-living and mobile (Hoeksema, 1989, 2004; Hoeksema and Yeemin, 2011). Some free-living mushroom coral species show a large detachment scar and their juveniles remain relatively long in the attached anthocaulus phase. A possible reason for postponed detachment is to avoid burial of the juvenile coral, especially if the coral remains vertically oriented so that sediment can more easily be shed than in a horizontal position (Chadwick-Furman and Loya, 1992). The evolutionary development of additional mouths over the upper surface in mushroom corals has resulted in the growth of larger coralla but also in a greater chance of survival during sedimentation—if one mouth is blocked by sediments, others remain intact (Hoeksema, 1991a; Gittenberger et al., 2011). In free-living mushroom corals, budding or fragmentation in combination with regeneration and mobility facilitates continuous growth and may result in large and dense accumulations of specimens on sandy surfaces (Pichon, 1974; Littler et al., 1997; Hoeksema, 2004; Hoeksema and Gittenberger, 2010; Hoeksema and Waheed, 2011).

3.3. Effects on sexual recruitment, larval survival and settlement

Sedimentation and turbidity not only influence the survival of adult corals, but also their reproductive success and probability of recruitment, as well as the survival and settlement of coral larvae (Babcock and Smith, 2000; Birrell et al., 2005). Sedimentation at a level that only partially covers the substrate and that is not directly harmful to adult colonies, and even suspended sediment, can significantly reduce larval recruitment by inhibiting settlement and reducing larval survival in the water column (Gilmour, 1999; Babcock and Smith, 2000; Birrell et al., 2005; Goh and Lee, 2008) although this is not always detectable in field studies (Fisk and Harriott, 1989). Settlement rates are near-zero on sediment-covered surfaces, and sedimentation tolerance in coral recruits is at least one order of magnitude lower than for adult corals (Fabricius, 2005).

Babcock and Davies (1991) evaluated effects on settlement rates of *Acropora millepora* larvae in aquaria under 0.5–325 mg cm^{-2} d^{-1} sedimentation. Higher sedimentation rates reduced the number of larvae settling on upper surfaces, but total numbers of settled larvae were not significantly affected by sedimentary regime. This was, however, likely an artefact since, in the field, accumulation of sediment on upward-facing surfaces would indeed greatly reduce the overall amount of suitable substratum available. Hodgson (1990b) investigated the larval settlement rate of *Pocillopora damicornis* on bare glass and on glass covered with measured amounts and area of fine sediment finding significant reduction due to sediment. Sediment cover of 95% completely prevented settlement. There was no increase in settlement when sediment cover was reduced from 90% to 50% of the glass surface area. In highly turbid conditions (>100 mg L^{-1}, which would not be unusual at sites in close proximity to a dredging operation), significant numbers of settled planulae of *Pocillopora damicornis* underwent reversed metamorphosis ("polyp bail-out"), indicating conditions were not appropriate for continued growth and development (Te, 1992). Chronic exposure

to sedimentation rates of 10–15 mg cm^{-2} d^{-1} caused a 50% decrease in fecundity in *Acropora palifera* in Papua New Guinea (Kojis and Quinn, 1984).

Elevated levels of suspended sediment (50 mg L^{-1}, 100 mg L^{-1}) affected fertilisation, larval survival, and larval settlement in *Acropora digitifera* (Gilmour, 1999). While post-fertilisation embryonic development was not inhibited by suspended sediments, larval survival and larval settlement were significantly reduced. Significant declines in fertilisation success were reported for *Acropora millepora* at suspended-sediment levels ⩾100 mg L^{-1} compared with lower levels ranging from 0 to50 mg L^{-1} with approximately 36% fertilisation at the highest tested suspended-sediment levels of 200 mg L^{-1} (Humphrey et al., 2008). Elevated concentrations of suspended sediment (43 mg L^{-1}, 159 mg L^{-1}) also significantly reduced fertilisation success in *Pectinia lactuca* compared with controls (Erftemeijer et al., 2012).

These findings imply that increased levels of suspended sediment and/or sedimentation due to dredging operations—especially when coinciding with the main spawning season of corals—may affect their reproductive success, compromise coral recruitment and thereby compromise the recovery of degraded reefs (Erftemeijer et al., 2012). The same issues are probably relevant in naturally or episodically turbid (higher stress) settings.

3.4. Nutrients and contaminants

The mucus coat that surrounds corals, which is moved off the coral by ciliary action and is replaced repeatedly, acts as their primary defence against precipitated sediment particles. A potentially problematic by-product of this abundant mucus production can be fertilisation of the nearby water potentially causing population explosions of bacteria (Mitchell and Chet, 1975; Coffroth, 1990; Ritchie and Smith, 2004; Brown and Bythell, 2005; Klaus et al., 2007). The metabolism of these bacteria can lead to local anoxic conditions and concomitant death of coral tissue in the immediate vicinity. Furthermore, high nutrient contents of silt can lead to microbial activity, eventually causing the underlying coral tissue to become necrotic (Weber et al., 2006; Hodgson, 1990a). Conversely, some coral species have been observed to exploit nutrient-rich suspended particles as a food source, thereby compensating for the stress caused by sedimentation (Fabricius and Wolanski, 2000).

Numerous kinds of terrestrial pollutants, including those from sewage and agricultural runoff, make their way into nearshore sediments that can be resuspended by dredging operations and subsequently cause eutrophication of coastal waters (Kenchington, 1985; Grigg and Dollar, 1990; San Diego-McGlone et al., 2008; Todd et al., 2010). As corals generally grow in oligotrophic waters, elevated nutrient levels can lead to a range of negative effects on coral health (Hawker and Connell, 1989), reduced fertilisation success (Harrison and Ward, 2001) and settlement rates (Hunte and Wittenberg, 1992). Increased phytoplankton concentrations reduce light penetration to the symbiotic zooxanthellae and increased organic sediment loads can smother corals (Bell, 1992). Eutrophication can also increase the severity of diseases (Bruno et al., 2003) and lead to competitive advantage for macroalgae that respond by rapid growth, smothering corals or blocking light (Lapointe, 1997; Walker and Ormond, 1982), although evidence for different trajectories also exists (McCook, 1999a, 1999b). Sediments that are influenced by outflow from industrial areas can contain relatively high levels of lead, cadmium, copper, tin, nickel and iron (Amin et al., 2009; Todd et al., 2010). In particular, copper is known to inhibit coral recruitment, fertilisation and development (Reichelt-Brushett and Harrison, 2005; Negri and Hoogenboom, 2011).

4. Responses among and within coral species

4.1. Responses to turbidity

Light-enhanced calcification is responsible for most of the skeletal growth of reef-building corals (Goreau, 1959). Low light decreases calcification in zooxanthellate scleractinian corals, being approximately three times lower in darkness than in light (Kawaguti and Sakumoto, 1948; Gattuso et al., 1999). Titlyanov (1991), however, noted that enhanced utilisation of light by zooxanthellae in three stony corals can result in stable levels of primary production in a wide light range (20–90% PAR). Low light levels may also inhibit the development of coral larvae (Rogers, 1990). Similar patterns of photo-acclimation (through photophysiological adaptations) across gradients of increased turbidity have been demonstrated by Hennige et al. (2008, 2010).

Although certainly also related to a variety of other environmental factors, species diversity of corals generally tends to decrease sharply with increasing (chronic) turbidity (Rogers, 1990; Becking et al., 2006; Cleary et al., 2008). Long-term turbidity stress can shift the species composition of reefs through the death of more light demanding corals and the subsequent replacement by usually deeper-living, more shade-tolerant ones at certain depths (Pastorok and Bilyard, 1985). Dikou and van Woesik (2006b) noted in Singapore the occurrence of deeper-water genera such as *Merulina*, *Pachyseris* and *Mycedium* found in relatively shallow (3–4 m) depths was most likely due to high turbidity levels. Also in Singapore, Goh et al. (1994) considered the sediment-impacted light environment to be the main factor controlling coral colony form. Foliose forms tended to dominate the shallow reef with more massive and encrusting forms found deeper.

4.2. Responses to sedimentation

Corals can react either actively or passively to sediments, which in many ways defines their capability to withstand prolonged sedimentation. Passive shedding refers to corals taking advantage primarily of their shape to allow increased runoff of sediment, to maintain parts of the corallum above sediment, or to use water currents to remove accumulated sediment (Stafford-Smith and Ormond, 1992; Stafford-Smith, 1993; Riegl, 1995; Riegl et al., 1995; Sanders and Baron-Szabo, 2005). It has long been known that coral shape correlates well to the environment, and in particular in paleo-ecological studies, corallum shape has frequently been equated to sedimentation conditions (Plusquellec et al., 1999; Sanders and Baron-Szabo, 2005). Colony shape plays an obvious role in aiding sediment runoff and hemispherical to columnar species have been found to be efficient passive shedders (Bak and Elgershuizen, 1976; Dodge and Vaisnys, 1977; Stafford-Smith, 1993; Riegl, 1995). Branching species retain little sediment, and many poritids are indeed very sediment-tolerant; however, some acroporids are inefficient sediment rejecters and do not appear well adapted to sedimentation despite an apparently advantageous growth form (Stafford-Smith, 1993). Thin, stick forms such as *Madracis mirabilis* or *Acropora cervicornis* are ideally suited passive shedders. Both species have little surface available for sediment accumulation and staghorn corals have polyps that are widely separated, further reducing the chance of sediment clogging (Meyer, 1989). Another efficient design for passive sediment rejection is the thin, platy and upright growth habit exhibited by *Agaricia tenuifolia* in shallow water. Only a small area is present at the top of each plate for sediment accumulation. This form, coupled with an erect growth habit, is very effective in letting sediment slide passively from the colony (Meyer, 1989). Gorgonians (Octocorallia), especially sea whips, were found to be among the most tolerant species to

sediment-loading and dredging-induced turbidity in Florida (Marszalek, 1981). Five species of gorgonians in the highly sedimented waters of Singapore showed growth rates ranging from 2.3 to 7.9 cm yr^{-1}, which are comparable to published growth rates from non-sedimented environments (Goh and Chou, 1995).

Riegl (1995), Riegl and Bloomer (1995) and Schleyer and Celliers (2003) found in zooxanthellate soft corals, which are generally inefficient and passive sediment shedders, that ridged morphology maintained sediment-free areas and thus maintained photosynthetic efficiency which allowed these corals to persist in relatively sand-laden environments. In scleractinian corals, calyx size, orientation, and degree of meandrisation have been found to correlate in some species with rejection efficiency (Hubbard and Pocock, 1972; Rogers, 1983; Johnson, 1992; Stafford-Smith, 1993; Philipp and Fabricius, 2003; Sanders and Baron-Szabo, 2005; Rachello-Dolmen and Cleary, 2007; Sorauf and Harries, 2010); however, such relationships appear to be dependent on sediment size (Riegl, 1995). A counter-intuitive mechanism of passive sediment rejection is that of funnel-shaped corals (*Acropora clathrata* and *Turbinaria peltata*) occurring in turbid, but also high-energy environments. Riegl et al. (1996) showed in field and laboratory experiments that funnel-opening angle and depth could control hydrodynamic clearance of sediment via generation of unstable vortices in the funnels under high-current (surge) conditions that efficiently removed sediment from corals.

Active sediment-shedding mechanisms include polyp inflation, tentacular action and polyp movement (Stafford-Smith and Ormond, 1992; Riegl, 1995; Bongaerts et al., 2012). The cue to this activity is likely irritation of surface receptors when ciliary motion alone is not capable of removing sediment. Tentacular motion can be coordinated to collect sediment, largely by the action of cilia on the tentacular surfaces, which is then pushed or made to slide off the polyp. In some species, sediment is moved to the centre of the oral disc and ingested. This may be correlated with the observed feeding for energy gain reported by Anthony (1999a, 2000). Tissue expansion is a regularly observed mechanism that consists either of expansion of the entire polyp with ensuing tentacular action, or of an inflation of the oral disc with retracted polyps. The first would be a reaction under light to moderate sediment load, the latter a reaction under heavier sediment load. The inflation of the polyp with retracted tentacles leads to the formation of a smooth colony surface, from which sediment can slide off easily. This mechanism is thus a combination of active and passive sediment-shedding.

In free-living stony corals, such as mushroom corals, tissue inflation can lead not only to the removal of sediments, but also to the relocation of the entire corallum which is capable of pushing itself over the substratum (Chadwick, 1988; Chadwick-Furman and Loya, 1992; Hoeksema and de Voogd, 2012), a dispersion mechanism leading to high densities of evenly distributed corals (Goreau and Yonge, 1968; Schuhmacher, 1979; Fisk, 1983; Hoeksema, 1988, 2004; Yamashiro and Nishihira, 1995). Furthermore, if a free-living mushroom coral is at risk of dying because of sedimentation, it may survive by budding, a mechanism of asexual reproduction in which an adult coral generates clonal polyps that continue to live after the parent coral's death. This mechanism may result in coral aggregations (Gilmour, 2002, 2004; Hoeksema, 2004), but high densities of free-living corals in sediment-rich habitats may also be the result of sexual reproduction to spread the risk of burial and subsequent mortality (Johnson, 1992).

Important for sediment rejection is the production of mucus sheets (Coffroth, 1990; Rogers, 1990; Stafford-Smith, 1993). Some corals produce copious amounts of mucus as their primary mechanism to remove silt (e.g. *Meandrina meandrites*), whereas other corals produce mucus more sparingly but then use additional clearing mechanisms such as ciliary action (*Montastraea annularis*)

(Dumas and Thomassin, 1977). Mucocytes, the cells producing mucus, are common in all coral tissues, but particularly so on the oral surface (Brown and Bythell, 2005). Together with ciliary action, mucus is used to move accumulated sediment off the coral (Schuhmacher, 1977). Mucus production, however, uses up an important part of a coral's daily photosynthetic production and its frequent replacement can lead to excessive demands on energy and a decrease in the number of mucus cells (Riegl and Bloomer, 1995; Vargas-Angel et al., 2006). Under severe sedimentation and turbidity stress, more than three times a coral's daily energy production can be used up for mucus production (Riegl and Branch, 1995)—mucus that is then sloughed off with the adhering sediment. Continued chronic sedimentation as well as frequent, repeated exposure to intermittent pulses of high sedimentation will lead to exhaustion of the sediment-clearing ability of corals, eventually leading to tissue thinning, loss of cilia and mucosecretory cells, and ultimately death (Fig. 4).

4.3. Within-species variation

It is clear that differences exist among species in their ability to withstand the effects of increased sedimentation. Do these differences also occur within species? As not all growth forms will survive equally under sediment stress, some environment-morphology matching can be expected. Certainly, many corals display
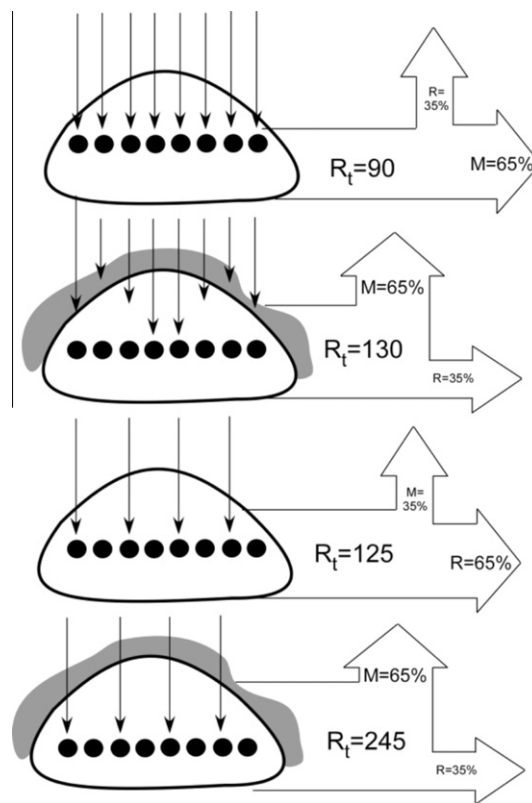
Fig. 4. Why corals starve to death when sedimented. Vertical arrows represent light, the black dots are zooxanthellae, the arrow coming from the coral represents energy use (measured by respirometry). Sediment is shown as grey cover on the coral. Under ~50% light (PAR$_s$) conditions, ~90% of productivity is respired, of which ~35% is due to mucus production and ~65% due to other metabolic functions. Under sedimentation, this is reversed and respiration due to mucus production now dominates. Also, more energy is respired than produced. Under increased turbidity (~25% PAR$_s$), the coral cannot function as autotroph anymore, and when sedimented uses more than two-days energy production in respiration, 65% of this for mucus alone. R$_t$ = total respiration; M = share of respiration due to mucus production; R = share of respiration due to regular metabolic activity. Modified from Riegl and Branch (1995). By permission of Elsevier.

a high degree of intraspecific morphological variation. This can be due to genetic differentiation (polymorphism), environment-induced changes (phenotypic plasticity) or a combination of both (Foster, 1979; Todd et al., 2002a,b; Todd, 2008). Various studies have shown that the ambient light environment (both turbidity and depth-related) can be correlated to intraspecifc colony, corallite, and sub-corallite morphology, but little is known about the within-species differences in relation to settling sediments.

Examples of intraspecific morphological variation that has been related to light include Jaubert (1977) who showed that colonies of *Porites convexa* (as *Synaraea convexa*) were hemispherical with many short branches in high light, flatter with longer branches in medium light, and explanate in the lowest light conditions. Graus and Macintyre (1982) modelled calcification rates and photosynthesis in *Montastraea annularis* and demonstrated that light had the greatest effect on its morphogenesis. Computer models based on light diffusion and light shelter effects accurately matched the dendritic form of *Merulina ampliata* (Nakamori, 1988) via reciprocal transplant experiments, Muko et al. (2000) determined that platy colonies of *Porites sillimaniani* developed branches within eight months when transplanted to high light conditions. Beltran-Torres and Carricart-Ganivet (1993) concluded that light was the principal physical factor influencing corallite diameter and septal number variation in *Montastraea cavernosa*, and Wijsman-Best (1974) suggested light reduction to cause a decrease with depth of both corallites per unit area and number of septa in various faviids. Todd et al. (2004a) concluded that irradiance was the main factor driving small-scale plastic responses in the massive corals *Favia speciosa* and *Diploastrea heliopora* and suggested that this response may enhance light capture by increasing surface area. The corallite shape of *Goniastrea pectinata* also changes in relation to light and Ow and Todd (2010), through modeling light capture, showed this response to be an adaptive response to the immediate light environment.

Some morphologies, both at colony and corallite level, are believed to encourage sediment-shedding (Lasker, 1980; Rogers, 1983, 1990). Marshall and Orr (1931), after smothering various coral taxa with sand, concluded that corals with large polyps were better at removing sediment than those with small polyps. Small polyps equate to less tissue-distension potential and thus to a reduced ability to remove coarse grains. Stafford-Smith and Ormond (1992) found that active-rejection capability was positively correlated with calyx size and Hodgson (1993) concluded that large corallites and extensible polyps were advantageous in his tests on 50 species of coral. Corals that move larger grains tend to have more septa, high relief and numerous septa teeth. The shape of the calyx is also important to sediment-shedding, with V or U floors apparently beneficial for mechanical reasons (Hubbard and Pocock, 1972). Todd et al. (2001) hypothesised that these features in *Favia speciosa* may be advantageous to this species in Singapore's sedimented waters. Further, they found that *Favia speciosa* polyps were significantly larger at their most sediment-impacted study site (Todd et al., 2001). Riegl (1995) also found corallum shape to be important while Dodge (1982) found no clear trend. Gleason (1998) noted green and brown morphs of *Porites astreoides* had different sediment-shedding abilities even though small-scale morphologies were very similar. Even intra-colonial variation can have a great effect on sediment removal; for instance, small differences in colony convexity can lead to areas where sediments accumulate and create anoxic conditions (Stafford-Smith, 1992, 1993).

In the only study to date to specifically examine whether sediment can induce change in coral morphology, Todd et al. (2004b) found a slight increase in rugosity (the height of the wall measured from the outside of the corallite) in fragments exposed to sediment treatment compared with controls (*Favia speciosa* control = 1.36 mm, sediment treatment = 1.53 mm; *Diploastrea heliopora*

control: 1.40 mm, sediment treatment = 1.54 mm). As passive rejection is enhanced by tall polyps with steep surfaces (Lasker, 1980), it is possible that this response would be beneficial to the two species tested. Any attempt to examine plastic responses of corals to chronic sediment is complicated by the reduction in light caused by sediment in the water. For instance, explanate *Porites sillimaniani* form branches under high light (Muko et al., 2000). It is easy to see how the branching form might be advantageous in high sediment conditions, but these are unlikely to develop as they require high light. Also, in *Turbinaria mesenterina*, convoluted forms (good for sediment rejection) became explanate (bad for sediment rejection) in low light and explanate forms became convoluted in high light conditions (Willis, 1985). The same problem also occurs at finer scales. Smaller corallites with fewer septa are likely related to decreased light in *Montastraea cavernosa* and some other faviids (Wijsman-Best, 1974; Beltran-Torres and Carricart-Ganivet, 1993) but the opposite traits are beneficial for sediment removal (Marshall and Orr, 1931; Hubbard and Pocock, 1972; Stafford-Smith and Ormond, 1992; Hodgson, 1993).

5. Tolerance levels and critical thresholds

All coral species are arranged along a gradient of relative tolerance to stress from sediment. Each coral species, therefore, has its own set of threshold values representing the concentrations of sediment which produce sublethal or lethal effects. After a certain maximum concentration, reduction of growth occurs due to smothering, reduced light levels and reduced zooxanthella photosynthesis. Ultimately, when sustained over a longer period, such concentrations can cause mortality.

5.1. Turbidity

There is a clear relationship between substratum cover by live corals and water transparency (K_{PAR}), which determines the compensation depth of corals (Yentsch et al., 2002). Values for the minimum light requirements of corals reported in the literature range from <1% to as much as 60% of surface irradiance (SI) (Table 3). Kleypas et al. (1999) suggested minimum light requirements to allow reef formation (40% SI) to differ from the minimum light requirements to allow survival of individual corals (10% SI). The sensitivity to reduced light is—at least in part—dependent on the growth form of corals, with branching species generally thriving only under at least 60% average SI, while most plocoid and meandroid massive species require only 20% average SI, and several platy corals can survive with as little as 0.15% (Jaap and Hallock, 1990). Typically, the reduced availability of light caused by increased turbidity is experienced more strongly by corals growing in deeper areas of a reef than by corals growing in shallower areas. Turbidity effects on corals depend on the grain size of the suspended sediment, with fine particles contributing most to light reduction while coarser particles may cause scouring and abrasion of coral tissue (PIANC, 2010).

Despite an impressive body of literature (see review by Hubbard, 1986), little quantitative information exists on the specific responses of reef organisms to suspended-sediment loading. There is a highly significant inverse relationship between coral growth rates and suspended-sediment yields (Miller and Cruise, 1995). Practical observations of coral mortality associated with turbidity plumes from dredging projects or increased runoff are inconsistent with laboratory experiments that have documented surprising tolerance by corals to high doses of sediment over short periods of time (Taylor and Saloman, 1978; Rogers, 1983). One of the factors responsible for this discrepancy may be the effect of the duration of exposure (Fig. 2). Tolerance limits of corals for total suspended

Table 3
Some published critical threshold of corals for light availability (% of surface irradiance SI).

Species/type of corals	Location	%SI	References
Plate corals	Florida,USA	0.15	Jaap and Hallock (1990)
Star corals	Curacao	1	Bak (1978)
Scleractinian corals	South China Sea	2–8	Titlyanov and Latypov (1991)
Individual corals	Worldwide	10	Achituv and Dubinsky (1990)
Star and brain corals	Florida, USA	20	Jaap and Hallock, 1990
Coral reefs	Worldwide	35	Achituv and Dubinsky (1990)
Branching corals	Florida, USA	60	Jaap and Hallock (1990)

matter (or suspended-sediment concentration) reported in the literature range from <10 mg L^{-1} in reef areas not subject to stresses from human activities to >100 mg L^{-1} in marginal reefs in turbid nearshore environments (Marshall and Orr, 1931; Roy and Smith, 1971; Mapstone et al., 1989; Hopley et al., 1993; Larcombe et al., 2001; Hoitink, 2003; Sofonia and Anthony, 2008) (Table 4). This wide range demonstrates that different coral species and corals in different geographic regions may respond differently to turbidity increases. Thermal tolerances in corals have also been reported to vary geographically (Weeks et al., 2008). Some corals have been shown to possess the ability to (temporarily) switch between autotrophy and heterotrophy or to make adjustments to their respiratory demands in response to episodic turbidity stress events (Telesnicki and Goldberg, 1995; Anthony and Fabricius, 2000) but these data are limited to a few coral species. Reduced photosynthetic capacity may lead to reduced energy reserves for maintenance and growth. Corals contain large lipid stores under normal (non-stressed conditions), but a recent study indicated that 30–50% depletion of those reserves may occur during stress events within a matter of weeks (Anthony et al., 2007).

In certain locations, coral reefs persist in highly turbid areas (Perry, 2005; Perry and Smithers, 2010). Larcombe et al. (1995) described the characteristics of suspended sediment concentrations of marine waters near inner-shelf fringing coral reefs in northern Australia and related these to the prevailing oceanographic and meteorological conditions. High temporal and spatial variation in near-bed SSCs corresponded to wind-generated swells, which,

Table 4
Some published critical thresholds of corals (reefs) for Total Suspended Sediment (mg L^{-1}).

Description	Location	mg L^{-1}	References
Coral reefs	Great Barrier Reef (GBR), Australia	3.3	Bell (1990)
Coral reefs	Fanning lagoon, Florida, USA	10	Roy and Smith (1971)
Coral reefs	Caribbean	10	Rogers (1990)
Coral reefs	Papua New Guinea	15	Thomas et al. (2003)
Coral reefs	Florida, USA	20	Bogers and Gardner (2004)
Corals	Dominican Republic	20	Van der Klis and Bogers (2004)
Marginal reef environments	Banten Bay, Java, Indonesia	40	Hoitink (2003)
Marginal reef environments	Paluma Shoals, QLD Australia	40	Larcombe et al. (2001)
Nearshore fringing reefs	Magnetic Island, GBR, Australia	75–120	Mapstone et al. (1989)
Nearshore fringing reefs	Cape Tribulation, GBR, Australia	100–260	Hopley et al. (1993)
Seven resistant coral species	Florida, USA	165	Rice and Hunter (1992)

within 1 km of the reefs, produced near-bed SSCs of well over 200 mg L^{-1}. At the fringing coral reefs SSCs ranged from 5 mg L^{-1} to 40 mg L^{-1}. Flushing of these bays by tidal currents was important to prevent the build-up of suspended sediment in the water around the coral reefs. Other extremely turbid reefs were described by Anthony and Larcombe (2000) from Halifax Bay, Australia, where "coastal turbid-zone reefs" occur in water less than 4 m deep, with turbidity sometimes over 100 NTU ($\sim$220 mg L^{-1}) as a result of wave-induced resuspension, and wind-driven longshore currents prevent accumulation of fine-grained sediment. In turbid situations, the key to sustained coral growth appears to be low sediment accumulation, frequently assured by strong tidal flushing, although recent studies from the GBR indicate that reefs in these settings can have quite high accretion rates. While reef growth was found to be possible under such conditions, these reefs hosted relatively moderate species numbers and sometimes had poorly consolidated frameworks (Hopley et al., 2007). Hoitink (2004) found that tidal currents around reefs in Indonesia resuspended sediments to give average Suspended-sediment concentrations between 2 and 10 mg L^{-1}, with maxima up to 50 mg L^{-1}. Riegl (1995) found surge-induced peak suspended-sediment concentrations of up to 389 mg L^{-1} in sandy gullies and 112 mg L^{-1} over coral on South African reefs; this, however, was local sediment stirred up and immediately re-deposited.

While the studies above demonstrate that coral reefs and turbidity/sedimentation can coexist, it also shows the danger of introducing sediment since it is likely to be remobilised repeatedly. All the reef systems discussed in the previous two paragraphs were clearly adapted to sedimentation and turbidity, with mostly low accretion rates demonstrated in South Africa (Ramsay and Mason, 1990; Riegl et al., 1995) and quite high accretion rates on inshore reefs from the Great Barrier Reef (Larcombe et al., 1995), comparable to those in "optimal" environments. Corals that are naturally exposed to high and variable background conditions of turbidity and sedimentation (e.g. due to storms and/or river influence) will show higher tolerances to short increases in turbidity or sedimentation caused by dredging (Nieuwaal, 2001). Corals from shallow-water environments, where they are frequently exposed to elevated temperatures, storms and wave action, are more likely to be tolerant of environmental stresses than corals in deeper waters (Brown and Howard, 1985; Hoeksema, 1991b; Hoeksema and Matthews, 2011).

A synthesis of literature data regarding the sensitivity of different coral species to turbidity is presented in Table 5. These data were reworked and related to a relative sensitivity index according to the response matrix presented in Table 6. Sensitivity classes were then given scores from 1 to 5, with 1 corresponding to "very tolerant" and 5 to "very sensitive". The scores for individual coral species were subsequently related to their dominant growth form and mean calyx diameter. Analysis of these data (90 entries for 46 species) confirmed that there is a significant relationship (Kruskal–Wallis, $P < 0.05$) between the growth form of corals and their sensitivity to turbidity (Fig. 5a). Most soft corals and many massive coral species are relatively sensitive to turbidity while laminar, plating and tabular corals as well as some morphologically variable corals are relatively tolerant. There was no significant relationship between the calyx diameter of corals and their sensitivity to turbidity (Fig. 5b).

5.2. Sedimentation

Most coral species are sensitive to enhanced sedimentation, even in the order of a few centimetres per year (Rogers, 1990). Pastorok and Bilyard (1985) suggested that sedimentation rates of >50 mg cm^{-2} d^{-1} (equivalent to 500 g m^{-2} d^{-1}) may be considered catastrophic for some coral communities, while 10–50 mg cm^{-2} d^{-1}

Table 5

Sensitivity of different coral species for turbidity. Overview of the response of different species of corals to various levels of turbidity tested, as reported in the literature. Nomenclature of coral species was updated according to the most recent taxonomic revisions. Growth forms (as stated or inferred): B = branching; C = columnar (incl. digitate); E = encrusting; F = foliaceous; L = laminar (incl. plate & tabular); M = massive; S = solitary (free-living); So = soft corals & gorgonians. Calyx diameter measured on museum specimen, supplemented with data from Stafford-Smith and Ormond (1992).

Coral species	Turbidity level (tested)	Response	Growth form	Calyx (mm)	References
Acropora cervicornis (Lamarck, 1816)	Severe light reduction (shading) for 5 weeks	Mass bleaching (3 weeks), mortality/algal cover (7 weeks), no recovery (8 months)	B	1.0	Rogers (1979)
Acropora cervicornis (Lamarck, 1816)	50 mg/l (96 h)	No effect	B	1.0	Thompson (1980b)
Acropora cervicornis (Lamarck, 1816)	150 mg/l (96 h)	Polyp retraction, mucus production but no mortality	B	1.0	Thompson (1980b)
Acropora cervicornis (Lamarck, 1816)	476 mg/l (96 h)	Partial mortality after 96 h.	B	1.0	Thompson (1980b)
Acropora cervicornis (Lamarck, 1816)	Total shading (3 weeks)	Bleaching and mortality, no recovery	B	1.0	Quoted in Nieuwaal (2001)
Acropora cervicornis (Lamarck, 1816)	25 mg/l (drilling mud) (24 h)	62% Decrease in calcification rate	B	1.0	Kendall et al. (1983)
Acropora cervicornis (Lamarck, 1816)	100 mg/l (drilling mud) (24 h)	50% Decline in soluble tissue protein	B	1.0	Kendall et al. (1983)
Acropora cervicornis (Lamarck, 1816)	50 and 100 mg/l (kaolin, 24 h)	Reduced calcification rate and free amino acids at 100 mg/l (recovery in 48 h)	B	1.0	Kendall et al. (1985)
Acropora cervicornis (Lamarck, 1816)	1000 mg/l (for 65 h)	Mortality of colonies	B	1.0	Thompson and Bright (1980)
Acropora digitifera (larvae)	50–100 mg/l (lab and feld tests)	Adverse effects on fertilisation, larval survival and settlement			Gilmour (1999)
Acropora millepora (Ehrenberg, 1834)	1–30 mg/l SPM (hours)	Increased feeding capacity at high SPM concentrations	B	1.0	Anthony (1999a)
Acropora millepora (Ehrenberg, 1834)	1–30 mg/l SPM (days)	Increasing contribution of heterotrophy at high SPM conc.	B	1.0	Anthony (2000)
Acropora millepora (Ehrenberg, 1834)	1, 3, 10, 30 and 100 mg/l TSS (16 weeks)	Full colony mortality at 100 mg/l for 12 weeks (50% mortality after 4 weeks)	B	1.0	Negri et al. (2009) and Flores et al. (2011)
Acropora nobilis (Dana, 1846)	10 mg/l (42 days)	Increased survival from high temperature treatment compared to control	L	1.5	Anthony et al. (2007)
Acropora spp.	170 mg/l (hours) of marine snow/SPM	Mucus production in response to flocculation			Fabricius and Wolanski (2000)
Agaricia agaricites (Linnaeus, 1758)	Severe light reduction (shading) for 5 weeks	Partial bleaching after 5 weeks, recovery within weeks	L	5.0	Rogers (1979)
Agaricia agaricites (Linnaeus, 1758)	50 mg/l (96 h)	No effect	L	5.0	Thompson (1980b)
Agaricia agaricites (Linnaeus, 1758)	150 mg/l (96 h)	Polyp retraction, mucus production but no mortality	L	5.0	Thompson (1980b)
Agaricia agaricites (Linnaeus, 1758)	476 mg/l (96 h)	Mortality after 65 h	L	5.0	Thompson (1980b)
Agaricia agaricites (Linnaeus, 1758)	<1% SI (several days)	33% Decrease in calcification rate (for >1 month), but survival	L	5.0	Bak (1978)
Agaricia agaricites (Linnaeus, 1758)	1000 mg/l (for 65 h)	Mortality of colonies	L	5.0	Thompson and Bright (1980)
Cladocora arbuscula (Lesueur, 1812)	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival (10 d), minor bleaching (20 d)	B	4.0	Rice and Hunter (1992)
Colpophyllia natans (Houttuyn, 1772)	Severe light reduction (shading) for 5 weeks	Partial bleaching (5 weeks), limited recovery & some algal growth (15 weeks)	M	25.0	Rogers (1979)
Dichocoenia stokesi Milne Edwards & Haime, 1848	0–2 NTU and 7–9 NTU (weeks)	No effect on P:R ratio	M	11.0	Telesnicki and Goldberg (1995)
Dichocoenia stokesi Milne Edwards & Haime, 1848	14–16 NTU (weeks)	Mucus production, P:R ratio <1 after 6 days exposure	M	11.0	Telesnicki and Goldberg (1995)
Dichocoenia stokesi Milne Edwards & Haime, 1848	28–30 NTU (weeks)	Mucus production, P:R ratio <1 after 3 days exposure	M	11.0	Telesnicki and Goldberg (1995)
Dichocoenia stokesi Milne Edwards & Haime, 1848	50–150–476 mg/l (96 h)	No effect at 50 and 150 mg/l; extreme sublethal stress but survival at 476 mg/l	M	11.0	Thompson (1980b)
Dichocoenia stokesi Milne Edwards & Haime, 1848	1000 mg/l (for 65 h)	No mortality	M	11.0	Thompson and Bright (1980)
Diploria labyrinthiformis (Linnaeus, 1758)	Severe light reduction (shading) for 5 weeks	Substantial bleaching (5 weeks), no recovery & some algal growth (15 weeks)	M	8.0	Rogers (1979)
Eusmilia fastigiata (Pallas, 1766)	severe light reduction (shading) for 5 weeks	No visible effects	M	12.0	Rogers (1979)
Favia favus (Forskål, 1775)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	M	14.0	Riegl and Branch (1995)
Favites pentagona (Esper, 1794)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	M	7.0	Riegl and Branch (1995)
Fungiidae (mushroom corals)		Adapted to highly turbid environments			Dikou and Van Woesik, (2006)
Galaxea fascicularis (Linnaeus, 1767)	>40 NTU (c.40 d), at times up to 175 NTU	Shift from autotrophy to heterotrophy (reversible)	C	8.0	Larcombe et al. (2001)
Goniastrea retiformis (Lamarck, 1816)	Shading (equivalent to 16 mg/l) – 2 months	Increased particle feeding & heterotrophy; survival and tissue gains	M	4.0	Anthony and Fabricius (2000)

(continued on next page)

Table 5 (continued)

Coral species	Turbidity level (tested)	Response	Growth form	Calyx (mm)	References
Goniastrea retiformis (Lamarck, 1816)	1–30 mg/l SPM (weeks)	Gained tissue & skeletal mass (all treatments); increasing heterotrophy	M	4.0	Anthony and Fabricius (2000)
Goniastrea retiformis (Lamarck, 1816)	1–16 mg/l suspended matter (8 weeks)	Increased growth rate as function of SPM concentration	M	4.0	Anthony (1999b)
Goniastrea retiformis (Lamarck, 1816)	Shading (equiv. 16 mg/l at 4 m) (8 weeks)	Significant reduction in growth rate	M	4.0	Anthony (1999b)
Gorgonia flabellum Linnaeus, 1758	Severe light reduction (shading) for 5 weeks	No visible effects	So		Rogers (1979)
Gorgonians & soft corals		Very tolerant to high turbidity			Fabricius and Dommisse (2000)
Gyrosmilia interrupta (Ehrenberg, 1834)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	M/E	16.0	Riegl and Branch (1995)
Isophyllia sinuosa (Ellis & Solander, 1786)	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival after 10 d, minor bleaching after 20 d	N	15.0	Rice and Hunter (1992)
Leptastrea sp.		Well adapted to turbid waters			Dikou and Van Woesik, (2006)
Lobophytum depressum Tixier-Durivault, 1966	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	So		Riegl and Branch (1995)
Lobophytum venustum Tixier-Durivault, 1957	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	So		Riegl and Branch (1995)
Madracis auretenra Locke, Weil & Coates, 2007	<1% SI (several days)	33% Decrease in calcification rate (for >1 month), but survival	B	1.0	Bak (1978)
Manicina areolata (Linnaeus, 1758)	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival after 10 d, minor bleaching after 20 d	M	14.0	Rice and Hunter (1992)
Meandrina meandrites (Linnaeus, 1758)	0–2 NTU and 7–9 NTU (weeks)	No effect on P:R ratio	M/E	15.0	Telesnicki and Goldberg (1995)
Meandrina meandrites (Linnaeus, 1758)	14–16 NTU (weeks)	Mucus production, P:R ratio < 1 after 6 days exposure	M/E	15.0	Telesnicki and Goldberg (1995)
Meandrina meandrites (Linnaeus, 1758)	28–30 NTU (weeks)	Mucus production, P:R ratio < 1 after 3 days exposure	M/E	15.0	Telesnicki and Goldberg (1995)
Millepora alcicornis Linnaeus, 1758	Severe light reduction (shading) for 5 weeks	Partial bleaching (5 weeks), algal growth (6 weeks), no recovery of damaged tissue	B	0.5	Rogers (1979)
Montastraea annularis (Ellis & Solander, 1786)	Severe light reduction (shading) for 5 weeks	Substantial bleaching (5 weeks), partial recovery (6–8 weeks), some algae/mucus	M/E	5.0	Rogers (1979)
Montastraea annularis (Ellis & Solander, 1786)	50 mg/l (96 h)	No effect	M/E	5.0	Thompson (1980b)
Montastraea annularis (Ellis & Solander, 1786)	150 mg/l (96 h)	Polyp retraction, mucus production but no mortality	M/E	5.0	Thompson (1980b)
Montastraea annularis (Ellis & Solander, 1786)	476 mg/l (96 h)	Mortality after 65 h	M/E	5.0	Thompson (1980b)
Montastraea annularis (Ellis & Solander, 1786)	100 mg/l (6-weeks)	Major sublethal effects (photosynthesis, respiration, calcification & nutr.uptake)	M/E	5.0	Szmant-Froelich et al. (1981)
Montastraea annularis (Ellis & Solander, 1786)	1–10 mg/l (6 weeks)	Only (some) effect on feeding response	M/E	5.0	Szmant-Froelich et al. (1981)
Montastraea annularis (Ellis & Solander, 1786)	525 mg/l	Decreased net production & tissue Chl, increased respiration & mucus	M/E	5.0	Dallmeyer et al. (1982)
Montastraea annularis (Ellis & Solander, 1786)	1000 mg/l (for 65 h)	Mortality of colonies	M/E	5.0	Thompson and Bright (1980)
Montastraea cavernosa (Linnaeus, 1767)	Severe light reduction (shading) for 5 weeks	No visible effects	M	11.0	Rogers (1979)
Montipora aequituberculata Bernard, 1897		Common on shallow, turbid inshore fringing reefs	F	0.6	Stafford-Smith (1993)
Montipora aequituberculata Bernard, 1897	1, 3, 10, 30 and 100 mg/l TSS (16 weeks)	Full colony mortality at 30 mg/l after 12 weeks (50% mortality after 4 weeks)	F	0.6	Negri et al. (2009) and Flores et al. (2011)
Montipora capitata Dana 1846	Light reduction from 57 to 44% SI (field; hours)	Photophysiological sublethal response; 1.4 times lower rETR, higher F_v/F_m	B	1.0	Piniak and Storlazzi (2008)
Montipora digitata (Dana, 1846)	1–30 mg/l SPM (hours)	Increased feeding capacity at high SPM concentrations	B	1.0	Anthony (1999a)
Montipora digitata (Dana, 1846)	>95% shading (transplanted into caves)	Survival/acclimation, reduced photosynthetic rate	L	1.0	Anthony and Hoegh-Guldberg (2003)
Montipora digitata (Dana, 1846)	70% light reduction (permanent transplantation)	Complete photoacclimation within 3 weeks	L	1.0	Anthony and Hoegh-Guldberg (2003)
Montipora verrucosa (Lamarck, 1816)	8 and 20 mg/l (modelling)	Reduced photosynthesis at 8 mg/l; negative energy balance at 20 mg/l	M/L	1.0	Te (1998)
Montipora sp.		Well adapted to turbid waters			Dikou and Van Woesik, (2006)
Mussa angulosa (Pallas, 1766)	Severe light reduction (shading) for 5 weeks	No visible effects (1 colony showing minor bleaching after 8 weeks)	M	40.0	Rogers (1979)
Pectinia lactuca (Pallas, 1766) (larvae)	6, 43 and 169 mg/l (lab test)	Adverse effects on fertilisation success and embryo development			Erftemeijer et al. (2012)
Pectinia sp.		Well adapted to turbid waters			Dikou and Van Woesik, (2006)
Phyllangia americana Milne Edwards & Haime, 1849	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival after 10 d, minor bleaching after 20 d	E	9.0	Rice and Hunter (1992)

Table 5 (continued)

Coral species	Turbidity level (tested)	Response	Growth form	Calyx (mm)	References
Platygyra daedalea (Ellis & Solander, 1786)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	M	5.0	Riegl and Branch (1995)
Pocillopora damicornis (Linnaeus, 1758)	1–30 mg/l SPM (hours)	Increased feeding capacity at high SPM concentrations	B	1.1	Anthony (1999a)
Pocillopora damicornis (Linnaeus, 1758)	1–30 mg/l SPM (days)	Increasing contribution of heterotrophy at high SPM conc.	B	1.1	Anthony (2000)
Pocillopora damicornis (Linnaeus, 1758) (larvae)	10, 100, 1000 mg/l (modelling)	Reverse metamorphosis (reduced settlement success) at 100 and 1000 mg/l	B	1.1	Te (1998)
Pocillopora damicornis (Linnaeus, 1758)		Characteristic of turbid waters	B	1.1	Dikou and Van Woesik, (2006)
Porites astreoides Lamarck, 1816	50–150–476 mg/l (96 h)	No effect at 50 and 150 mg/l; extreme sublethal stress (but survival) at 476 mg/l	M/E	1.5	Thompson (1980b)
Porites astreoides Lamarck, 1816	<1% SI (several days)	Bleaching and mortality	M/E	1.5	Bak (1978)
Porites astreoides Lamarck, 1816	1000 mg/l (for 65 h)	No mortality	M/E	1.5	Thompson and Bright (1980)
Porites cylindrica Dana, 1846	Shading (equivalent to 16 mg/l) – 2 months	Energy deficiency/C-loss not compensated by particle feeding; sublethal stress	M	1.5	Anthony and Fabricius (2000)
Porites cylindrica Dana, 1846	1–30 mg/l SPM (weeks)	Skeletal growth sustained, tissue biomass decreased at high SPM	M	1.5	Anthony and Fabricius (2000)
Porites cylindrica Dana, 1846	1–30 mg/l SPM (hours)	Increased feeding capacity at high SPM concentrations	M	1.5	Anthony (1999a)
Porites cylindrica Dana, 1846	1–16 mg/l suspended matter (8 weeks)	No effect on growth rates	M	1.5	Anthony (1999b)
Porites cylindrica Dana, 1846	Shading (equiv. 16 mg/l at 4 m) (8 weeks)	Significant reduction in growth rate	M	1.5	Anthony (1999b)
Porites divaricata Lesueur, 1821	50–150–476 mg/l (96 h)	No effect at 50 and 150 mg/l; extreme sublethal stress (but survival) at 476 mg/l	B	1.2	Thompson (1980b)
Porites divaricata Lesueur, 1821	1000 mg/l (for 65 h)	No mortality	B	1.2	Thompson and Bright (1980)
Porites furcata Lamarck, 1816	50–150–476 mg/l (96 h)	No effect at 50 and 150 mg/l; extreme sublethal stress (but survival) at 476 mg/l	B	2.0	Thompson (1980b)
Porites furcata Lamarck, 1816	1000 mg/l (for 65 h)	No mortality	B	2.0	Thompson and Bright (1980)
Porites lobata Dana, 1846		Dominant in turbid waters			Stafford-Smith (1993)
Porites lutea Milne Edwards & Haime, 1851		Dominant in turbid waters	M	1.5	Stafford-Smith (1993)
Porites lutea Milne Edwards & Haime, 1851	Increased turbidity up to 286 mg/l (4 months)	Partial mortality of 25% of colonies, recovery within 22 months	M	1.5	Brown et al. (1990)
Porites porites (Pallas, 1766)	Significant light reduction due to eutrophication	Reduced reproductive success (ova maturation, larval development)	M	2.0	Tomascik and Sander (1987)
Porites sp.	General increase in SPM	Decreasing tissue thickness from nearshore to offshore			Barnes and Lough (1992)
Porites sp.	General increase in SPM	Decreasing skeletal density, linear extension, increasing calcification			Lough and Barnes (1992, 2000)
Porites sp.		Well adapted to turbid waters			Dikou and Van Woesik, (2006)
Sarcophyton glaucum (Quoy & Gaimard, 1833)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	So		Riegl and Branch (1995)
Scolymia cubensis (Milne Edwards & Haime, 1849)	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival after 10 d, minor bleaching after 20 d	S	91.0	Rice and Hunter (1992)
Scolymia cubensis (Milne Edwards & Haime, 1849)	49–199 mg/l (10 days)	Partial polyp death and partial bleaching (in some indivduals)	S	91.0	Rice (1984)
Siderastrea radians (Pallas, 1766)	49–199 mg/l (10 days)	Partial polyp death and partial bleaching (in some indivduals)	M/E'	5.0	Rice (1984)
Siderastrea siderea (Ellis & Solander, 1786)	Severe light reduction (shading) for 5 weeks	Partial bleaching after 5 weeks, partial recovery in 6–8 weeks	M	3.0	Rogers (1979)
Sinularia dura (Pratt, 1903)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	So		Riegl and Branch (1995)
Sinularia leptoclados (Ehrenberg, 1834)	Light reduced to 50% and 25% PAR (surface)	Severely diminished productivity, increased carbon loss and mucus	So		Riegl and Branch (1995)
Solenastrea hyades (Dana, 1846)	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival after 10 d, minor bleaching after 20 d	M	5.0	Rice and Hunter (1992)
Solenastrea hyades (Dana, 1846)	49–199 mg/l (10 days)	Partial polyp death and partial bleaching (in some individuals)	M	5.0	Rice (1984)
Stephanocoenia intersepta (Lamarck, 1816)	49, 101, 165 and 199 mg/l (10–20 days)	No effect on growth rate or survival after 10 d, minor bleaching after 20 d	M	3.0	Rice and Hunter (1992)
Stephanocoenia intersepta (Lamarck, 1816)	49–199 mg/l (10 days)	Partial polyp death and partial bleaching (in some individuals)	M	3.0	Rice (1984)
Turbinaria mesenterina (Lamarck, 1816)		Tolerant to high turbidity	L	1.5	Quoted in Nieuwaal (2001)
Turbinaria reniformis Bernard, 1896		Tolerant to high turbidity	L	2.0	Quoted in Nieuwaal (2001)
Turbinaria spp.		Most tolerant to high turbidity and sedimentation			Stoddart and Stoddart (2005)

Table 6
Response matrix ranking the relative sensitivity of corals according to their type of response to different levels of turbidity (mg L^{-1}). Severe shading, total shading and <1%SI were categorised as >100 mg L^{-1}, NTU values were categorised as follows: 0–2 NTU:<10 mg L^{-1}, 7–9 NTU: 10–20 mg L^{-1}, 14–16 NTU: 20–40 mg L^{-1}, 28–30 NTU: 40–100 mg L^{-1}, >40 NTU: >100 mg L^{-1}.

Response category	Turbidity level (mg L^{-1}) tested				
	<10	10–20	20–40	40–100	>100
No effect	(most spp.)	Intermediate	Tolerant	Very tolerant	Very tolerant
Sublethal effects (minor) (reduced growth/calcification, mucus production etc.)	Sensitive	Sensitive	Intermediate	Tolerant	Very tolerant
Sublethal effects (major) (bleaching, tissue damage)	Very sensitive	Sensitive	Intermediate	Tolerant	Tolerant
Lethal effects (partial mortality)	Very sensitive	Very sensitive	Sensitive	Intermediate	Tolerant
Major lethal effects (mass mortality)	Very sensitive	Very sensitive	Sensitive	Intermediate	(most spp.)

could be classified as moderate to severe. Other studies, however, revealed how many coral species and reefs are capable of surviving sedimentation rates as high as 100 mg cm^{-2} d^{-1} for several days to weeks without any major negative effects, while some (nearshore) reefs naturally experience sedimentation rates well over 200 mg cm^{-2} d^{-1} (Table 7). Nearshore fringing reefs in the Great Barrier Reef region that are characterised by high and variable sedimentation rates, ranging from 2 to 900 mg cm^{-2} d^{-1} (short-term rates) with long-term means of 50–110 mg cm^{-2} d^{-1}, were found to harbour highly diverse coral growth with a mean coral cover of 40–60% (Ayling and Ayling, 1991). A few coral species, such as *Montastraea cavernosa* and *Astrangia poculata*, can tolerate sedimentation rates as high as 600–1380 mg cm^{-2} d^{-1} (Lasker, 1980; Peters and Pilson, 1985). This wide range demonstrates that different coral

Fig. 5. Relationship between the sensitivity of corals to turbidity and [A] their growth form, and [B] their calyx size. Sensitivity (mean score ± SD) was determined by ranking corals according to their type of response to different levels of turbidity (see text and Table 6). Legend (growth forms): B = branching; C = columnar (incl. digitate); E = encrusting; F = foliaceous; L = laminar (incl. plate & tabular); M = massive; S = solitary (free-living); So = soft corals & gorgonians.

species and corals in different geographic regions may respond differently to increased amounts and rates of sedimentation.

Frequent short-term exposure to high sedimentation events or chronic (long-term) exposure to relatively high sedimentation rates results in increased mortality rates in populations of many coral species (Tomascik and Sander, 1985). If moderate levels of increased turbidity and sedimentation on a reef persist for particularly long periods of time (years or decades), the coral reef may undergo changes in diversity, with the most sensitive coral species (gradually) disappearing as can be seen on reefs in the proximity of big cities such as Singapore and Jakarta (Chou, 1988, 1996; Hoeksema and Koh, 2009; van der Meij et al., 2010; Hoeksema et al., 2011). These losses may also affect other species that depend on coral reefs, such as molluscs (van der Meij et al., 2009), especially if these live in close associations with specific coral hosts (Stella et al., 2011; Hoeksema et al., 2012). Such changes in species composition may cause (sometimes catastrophic) shifts in the coral reef ecosystem, resulting in a loss of ecological functions and ecosystem stability (Scheffer et al., 2001).

Stafford-Smith and Ormond (1992) summarised the conventional wisdom regarding sediment particle size and rejection, i.e. that silts and small particles are generally transported off the colony by ciliary currents whereas larger particles are moved by tissue expansion. Fine grain sizes flow off a colony more easily than coarse grains (Lasker, 1980) but nutrient-rich silts in calm waters can still be very stressful (Fabricius, 2005). Stafford-Smith and Ormond (1992) also explained the energetic costs of different sediment inputs, noting that sporadic downward fluxes of sediment are less costly than a continual light rain of particles. This is because short bursts of sediment leave accumulations in only a few colony areas, such as concave or flat surfaces, whereas a continual rain of particles affects a much larger expanse of tissue.

Some of the variation in sensitivity of corals to sedimentation reported in the literature may have been caused by differences in the particle size of sediments applied in the respective experiments, which calls for a more standardised approach in future experiments. Mud- and silt-sized sediments frequently have a more adverse impact than sand because of different physical and chemical properties (Thompson, 1980a,b; Weber et al., 2006; Piniak, 2007). Mud- and silt-sized sediments are more cohesive and colloidally bind nutrients better than sand. Therefore, a more active bacterial community is likely to develop in silt sheets causing damage to the corals. Ciliary action accompanies more or less all sediment-clearing activity, but is sensitive to grain size. Some of the fungiid corals and *Solenastrea hyades* appear to depend on ciliary action alone to rid the colony of fine sediment (Meyer, 1989). Tentacular action is especially effective for removing larger sediment particles. Surprisingly few coral species can use their tentacles to remove sediment, with *Porites porites* and *P. astreoides* being two notable exceptions (Meyer, 1989). Corals using ciliary action or mucus are more sensitive to continuous siltation. Some of these species simply quit their cleaning action after a short period of repeated sedimentation. A continuous rain of sediment
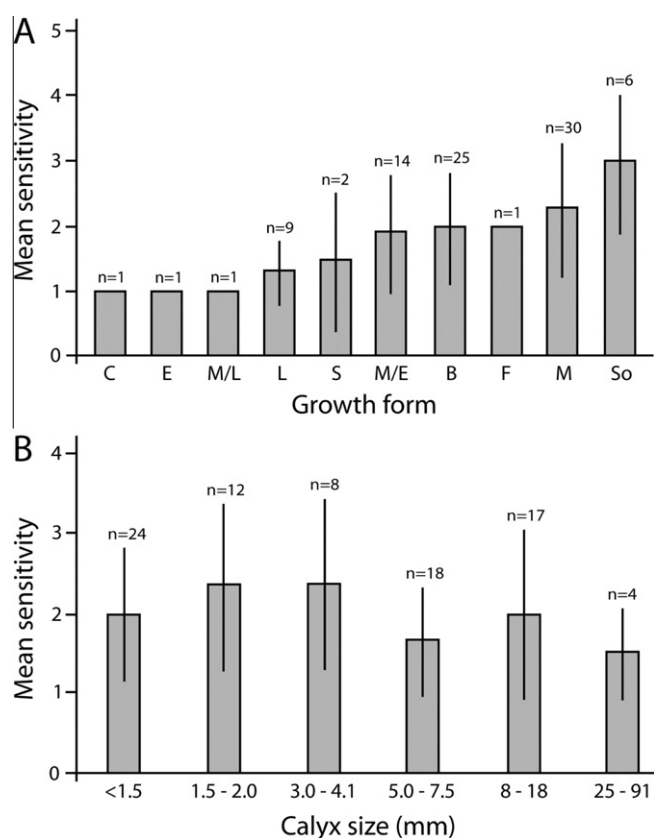
Table 7
Some published critical thresholds of coral reefs for sedimentation (mg cm^{-2} day^{-1}).

Species/type of corals	Location	mg cm^{-2} day^{-1}	References
Coral reefs	Worldwide (moderate to severe)	10	Pastorok and Bilyard (1985)
Coral reefs	Caribbean	10	Rogers (1990)
Coral reefs	Caribbean	37	Pastorok and Bilyard (1985)
Coral reefs	Worldwide (catastrophic)	50	Pastorok and Bilyard (1985)
Coral reefs	Puerto Rico	90	Miller and Cruise (1995)
Coral reefs	Indo-Pacific	228	Pastorok and Bilyard (1985)
Most coral species	Worldwide	300	Bak and Elgershuizen (1976)

Table 8
Some examples of the duration coral species can survive very high sedimentation rates (burial).

Species	Survival characteristics	Reference
Porites sp.	90% Bleaching after 68 h burial, recovery within 4 weeks	Wesseling et al. (1999)
Acropora sp.	100% Mortality after 20 h burial, no recovery	Wesseling et al. (1999)
Galaxea sp.	Sublethal stress after 20–68 h burial, recovery within 3–4 weeks	Wesseling et al. (1999)
Heliopora coerule	Sublethal stress after 20–68 h burial, recovery within 3–4 weeks	Wesseling et al. (1999)
Scolomia cubensis	LT50 after 7 days (complete burial)	Rice and Hunter (1992)
Isophyllia sinuosa	LT50 after 7.2 days (complete burial)	Rice and Hunter (1992)
Manicina areolata	LT50 after 10 days (complete burial)	Rice and Hunter (1992)
Siderastrea radians	LT50 after 13.6 days (complete burial)	Rice and Hunter (1992)
Cladocora arbuscula	LT50 after 15 days (complete burial)	Rice and Hunter (1992)
Solenastrea hyades	LT50 after 15 days (complete burial)	Rice and Hunter (1992)
Stephanocoenia intersepta	LT50 after 16.2 days (complete burial)	Rice and Hunter (1992)

temporarily exhausts both the mucus-secreting and ciliary drive for a period of one or two days. Recovery is possible only if siltation stops during the recovery period (Schuhmacher, 1977; Fortes, 2001).

Extreme sediment loads can lead to burial and eventual mortality (Rogers, 1983; Stafford-Smith, 1992). Wesseling et al. (1999) completely buried corals of the genera *Acropora, Porites, Galaxea* and *Heliopora* and found that, even after 68 h, all corals except *Acropora* eventually recovered. Rice and Hunter (1992) also determined that seven species near Florida were highly resistant to sediment burial. However, a heavy influx of sediment from a dredging operation resulted in complete or partial mortality in explanate colonies of *Porites astreoides* (Bak, 1978). Upland forest logging caused a nearly 100-fold increase in suspended sediment loads of Manlag River, resulting in prolonged sediment deposition at rates of 20 mg cm^{-2} d^{-1} in Bacuit Bay (Philippines), injuring and killing many of the ~50 coral species in the area, reducing species diversity, coral cover and average colony size (Hodgson, 1993; Birkeland, 1997; Hodgson and Dixon, 2000).

Heavy sedimentation is associated with fewer coral species, less live coral, lower coral growth rates, greater abundance of branching forms, reduced coral recruitment, decreased calcification, decreased net productivity of corals, and slower rates of reef accretion (Rogers, 1990). Tolerance of corals to high sediment loads varies considerably among species, with some corals being fairly resistant to low light levels and/or sedimentation effects (Rice and Hunter, 1992).

Field and laboratory experiments in Florida (USA) have shown that some of the most tolerant coral species in the Caribbean can survive complete burial with sediment for periods ranging from 7 to 15 days (Rice and Hunter, 1992) (Table 8). Burial with sediment of several Philippine corals caused sublethal effects (bleaching) and mortality within 20 to 68 h (Wesseling et al., 1999). Polyp inflation is an effective means of actively shedding sediment and corals with large inflation ratios are among the best sediment rejecters. Inflators are not only capable of (re)moving sediment continuously, but they also can endure siltation rates 5–10 times higher than regularly found on coral reefs. Many of these coral species are small forms, living attached or loose in sand bottoms, such as the Caribbean faviid *Manicina areolata* and the Pacific fungiid corals (Schuhmacher, 1977, 1979; Hoeksema, 1993; Johnson, 1992; Hubmann et al., 2002; Uhrin et al., 2005; Sorauf and Harries, 2010; Bongaerts et al., 2012).

A synthesis of literature data regarding sensitivity of different coral species to sedimentation is presented in Table 9. These data were reworked and related to a relative sensitivity index according to the response matrix presented in Table 10. Sensitivity classes were then given scores from 1 to 5, with 1 corresponding to "very tolerant" and 5 to "very sensitive". The scores for individual coral species were subsequently related to their dominant growth form and mean calyx diameter. Analysis of these data (102 entries for 71 species) confirmed that there is a significant relationship (Kruskal–Wallis, $P < 0.05$) between the growth form of corals and their sensitivity to sedimentation (Fig. 6a). Free-living corals (such as mushroom corals), branching corals and many massive corals (especially with fleshy polyps) are quite tolerant to high rates of sedimentation, while laminar, plating and tabular corals as well as several soft corals are relatively sensitive. There was no significant relationship between the calyx diameter of corals and their sensitivity to sedimentation (Fig. 6b).

This relatively straightforward relationship (Figs. 5 and 6) can of course be complicated and altered by the interaction of several other factors such as active or passive sediment-clearing mechanisms, turbulence and exposure to wave action, colony orientation, morphological variability and adaptation within species, depth distribution, and the cumulative effects of extreme temperatures and salinities. However, despite some variability, complication by other factors and even some potential contradictions, it is clear from the overall findings that corals can indeed be roughly categorised according to their relative sensitivity to turbidity and sedimentation based on their growth form and morphology (Fig. 5 and 6).

6. Mitigating factors and potential for recovery

The sensitivity of corals to, and their ability to recover from, the impacts of dredging and related activities depends on a range of factors, including the ecological state or condition of the reef (e.g. degraded or pristine; dominated by algae, bio-eroders or reef-builders; level of fishing; and temperature anomalies), its resilience (species diversity; presence of keystone species; loss and replacement of keystone species; spatial heterogeneity; presence of refugia and connectivity to nearby unaffected reefs) and the

Table 9

Sensitivity of different coral species for sedimentation. Overview of the response of different species of corals to various sedimentation rates tested, as reported in the literature. Nomenclature of coral species was updated according to the most recent taxonomic revisions. Growth forms (as stated or inferred): B = branching; C = columnar (incl. digitate); E = encrusting; F = foliaceous; L = laminar (incl. plate & tabular); M = massive; S = solitary (free-living); So = soft corals & gorgonians. Calyx diameter taken from Stafford-Smith and Ormond (1992) supplemented with own measurements (BWH – Naturalis).

Coral species	Sedimentation rate (tested)	Response	Growth form	Calyx (mm)	References
Acropora cervicornis (Lamarck, 1816)	200 mg/m^2/d (daily for 45 days)	No effect (not even on growth rate) even after 45 days	B	1.0	Rogers (1979)
Acropora cervicornis (Lamarck, 1816)	200 mg cm^{-2} d^{-1} (daily)	No effect	B	1.0	Rogers (1990)
Acropora cervicornis (Lamarck, 1816)	430 mg cm^{-2} d^{-1} (>1 day)	Physiological stress	B	1.0	Bak and Elgershuizen (1976)
Acropora cervicornis (Lamarck, 1816)	Burial (10–12 cm of reef sand)	Sublethal stress within 12 h; 100% mortality within 72 h	B	1.0	Thompson (1980a)
Acropora formosa (Dana, 1846)	Up to 14.6 mg/m^2/d (fine silt) due to dredging	No effect on growth rate (in situ)	B	1.2	Chansang et al. (1992)
Acropora formosa (Dana, 1846)	200–300 mg cm^{-2} d^{-1} (up to 7 days)	Decreased growth	B	1.2	Simpson (1988)
Acropora millepora (Ehrenberg, 1834) (larvae)	0.5–325 mg cm^{-2} d^{-1} (2 days)	Reduction of larval settlement			Babcock (1991)
Acropora millepora (Ehrenberg, 1834)	83 mg cm^{-2} d^{-1} (up to 16 weeks)	Onset mortality after 4 weeks, full mortality after 12 weeks	B	1.0	Negri et al. (2009) and Flores et al. (2011)
Acropora palifera (Lamarck 1816)	Field site comparison (<1 versus 13.5 mg cm^{-2} d^{-1})	Reduced fecundity at site with higher sedimentation	L	2.0	Kojis and Quinn (1984)
Acropora palmata (Lamarck, 1816)	Up to 600 mg cm^{-2} d^{-1} (natural events)	Poor rejection ability; sediment accumulation	B	2.0	Abdel-Salam and Porter (1988)
Acropora palmata (Lamarck, 1816)	430 mg cm^{-2} d^{-1} (>1 day)	Physiological stress	B	2.0	Bak and Elgershuizen, 1976
Acropora palmata (Lamarck, 1816)	200 mg cm^{-2} d^{-1} (once)	Partial mortality	B	2.0	Rogers (1977)
Acropora palmata (Lamarck, 1816)	200 mg cm^{-2} d^{-1} (field application)	Death of underlying tissue	B	2.0	Rogers (1990)
Acropora palmata (Lamarck, 1816)	Burial (10–12 cm of reef sand)	100% mortality within 72 h	B	2.0	Thompson (1980a)
Acropora sp.	5 mg cm^{-2} d^{-1}	Massive mucus production (within 1 h), sublethal			Fabricius and Wolanski (2000)
Acropora sp.	Burial for 20 h	Mortality of all colonies			Wesseling et al. (1999)
Acropora spp.	39.6 mg cm^{-2} d^{-1} (for 2 weeks)	Partial bleaching (less affected)			Fabricius et al. (2007)
Agaricia agaricites (Linnaeus, 1758)	Heavy sedimentation event (>1 cm)	Reduced growth but survival	L	5.0	Bak (1978)
Agaricia agaricites (Linnaeus, 1758)	430 mg cm^{-2} d^{-1} (sand)	Mortality after 1 day	L	5.0	Bak and Elgershuizen (1976)
Agaricia agaricites (Linnaeus, 1758)	Burial (10–12 cm of reef sand)	60% Tissue loss within 24 h; 100% mortality after 72 h	L	5.0	Thompson (1980a)
Agaricia lamarcki Milne Edwards & Haime, 1851	140 mg/m^2/d (mean) for several weeks	Mass mortality (4 years after steep decline in growth)	L	8.0	van 't Hof (1983)
Agaricia sp.	30 mg/m^2/d (natural)	No effect; dominant species			Loya (1976)
Alveopora spp.		Can survive high sedimentation rates			Stafford-Smith and Ormond (1992)
Astrangia poculata (Ellis & Solander, 1786)	<600 mg cm^{-2} d^{-1}	Survival	S	6.0	Peters and Pilson (1985)
Catalaphyllia jardinei (Saville-Kent, 1893)		Survive high sedimentation rates	M	40.0	Stafford-Smith and Ormond (1992)
Cladocora arbuscula (Lesueur, 1812)	Complete burial	50% Survival after 15 days	B	4.0	Rice and Hunter (1992)
Ctenactis echinata (Pallas, 1766)	Continuously repeated burial (sand)	Tissue mortality and colony death after 24–72 h	S	200.0	Schuhmacher (1977)
Cycloseris costulata (Ortmann, 1889)	Continuously repeated burial (sand)	Survival (endurance with no apparent effect)	S	15.0	Schuhmacher (1977)
Cycloseris costulata (Ortmann, 1889)	40 mm3/cm^2/d	Maximum rate tolerated (field gradient)	S	15.0	Schuhmacher (1977)
Cycloseris distorta (Michelin, 1842)		Efficient sediment rejector (polyp inflation)	S	7.5	Schuhmacher (1977)
Cycloseris spp.		Can actively dig through overlying sediment			Stafford-Smith and Ormond (1992)
Danafungia horrida (Dana, 1846)	Continuously repeated burial (sand)	Tissue mortality and colony death after 24–72 h	S	215.0	Schuhmacher (1977)
Danafungia scruposa (Klunzinger, 1879)	Continuously repeated burial (sand)	Tissue mortality and colony death after 24–72 h	S	380.0	Schuhmacher (1977)
Dichocoenia stokesi Milne Edwards & Haime, 1848	430 mg cm^{-2} d^{-1} (sand + oil)	Mortality after 1 day	M	11.0	Bak and Elgershuizen (1976)
Diploastrea heliopora (Lamarck, 1816)	20 mg cm^{-2} d^{-1} (mixed sand)	Survival (4 months)	M	14.0	Todd et al. (2004a)
Diploria clivosa (Ellis & Solander, 1786)	Repeated application of 200 mg/cm^2	Extensive damage	M	9.0	Rogers (1983)
Diploria labyrinthiformis (Linnaeus, 1758)	High sedimentation rates (dredging)	Survival (no effect)	M	8.0	Dodge and Vaisnys (1977)
Diploria strigosa (Dana, 1846)	Up to 600 mg cm^{-2} d^{-1} (natural events)	High sediment clearing rate	M	8.0	Abdel-Salam and Porter (1988)
Diploria strigosa (Dana, 1846)	200 mg cm^{-2} d^{-1} (daily)	No effect	M	8.0	Rogers (1990)
Diploria strigosa (Dana, 1846)	High sedimentation rates (dredging)	Mass mortality (4 years after steep decline in growth)	M	8.0	Dodge and Vaisnys (1977)

Table 9 (*continued*)

Coral species	Sedimentation rate (tested)	Response	Growth form	Calyx (mm)	References
Diploria strigosa (Dana, 1846)	Burial (10–12 cm of reef sand)	Partial bleaching and sublethal stress within 24 h	M	8.0	Thompson (1980a)
Duncanopsammia axifuga (Milne Edwards & Haime, 1848)		Can survive high sedimentation rates	B	14.0	Stafford-Smith and Ormond (1992)
Echinopora spp.		Active sediment rejector			Stafford-Smith and Ormond (1992)
Echinopora mammiformis (Nemenzo, 1959)		Active sediment rejector	L	5.0	Stafford-Smith and Ormond (1992)
Euphyllia spp.		Can survive high sedimentation rates			Stafford-Smith and Ormond (1992)
Favia favus (Forskal, 1775)	200 mg cm^{-2} d^{-1} (6 weeks)	Minor tissue damage, mucus production, no bleaching	M	14.0	Riegl (1995) and Riegl and Bloomer (1995)
Favia speciosa (Dana, 1846)	20 mg cm^{-2} d^{-1} (mixed sand)	Survival (4 months)	M	12.0	Todd et al. (2004a)
Favia spp.	(0.9–1.3 mg/m^2/day)	Described as relatively 'sensitive' to sedimentation			McClanahan and Obura (1997)
Favia stelligera (Dana, 1846)	200 mg cm^{-2} d^{-1}	Mortality within 1–2 days	M	6.0	Stafford-Smith (1993)
Favites pentagona (Esper, 1794)	200 mg cm^{-2} d^{-1} (6 weeks)	Tissue damage, mucus production	M	7.0	Riegl (1995) and Riegl and Bloomer (1995)
Favites spp.	(between 1.3 and 4 mg cm^{-2} d^{-1}; not quoted)	tolerance to sedimentation described as 'intermediate'			McClanahan and Obura (1997)
Fungia fungites (Linnaeus, 1758)	Continuously repeated burial (sand)	Tissue mortality and colony death after 24–72 h	S	310.0	Schuhmacher (1977)
Fungia fungites (Linnaeus, 1758)	10 mm^3/cm^2/d	Maximum rate tolerated	S	310.0	Schuhmacher (1977)
Galaxea fascicularis (Linnaeus, 1767)	39.6 mg cm^{-2} d^{-1} (for 2 weeks)	Sublethal (sed.accum.), act.removal (polyp), recovery	M	8.0	Fabricius et al. (2007)
Galaxea fascicularis (Linnaeus, 1767)	Burial for 20 h	Tissue bleaching, recovery after 4 weeks	M	8.0	Wesseling et al. (1999)
Galaxea spp.	(4 mg/m^2/day)	Tolerance to sedimentation described as 'intermediate'			McClanahan and Obura (1997)
Gardineroseris planulata (Dana, 1846)	200 mg cm^{-2} d^{-1}	Partial mortality after 6 days	M	7.0	Stafford-Smith (1993)
Goniastrea retiformis (Lamarck, 1816)		Common on reefs affected by sedimentation	M	4.0	Brown and Howard (1985)
Goniopora lobata Milne Edwards & Haime, 1860		Active sediment rejector	[c]	4.0	Stafford-Smith and Ormond (1992)
Goniopora spp.		Survive high sedimentation rates			Stafford-Smith and Ormond (1992)
Gyrosmilia interrupta (Ehrenberg, 1834)	200 mg cm^{-2} d^{-1} (6 weeks)	Tissue damage, mucus production, no bleaching	M/E	16.0	Riegl (1995) and Riegl and Bloomer (1995)
Heliofungia actiniformis (Quoy & Gaimard, 1833)		Efficient sediment rejector (polyp inflation)	S	210.0	Schuhmacher (1977)
Heliopora coerulea (Pallas, 1766)	Burial for 20 h	Tissue bleaching, recovery after 4 weeks	B	0.8	Wesseling et al. (1999)
Heteropsammia cochlea (Spengler, 1783)		Obligate commensal sipunculid prevents burial	S	7.0	Stafford-Smith and Ormond (1992)
Hydnophora spp.	(4 mg/m^2/day)	Tolerance to sedimentation described as 'intermediate'			McClanahan and Obura (1997)
Isopora palifera (Lamarck, 1816)	10–15 mg cm^{-2} d^{-1}	50% Reduction in fecundity	C	2.0	Kojis and Quinn (1984)
Isophyllia sinuosa (Ellis & Solander, 1786)	Complete burial	50% Survival after 7.2 days	M	15.0	Rice and Hunter (1992)
Leptoria phrygia (Ellis & Solander, 1786)	25 mg cm^{-2} d^{-1}	Minor tissue damage within 3 weeks	M	4.1	Stafford-Smith (1992)
Leptoria phrygia (Ellis & Solander, 1786)	50–100 mg cm^{-2} d^{-1}	Major tissue damage and bleaching after 4 days	M	4.1	Stafford-Smith (1992)
Leptoria phrygia (Ellis & Solander, 1786)	100–200 mg cm^{-2} d^{-1}	Partial mortality and bleaching after 4 days	M	4.1	Stafford-Smith (1992)
Leptoria phrygia (Ellis & Solander, 1786)	>200 mg cm^{-2} d^{-1}	Mortality within 1–2 days	M	4.1	Stafford-Smith (1992, 1993)
Lobophytum depressum Tixier-Durivault, 1966	200 mg cm^{-2} d^{-1} (6 weeks)	Tissue damage, bleaching and partial mortality	So		Riegl (1995) and Riegl and Bloomer (1995)
Lobophytum venustum Tixier-Durivault, 1957	200 mg cm^{-2} d^{-1} (6 weeks)	Minor tissue damage and bleaching	So		Riegl (1995) and Riegl and Bloomer (1995)
Madracis auretenra Locke, Weil & Coates, 2007	Heavy sedimentation event (>1 cm)	Reduced growth but survival	B	1.0	Bak (1978)
Manicina areolata (Linnaeus, 1758)	Complete burial	50% Survival after 10 days	M	23.0	Rice and Hunter (1992)
Meandrina meandrites (Linnaeus, 1758)		Produces copious amounts of mucus to remove silt	M	15.0	Dumas and Thomassin (1977)
Millepora spp.	(4 mg/m^2/day)	Tolerance to sedimentation described as 'intermediate'			McClanahan and Obura (1997)
Montastraea annularis (Ellis & Solander, 1786)		High sediment clearing rate	M/E	5.0	Abdel-Salam and Porter (1988)
Montastraea annularis (Ellis & Solander, 1786)	200 mg cm^{-2} d^{-1} (daily applications)	Tolerant for at least 38 days	L/E	5.0	Rogers (1979)
Montastraea annularis (Ellis & Solander, 1786)	400–800 mg cm^{-2} d^{-1} (single application)	Mortality	M	5.0	Rogers (1979)

Table 9 (continued)

Coral species	Sedimentation rate (tested)	Response	Growth form	Calyx (mm)	References
Montastraea annularis (Ellis & Solander, 1786)	19 mg cm^{-2} d^{-1} (permanent)	Reduced growth rate	M/E	5.0	Torres (1998)
Montastraea annularis (Ellis & Solander, 1786)	200 mg cm^{-2} d^{-1} (daily)	No effect	M/E	5.0	Rogers (1990)
Montastraea annularis (Ellis & Solander, 1786)	400 mg cm^{-2} d^{-1}	Temporary bleaching	M/E	5.0	Rogers (1990)
Montastraea annularis (Ellis & Solander, 1786)	800 mg cm^{-2} d^{-1}	Death of underlying tissue	M/E	5.0	Rogers (1990)
Montastraea annularis (Ellis & Solander, 1786)	800 mg cm^{-2} d^{-1} (single application)	Mortality	M/E	5.0	Rogers (1977)
Montastraea annularis (Ellis & Solander, 1786)	430 mg cm^{-2} d^{-1} (sand + oil)	Mortality after 1 day	L/M	5.0	Bak and Elgershuizen (1976)
Montastraea annularis (Ellis & Solander, 1786)	10 mg cm^{-2} d^{-1} (natural)	Reduced %cover	M	5.0	Torres and Morelock (2002)
Montastraea annularis (Ellis & Solander, 1786)	19 mg cm^{-2} d^{-1} (resuspended carbonate mud)	Reduced growth rate	M	5.0	Dodge et al. (1974)
Montastraea annularis (Ellis & Solander, 1786)	Burial (10–12 cm of reef sand)	40% Tissue loss within 24 h; 90% tissue loss within 72 h	M	5.0	Thompson (1980a)
Montastraea annularis (Ellis & Solander, 1786)		Produces little mucus; removes silt by by ciliary action	M	5.0	Dumas and Thomassin (1977)
Montastraea cavernosa (Linnaeus, 1767)	<1390 mg cm^{-2} d^{-1}	Survival	M	11.0	Lasker (1980)
Montastraea cavernosa (Linnaeus, 1767)	150 mg/m^2/d (natural)	Survival/dominance	M	11.0	Loya (1976)
Montastraea cavernosa (Linnaeus, 1767)	Burial (10–12 cm of reef sand)	30% Tissue loss after 72 h; remaining tissue in decay	M	11.0	Thompson (1980a)
Montipora aequituberculata Bernard, 1897	200 mg cm^{-2} d^{-1}	Bleaching after 6 days (but no mortality)	L	0.6	Stafford-Smith (1993)
Montipora aequituberculata Bernard, 1897	25 mg cm^{-2} d^{-1} (up to 16 weeks)	Onset mortality after 4 weeks, full mortality after 12 weeks	F	0.6	Negri et al. (2009) and Flores et al. (2011)
Montipora capitata Dana, 1846	Burial (2.2–2.8 g/cm^2 for 45 h)	sublethal effects after 30 h, little recovery after 90 h	B	2.0	Piniak (2007)
Montipora foliosa (Pallas, 1766)		Active sediment rejector	L	0.7	Stafford-Smith and Ormond (1992)
Montipora peltiformis Bernard, 1897	33–160 mg/cm^2 (silt) exposure for 36 h	Reduced photosynthesis within 12–60 h	F	1.0	Weber et al. (2006)
Montipora peltiformis Bernard, 1897	79–234 mg/cm2 (up to 36 h)	Signifcant decline in photosynthesis (quantum yield)	M/L	1.0	Philipp and Fabricius (2003)
Montipora spp.	(0.9–1.3 mg/m^2/day)	Described as 'sensitive' to sedimentation			McClanahan and Obura (1997)
Montipora verrucosa (Lamarck, 1816)	30 mg cm^{-2} d^{-1} (daily applications)	Survived (10 days of application)	M	1.5	Hodgson (1990a)
Mycetophyllia aliciae Wells, 1973	430 mg cm^{-2} d^{-1} (sand + oil)	Mortality after 1 day	L	14.0	Bak and Elgershuizen (1976)
Oxypora glabra Nemenzo, 1959	30 mg cm^{-2} d^{-1} (daily applications)	Total mortality within 10 days	L/E	5.0	Hodgson (1990a)
Pectinia lactuca (Pallas, 1766)		Active sediment rejector	L	18.0	Stafford-Smith and Ormond (1992)
Pectinia paeonia (Dana, 1846)		Active sediment rejector	L	15.0	Stafford-Smith and Ormond (1992)
Pectinia sp.		Active sediment rejector			Stafford-Smith and Ormond (1992)
Platygyra daedalea (Ellis & Solander, 1786)	200 mg cm^{-2} d^{-1} (6 weeks)	Minor tissue damage, mucus production, no bleaching	M	5.0	Riegl (1995) and Riegl and Bloomer (1995)
Platygyra sinensis (Milne Edwards & Haime, 1849)	Complete burial	Bleaching and tissue damage after 48 h	M	4.0	Wong (2001)
Platygyra spp.	(4 mg/m^2/day)	Tolerance to sedimentation described as 'intermediate'			McClanahan and Obura (1997)
Pleuractis granulosa (Klunzinger, 1879)	Continuously repeated burial (sand)	Survival (high endurance with no apparent effect)	S	185.0	Schuhmacher (1977)
Pleuractis granulosa (Klunzinger, 1879)	15 mm^3/cm^2/d	Maximum rate tolerated	S	185.0	Schuhmacher (1977)
Pleuractis moluccensis (Van der Horst, 1919)		Adapted to withstand considerable sedimentation rates	S	19.0	Schuhmacher (1977)
Pocillopora damicornis (Linnaeus, 1758)	50–95% sediment cover	Complete inhibition of larval settlement	B	1.0	Hodgson (1990b)
Pocillopora damicornis (Linnaeus, 1758)	67 and 186 mg cm^{-2} d^{-1} (fine silt; 83 days)	50–100% Mortality of transplanted fragments (esp. small)	B	1.0	Sakai et al. (1989)
Pocillopora damicornis (Linnaeus, 1758)	11–490 mg cm^{-2} d^{-1}ay (11 months)	Reduced growth rate of transplanted fragments	B	1.0	Piniak and Brown (2008)
Pocillopora meandrina Dana, 1846	30 mg cm^{-2} d^{-1} (daily applications)	Mortality within 10 days	B	1.0	Hodgson (1990a)
Pocillopora sp.	Increased sedimentation (dredging)	Considerable mortality			Hudson et al. (1982)

Table 9 (*continued*)

Coral species	Sedimentation rate (tested)	Response	Growth form	Calyx (mm)	References
Pocillopora spp.	(0.9–1.3 mg/m²/day)	Described as 'sensitive' to sedimentation			McClanahan and Obura (1997)
Porites astreoides Lamarck, 1816	Heavy sedimentation event (>1 cm)	Mortality (inability to reject sediment)	L	1.5	Bak (1978)
Porites astreoides Lamarck, 1816		Abundant in heavily sedimented areas	M	1.5	Cortes and Risk (1985)
Porites astreoides Lamarck, 1816	430 mg cm⁻² d⁻¹ (sand)	Mortality after 1 day	M/E	1.5	Bak and Elgershuizen (1976)
Porites astreoides Lamarck, 1816	10 mg cm⁻² d⁻¹ (natural)	No effect	M/E	1.5	Torres and Morelock (2002)
Porites astreoides Lamarck, 1816	Burial (10–12 cm of reef sand)	Bleaching within 24 h; 70% tissue loss after 72 h	M/E	1.5	Thompson (1980a)
Porites lobata Dana, 1846	30 mg cm⁻² d⁻¹ (daily applications)	Mortality within 10 days	M	1.5	Hodgson (1990a)
Porites lobata Dana, 1846	Burial (1.5–1.6 g/cm² for 45 h)	Sublethal effects after 30 h, little recovery after 90 h	M	1.5	Piniak (2007)
Porites lobata Dana, 1846	200 mg cm⁻² d⁻¹	Bleaching after 6 days (but no mortality)	M	1.5	Stafford-Smith (1993)
Porites lobata Dana, 1846	Complete burial (48 h)	Bleaching; complete recovery after sediment removal	M	1.5	Yeung (2000)
Porites lutea Milne Edwards & Haime, 1851	200 mg cm⁻² d⁻¹	Bleaching after 6 days (but no mortality)	M	1.5	Stafford-Smith (1993)
Porites lutea Milne Edwards & Haime, 1851		Common on reefs affected by sedimentation	M	1.5	Brown and Howard (1985)
Porites lutea Milne Edwards & Haime, 1851	Increased sedimentation (dredging)	Survival	M	1.5	Hudson et al. (1982)
Porites lutea Milne Edwards & Haime, 1851	Up to 14.6 mg/m²/d (fine silt) due to dredging	No effect on growth rate (in situ)	M	1.5	Chansang et al. (1992)
Porites porites (Pallas, 1766)		Uses tentacles to remove larger sediment particles	M	2.0	Meyer (1989)
Porites porites (Pallas, 1766) *forma* furcata	Burial (10–12 cm of reef sand)	90% bleaching within 24 h; 70% tissue loss after 72 h	B	2.0	Thompson (1980a)
Porites rus (Forskal, 1775)	39.6 mg cm⁻² d⁻¹ (for 2 weeks)	Massive mortality (anoxia)	M	0.5	Fabricius et al. (2007)
Porites sp.		Persists in areas of heavy sedimentation			Fabricius (2005)
Porites sp.	Burial for 6 h	No effect			Wesseling et al. (1999)
Porites sp.	Burial for 20 h	Discoloration & bleaching after 3 weeks			Wesseling et al. (1999)
Porites sp.	39.6 mg cm⁻² d⁻¹ (for 2 weeks)	Mucus production, survival (most tolerant)			Fabricius et al. (2007)
Porites spp.	(between 1.3 and 4 mg cm⁻² d⁻¹; not quoted)	Tolerance to sedimentation described as 'intermediate'			McClanahan and Obura (1997)
Sarcophyton glaucum (Quoy & Gaimard, 1833)	200 mg cm⁻² d⁻¹	Tissue damage and partial mortality within 6 weeks	So		Riegl (1995)
Scolymia cubensis (Milne Edwards & Haime, 1849)	Complete burial	50% Survival after 7 days	S	75.0	Rice and Hunter (1992)
Scolymia cubensis (Milne Edwards & Haime, 1849)	3 g of 3 grain-sizes: 62 µm, 250 µm, 2 mm (24 h)	Sediment-shedding efficiency related to calical angle	S	75.0	Logan (1988)
Siderastrea radians (Pallas, 1766)	Complete burial	50% Survival after 13.6 days	M/E	5.0	Rice and Hunter (1992)
Siderastrea radians (Pallas, 1766)	Total burial	Survival for more than 73 h	M/E	5.0	Mayer (1918)
Siderastrea radians (Pallas, 1766)	Burial (chronic)	Reduced growth and some mortality	M/E	5.0	Lirman et al. (2003)
Siderastrea siderea (Ellis & Solander, 1786)	10 mg cm⁻² d⁻¹ (natural)	No effect	M	3.0	Torres and Morelock (2002)
Siderastrea siderea (Ellis & Solander, 1786)	0.3–64 mg cm⁻² d⁻¹	Partial mortality	M	3.0	Nugues and Roberts (2003)
Siderastrea siderea (Ellis & Solander, 1786)	Burial (10–12 cm of reef sand)	50% Bleaching and sublethal stress within 24 h	M	3.0	Thompson (1980a)
Sinularia dura (Pratt, 1903)	200 mg cm⁻² d⁻¹ (6 weeks)	Minor tissue damage and bleaching	So		Riegl (1995) and Riegl and Bloomer (1995)
Sinularia leptoclados (Ehrenberg, 1834)	200 mg cm⁻² d⁻¹ (6 weeks)	Minor tissue damage and bleaching	So		Riegl (1995), Riegl and Bloomer (1995)
Solenastrea hyades (Dana, 1846)	Complete burial	50% Survival after >15 days	M	5.0	Rice and Hunter (1992)
Stephanocoenia intersepta (Lamarck, 1816)	Complete burial	50% Survival after 16.2 days	M	3.0	Rice and Hunter (1992)
Trachyphyllia geoffroyi (Audouin, 1826)		Actively dig through overlying sediment	S	45.0	Stafford-Smith and Ormond (1992)
Turbinaria mesenterina	110 mg/cm² (5 weeks)	No significant sublethal physiological effects	L	1.5	Sofonia and Anthony (2008)
Turbinaria (several spp.)		Active sediment rejector			Stafford-Smith and Ormond (1992)

typical ambient conditions experienced by the reef (McClanahan et al., 2002; Marshall and Schuttenberg, 2006). Reefs with effective management that minimises anthropogenic stresses are likely to have higher resilience than reefs that are already experiencing multiple stressors (West and Salm, 2003). Cumulative effects from or on related (adjacent) ecosystems such as mangroves and seagrass meadows (including effects from maintenance dredging cycles) may also have indirect consequences for the coral reef

Table 10
Response matrix ranking the relative sensitivity of corals according to their type of response to different rates of sedimentation.

| Response category	Sedimentation rate (mg cm^{-2} d^{-1}) tested:				
	<10	10–50	50–200	>200	Complete burial
No effect	(most spp.)	Intermediate	Tolerant	Very tolerant	Very tolerant
Sublethal effects (minor)	Sensitive	Intermediate	Tolerant	Very tolerant	Very tolerant
Sublethal effects (major) (bleaching, tissue damage)	Sensitive	Sensitive	Intermediate	Tolerant	Tolerant
Lethal effects (partial mortality)	Very sensitive	Sensitive	Intermediate	Tolerant	Tolerant
Major lethal effects (mass mortality)	Very sensitive	Very sensitive	Sensitive	(most spp.)	(most spp.)
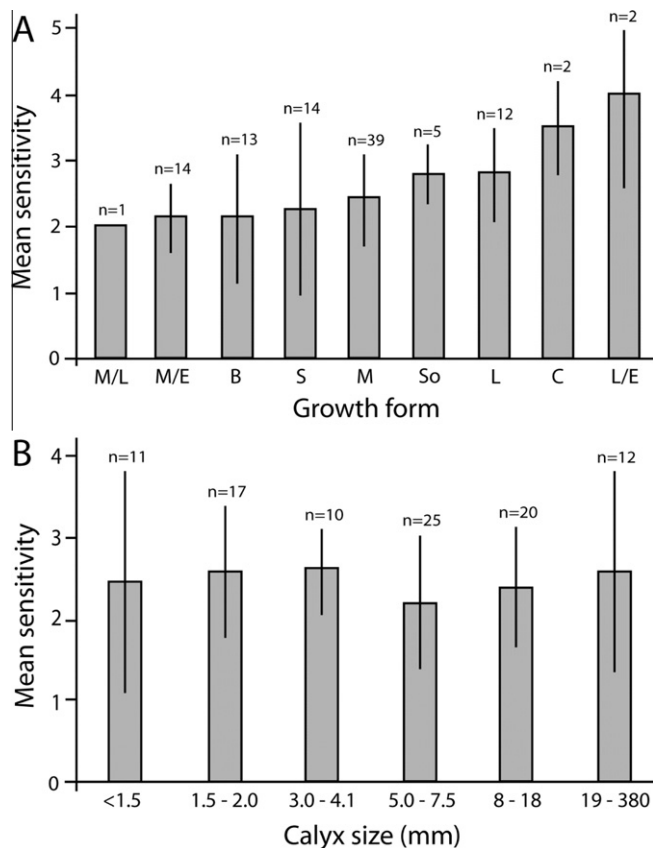
Fig. 6. Relationship between the sensitivity of corals to sedimentation and [A] their growth form, and [B] their calyx size. Sensitivity (mean score ± SD) was determined by ranking corals according to their type of response to different rates of sedimentation (see text and Table 10). Legend (growth forms): B = branching; C = columnar (incl. digitate); E = encrusting; L = laminar (incl. plate & tabular); M = massive; S = solitary (free-living); So = soft corals & gorgonians.

ecosystem. This is particularly so for ecological processes, functions and reef species that have important inter-linkages with mangrove and seagrass systems (Hemminga et al., 1994; Adams et al., 2006; Pollux et al., 2007). The timing of the dredging and construction activities may also affect the severity of impact, depending on the degree of seasonality and day–night cycles characterising the particular reef. Impacts during, or shortly prior to and after spawning events are of particular concern, since not only adult organisms may be negatively affected, but recruitment for the entire season may be jeopardised.

While sedimentation certainly is a major stressor that can lead to significant coral mortality, strong, isolated sediment pulses need not necessarily kill a reef. Many reefs, and certainly corals in most settings, can indeed survive repeated, even severe, sediment input (Browne et al., 2010). One of the most important factors mitigating against permanent damage is strong water motion, either by surge or by currents, that serves to re-suspend and remove the sediment

from the corals (Stafford-Smith and Ormond, 1992; Riegl, 1995; Riegl et al., 1996; Schleyer and Celliers, 2003). As long as the coral's surface is free from sediment, regeneration is relatively easily achieved, even if damage occurred. A continuous cover of sediment on corals may lead to beginning tissue necrosis within 24 h in sensitive coral species, while in tolerant species there may still be no signs of necrosis after 14 days (Table 8). This process is particularly readily observed in soft corals. Once the sediment has been removed, however, even if tissue necroses have occurred, regeneration can take place in the space of only a few weeks (Meesters et al., 1992). Strong currents can aide passive sediment-clearing. Purely oscillating currents or surge, while temporarily cleaning colonies, may not help overall since sediments will build up around the corals and eventually smother them.

Provided that environmental conditions return to the pre-impact situation and that these conditions are not hampering recovery, time-scales for natural recovery of coral reefs are in the order of a few years to several decades, depending on the degree of damage, types of species affected, and possibilities for recruitment (Pearson, 1981; Moberg and Rönnbäck, 2003). Recovery of corals from sublethal stress can be rapid (weeks to months), while recovery from partial mortality takes several years. Reef recovery from mass mortality is generally slow and may take many years to decades, while in some cases recovery has not occurred at all. Few examples of recovery of coral reefs after severe sediment damage have been documented. Increased sedimentation is sometimes accompanied by other stresses, prolonging or inhibiting recovery, making it difficult to generalise or make predictions about recovery (Rogers, 1990). Of 65 examples for which sufficient data exist to make a judgment, coral cover recovered in 69% of cases after acute, short-term disturbances, but only in 27% of cases after chronic, long-term disturbance (Connell, 1997).

Wesseling et al. (1999) noted that the recovery time of corals following experimental short-term burial varied among coral species, ranging from several weeks to months, and also depended on the duration of the sedimentation event. In larger massive corals, sediment burial may cause bleaching and damaged patches, which—if larger than about 2 cm in diameter—do not recover, but will be colonised by algae or sponges preventing recovery of the coral (Hodgson, 1994). Brown et al. (1990) reported a 30% reduction in living coral cover 1 year after the start of dredging operations at Phuket (Thailand). After the dredging event had ceased, the reef recovered rapidly with coral cover values and diversity indices restored to former levels around 22 months after dredging began. The domination of this reef by massive coral species, which are physiologically adapted to intertidal living and which display partial rather than total colony mortality, may have contributed to its apparent resilience (Brown et al., 2002). Maragos (1972) estimated that 80% of the coral communities in the lagoon of Kaneohe Bay (Hawaii) died because of a combination of dredging, increased sedimentation and sewage discharge. Six years after discharge of sewage into Kaneohe Bay ceased, a dramatic recovery of corals and a decrease in the growth of smothering algae was reported (Maragos et al., 1985).

Coastal coral reefs adjacent to population centers often do not recover from disturbances, in contrast to remote reefs in relatively pristine environments, because chronic human influences have degraded water and substratum quality, thereby inhibiting recovery (McCook, 1999a; Wolanski et al., 2004). In the Seychelles, where corals had to recover from an intense bleaching event, *Acropora* species—usually the first to rapidly colonise new empty spaces—recovered substantially more slowly due to recruitment limitation, because these species were virtually eliminated throughout almost the entire Indian Ocean (Goreau, 1998). As a result, these species will not be able to re-establish themselves for many years or even decades. Poor water quality and excessive algal growth in some areas hampered recovery even when coral larvae were available (Goreau, 1998).

7. Management of dredging operations near coral reefs

For an overview of best practices for the management of dredging operations near coral reefs, reference is made to the recent PIANC report No. 108 (PIANC, 2010). Setting realistic and ecologically meaningful thresholds for model interrogation, as permit conditions to dredging contractors and for use as triggers in a reactive monitoring and management program, can be a challenge in coral reef environments. One of the problems encountered when trying to determine realistic thresholds for dredging near coral reefs includes a lack of knowledge, since only 10% of coral species has ever been studied with respect to their response to sediment disturbance. There is still a rather poor understanding of the relationship between sediment stress and the response of most corals. While meaningful sets of thresholds or criteria would ideally have to incorporate the intensity, duration and frequency of turbidity (or sedimentation) events generated by the dredging activities, actual values are difficult to determine with confidence and at present remain little more than estimates.

In some cases, uncertainties in model predictions of dredging plumes and a conservative approach by regulators applying the precautionary principle may have led to overestimation of impacts of dredging operations on corals while field monitoring suggested less coral mortality than predicted (Hanley, 2011). In other cases, the opposite situation may have led to unnecessary and avoidable damage on coral reefs. To prevent coral mortality, there is clearly a need for reliable sublethal coral health indicators as early warning for stress but the science for this is still in its infancy (Jameson et al., 1998; Vargas-Angel et al., 2006; Cooper and Fabricius, 2007; Cooper et al., 2009). Such bio-indicators, some of which can show remarkable temporal dynamics in response to variations in water quality (Cooper et al., 2008), require on-site validation before use in monitoring programs (Fichez et al., 2005).

Recently, some significant advances have been made in establishing reactive (feedback) monitoring programs that have proven a meaningful tool for minimising coral mortality during large-scale dredging operations in Singapore and Australia (Koskela et al., 2002; Doorn-Groen, 2007; Sofonia and Unsworth, 2010). The design of such monitoring programs should guarantee sufficient statistical power to detect a required effect size, which can be as much a challenge as the availability of suitable reference sites. Seasonal restrictions during mass coral spawning are sometimes placed on dredging programs, but the effectiveness of such mitigating measures on long-term coral reef resilience is not well understood. Given the wide variation in sensitivity among coral species, meaningful criteria to limit the extent and turbidity of dredging plumes and their effects on corals will always require site-specific evaluations. We emphasise the importance of taking into account the species assemblage present at any given site and understanding the dynamics of local ambient background conditions, including spatial and temporal variability of turbidity and sedimentation, before setting thresholds in any dredging operation near coral reefs. A combination of reactive (feedback) monitoring of water quality and coral health during dredging activities and spill-budget modelling of dredging plumes to guide decisions on when to modify (or even stop) dredging appears to be the most promising approach to effectively minimise negative impacts on corals and coral reefs.

Acknowledgments

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Effects of terrestrial runoff on the ecology of corals and coral reefs: review and synthesis

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Abstract

This paper reviews and evaluates the current state of knowledge on the direct effects of terrestrial runoff on (1) the growth and survival of hard coral colonies, (2) coral reproduction and recruitment, and (3) organisms that interact with coral populations (coralline algae, bioeroders, macroalgae and heterotrophic filter feeders as space competitors, pathogens, and coral predators). The responses of each of these groups are evaluated separately against the four main water quality parameters: (1) increased dissolved inorganic nutrients, (2) enrichment with particulate organic matter, (3) light reduction from turbidity and (4) increased sedimentation. This separation facilitates disentangling and understanding the mechanisms leading to changes in the field, where many contaminants and many responses co-occur. The review also summarises geographic and biological factors that determine local and regional levels of resistance and resilience to degradation. It provides a conceptual aid to assess the kind of change(s) likely to occur in response to changing coastal water quality.
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Keywords: Nutrients; Particulate organic matter; Turbidity; Sedimentation; Coral reef; Calcification; Recruitment; Competition

1. Introduction

Around the world, water quality in coastal areas is changing in response to rapidly increasing fertiliser use and land clearing (Vitousek et al., 1997; Tilman et al., 2001; Smith et al., 2003). Annual nitrogen fertiliser use has increased globally more than sixfold since 1960 (Matson et al., 1997), land clearing continues at a rate of 1% of the earth's surface per year (GESAMP, 2001), and coastal urbanisation is expanding disproportionally to human population growth. Oxygen-depleted seafloor zones, caused primarily by river-borne agricultural nitrogen and phosphorus, have doubled in number and expanded in size since 1990, presenting clear evidence that many coastal waters are becoming more eutrophic (GESAMP, 2001). Coastal coral reefs, like other marine coastal ecosystems, are increasingly exposed to growing loads of nutrients, sediments and pollutants discharged from the land. Terrestrial runoff is therefore a growing concern for most of the 104 nations endowed with coral reefs (Bryant et al., 1998; Spalding et al., 2001).

Field studies have provided a large body of information showing that sedimentation, nutrient enrichment and turbidity can degrade coral reefs at local scales (Table 1). At regional scales, it has often been difficult to assess causal relationships between increasing terrestrial runoff and reef degradation, because pollution effects and other disturbances are typically confounded, historical data are often missing, and reef communities change naturally along gradients from oceanic conditions (low siltation, high water clarity, generally low nutrient levels except during upwelling periods) to terrestrially influenced conditions (fluctuating salinity, variable or high silt and nutrient levels, variable or reduced water clarity). As nutrients increase, coral reef communities change from dominance of nutrient-recycling symbiotic organisms such as corals (in oligotrophic oceanic waters), to

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Table 1
List of some of the more comprehensively documented field assessments on the effects of enhanced terrestrial runoff, and other forms of pollution, on the ecology of coral reefs

Location	Agent	Response	Source
Northern Gulf of Aqaba (Eilat), Red Sea	50% increase in nutrients from floating fish farms	50% coral mortality from benthic algal blooms, 3–4 fold reduced reef calcification, 50% increased P/R ratio	Loya (2004)
	Sewage discharge, spillage of phosphate dust	Increased algal growth trapping sediment; fourfold increased mortality in *Stylophora pistillata*, possibly from reduced light, inhibition of calcification, and increased sedimentation	Walker and Ormond (1982)
Reunion Island, Indian Ocean	Coastal urbanisation, groundwater enriched with nutrients from untreated sewage	Higher coral cover, coral diversity, fish diversity and density of sea urchins, and lower macroalgal density on reefs away from nutrient enrichment and in the 1970s before nutrient enrichment, than on nutrient-enriched reefs. High bioerosion, calcification slower than reef erosion on nutrient enriched reefs	Cuet et al. (1988), Montaggioni et al. (1993), Naim (1993) and Chazottes et al. (2002)
Hong Kong	Excess pollutants, nutrients, sediment dredging	Low coral recruitment, few zooxanthellate octocorals, disappearance of giant clams (*Tridacna* spp.), high bioerosion	Morton (1994) and Hodgson and Yau (1997)
Japan	Eutrophication and sedimentation	Declining coral cover	Shimoda et al. (1998)
	Gradients away from rivers	Change in coral community composition away from source	West and Van Woesik (2001)
Philippines	Excess sedimentation from logging	Declining coral cover, declining biodiversity due to disappearance of sediment-sensitive species over 12 months, inhibition of coral settlement	Hodgson (1990a) and Hodgson and Walton Smith (1993)
Indonesia	Excess nutrients and sedimentation	Low coral cover, reduced coral diversity, unaltered vertical extension but low skeletal density in massive corals, increased bioerosion	Edinger et al. (2000), Tomascik et al. (1997), Edinger et al. (1998) and Holmes et al. (2000)
Great Barrier Reef	Gradient in nutrients and turbidity	Increased macroalgal cover and richness (esp. red and green macroalgae), reduced octocoral richness	Fabricius et al. (in press) and Fabricius and De'ath (2004)
	Gradient away from river	Reduced coral cover, richness; increased filter feeders and macroalgae near source	van Woesik et al. (1999)
	Turbidity	Decreasing richness of zooxanthellate octocorals	Fabricius and De'ath (2001b)
	Inshore–offshore gradient, terrestrial runoff	Increasing density of internal macrobioeroders towards the coast	Hutchings et al. (in press)
	Sedimentation gradient	Decreasing cover of crustose coralline algae	Fabricius and De'ath (2001a)
Kanehoe Bay, Hawaii	Nutrients	Reduced coral cover, increased filter feeders, increased macroalgal cover	Smith et al. (1981), Hunter and Evans (1995), Stimson and Larned (2000) and Stimson et al. (2001)
Barbados	Eutrophication gradient	Photosynthetic pigments increase with increasing nutrient enrichment. Convex modal responses in gross photosynthesis, respiration, linear extension, calcification (enhanced by nutrients, depressed by turbidity) Reduced species diversity, probably due to differences in sediment rejection abilities, combined with feeding and reproductive strategies, altered community structure; increased bioerosion in coral rubble	Marubini (1996), Tomascik and Sander (1985) and Tomascik (1990) Tomascik and Sander (1987b) and Holmes (2000)

Table 1 (*continued*)

Location	Agent	Response	Source
		Reduced gamete formation, larval development and settlement, reduced recruit and juvenile density and diversity, juveniles larger, increased juvenile mortality	Tomascik and Sander (1987a), Tomascik (1991), Hunte and Wittenberg (1992) and Wittenberg and Hunte (1992)
Grand Cayman Island	Untreated fecal sewage, sixfold increased bacterial biomass	Fivefold increased internal bioerosion by the boring sponge *Cliona delitrix*	Rose and Risk (1985)
Costa Rica (2 sites)	Sedimentation	Low live coral cover, low species diversity, and large average colony diameters, high acid-insoluble residues incorporated in skeleton on exposed reef	Cortes and Risk (1985)
Brazil (2 sites)	Eutrophication	High macroalgal abundances, high density of heterotrophs	Costa Jr et al. (2000)

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increasing proportions of macroalgae (on eastern continental margins naturally exposed to river runoff), and further to heterotrophic filter feeders (in nutrient-enriched areas of upwelling or lagoons) (Birkeland, 1987). Although coastal coral reefs can flourish at relatively high levels of particulate matter and siltation (Anthony, 1999), they tend to be restricted to the upper 10 m depth (in extreme cases 4 m depth) in turbid water, while extending to >40 m in clear oceanic waters (Yentsch et al., 2002).

This review compiles the current state of knowledge on runoff-specific responses in coral reefs, in order to aid assessment of the effects of terrestrial runoff at regional scales. Inorganic nutrients and particulate material, although not 'classical' pollutants, are arguably the most important contaminants at national and regional levels (GESAMP, 2001), and this review will focus on assessing the effects of these materials on reef communities. However, contamination by pesticides, heavy metals, hydrocarbons or other human-made pollutants can also significantly affect the health of reefs at local scales (Guzman and Holst, 1993). For example, heavy metals such as copper and zinc and some hydrocarbons have been linked to reduced fertilization, fecundity and growth in adult corals (Heyward, 1988; Brown, 1987; Loya and Rinkevich, 1987; GESAMP, 2001). Some herbicides (e.g., diuron and atrazin) cause rapid (but reversible) photophysiological stress in corals after short-term exposure at environmentally relevant concentrations of <1 µg l^{-1} (Owen et al., 2003; Jones and Kerswell, 2003; Jones et al., 2003; Negri et al., in press); their effects at chronic low-level exposures are still largely unknown. Other studies, too numerous to be listed here, document the uptake of a variety of human-made pollutants by adult corals; the effects of these substances on coral reefs are beyond the scope of this review.

This paper systematically reviews and synthesises the available information on the direct effects of terrestrial runoff on (1) calcification, tissue growth, zooxanthellae populations and photosynthesis in adult hard corals, (2) the six main stages of coral reproduction and recruitment, and (3) six groups of other reef organisms that affect hard coral abundances. The latter group includes those organisms that affect coral larval settlement, bioeroding filter feeders that weaken the structural strength of reefs, macroalgae, heterotrophic filter feeders and octocorals competing for space with corals, disease pathogens, and coral predators. Responses of each of these groups are assessed separately against exposure to the four main water quality parameters, namely: (1) dissolved inorganic nutrients, (2) suspended particulate organic matter, (3) light reduction from turbidity and (4) sedimentation. This separation disregards additive or synergistic effects, but helps to understand the mechanisms for change in the field where many contaminants and responses co-occur. Furthermore, the paper identifies geographic and biological properties influencing the level of resistance and resilience of reefs to degradation.

2. Direct effects of terrestrial runoff on hard corals

2.1. Colony calcification, tissue growth and symbiosis

2.1.1. Dissolved inorganic nutrients

Considerable effort has gone into experiments studying the direct effects of elevated dissolved inorganic

Table 2
List of some representative studies of direct effects of terrestrial runoff on adult corals (see also Figs. 1 and 2)

Parameter	Response	Source
(a) *Enrichment with dissolved inorganic nutrients*		
NH_4, NH_4 plus PO_4^{3-}	Increased zooxanthellae density, increased protein synthesis by zooxanthellae	Muscatine et al. (1989)
NH_4 (15 μM)	After 8 weeks, increased zooxanthellae density, increased chlorophyll and N per zooxanthella	Snidvongs and Kinzie (1994)
NO_3 (0, 1, 2, 5, 20 μM)	Calcification decreases with increasing NO_3 to 50% of controls, effects significant at ≥ 1 μM. After 30–40 days: at ≥ 1 μM, increased N per zooxanthellae, increased zooxanthellae density. At ≥ 5 μM NO_3, increased zooxanthellae size, chlorophyll per zooxanthellae, photosynthesis, increased coral protein through greater zooxanthellae biomass. At 20 μM NO_3, 30% increased chlorophyll and zooxanthellae density, reduced respiration per unit protein	Marubini (1996)
NH_4 (10 μM and 20 μM)	After 9 weeks: unaltered buoyant weight gain at 10 μM, reduced buoyant weight gain (−60%) at 20 μM	Ferrier-Pages et al. (2000)
NO_3 (2 μM)	No change in zooxanthellae density or rate of photosynthesis. Reduced buoyant weight gain (−34%) after 3 weeks	Ferrier-Pages et al. (2001)
NH_4 (10 or 20 μM)	Inconsistent effects on linear extension and buoyant weight after 1 year: 10–20% reduction, or no effect, or slight increase. Reduced lipids	Koop et al. (2001)
NH_4	Increased zooxanthellae density, chlorophyll concentration. Decreased linear extension	Stambler et al. (1991)
NO_3 (15 μM)	After 2 weeks, reduced primary production, unaltered zooxanthellae density and chlorophyll concentrations. Temperature effects enhanced by presence of nitrate	Nordemar et al. (2003)
PO_4^{3-} (2 μM)	Increased photosynthesis, reduced calcification	Kinsey and Davies (1979)
PO_4^{3-}	No effect on zooxanthellae density or their protein production	Muscatine et al. (1989)
PO_4^{3-} (1.2 μM)	Slowed calcification, unaltered zooxanthellae density, lower C and P per zooxanthella	Snidvongs and Kinzie (1994)
PO_4^{3-} (0, 0.2, 1, 5 μM)	After 30 days: no change in photosynthesis, organic productivity, zooxanthellae density or size, tissue biomass; calcification up to 20% decreased in one species with increasing PO_4, unaltered in another	Marubini (1996)
PO_4^{3-} (2 μM)	After 9 weeks, reduced buoyant weight gain (−60%), increased gross photosynthesis (up to +150% increase)	Ferrier-Pages et al. (2000)
PO_4^{3-} (2 or 4 μM)	Inconsistent effects on growth rates after 1 year: increased calcification, linear extension and/or reduced skeletal density in some species. Increased lipids	Koop et al. (2001)
PO_4^{3-}	No effects on zooxanthellae density or linear extension	Stambler et al. (1991)
NH_4 (10 or 20 μM) plus PO_4^{3-} (2 μM)	Reduced buoyant weight gain (−60%), increased gross photosynthesis (up to +150% increase)	Ferrier-Pages et al. (2000)
NH_4 plus PO_4^{3-} (20 and 4 μM)	Increased mortality in *Pocillopora damicornis* after 1 year	Koop et al. (2001)
(b) *Enrichment with suspended particulate matter*		
Increased particulate and dissolved nutrients from fish excretions	Increased linear extension	Meyer and Schultz (1985)
Artemia food	No effect on density of zooxanthellae	Muscatine et al. (1989)
Particulate and dissolved nutrients released from fish farm	In adult corals, increased growth, oocyte and testes numbers, unaltered survival. In small coral fragments, reduced growth probably due to physical effects (burial by settled particulate matter, light reduction)	Bongiorni et al. (2003b) and Bongiorni et al. (2003a)
Suspended particulate matter (SPM), sedimentation, eutrophication gradient	Increased linear extension at moderate SPM, reduced linear extension at high SPM due to smothering, reduced light levels and reduced zooxanthellae photosynthesis. Small average colony size. No effect on partial mortality	Tomascik and Sander (1985) and Lewis (1997)

Table 2 (*continued*)

Parameter	Response	Source
1–32 mg l^{-1} SPM	Increased SPM feeding, covering up to 50% carbon and 30% nitrogen required for tissue growth at high particle concentrations. No effect on calcification	Anthony (1999)
1–16 mg l^{-1} SPM	After 4 weeks exposure: unaltered calcification. Increased tissue biomass but unaltered lipids in one species; convex modal change in tissue biomass and lipids in response to SPM in a second species	Anthony and Fabricius (2000)
Cross-shelf gradient	Increased linear extension, reduced skeletal density towards inshore environments. Highest annual calcification inshore, lowest offshore	Lough and Barnes (1992)
(c) Light reduction from turbidity		
Reduced light, excess phosphate, sedimentation	Reduced calcification, increased mortality	Walker and Ormond (1982)
Turbidity	Changed coral community structure and life forms, reduced species richness, compressed depth zonation	Loya (1976), Acevedo and Morelock (1988), Fabricius and De'ath (2001b) and Crabbe and Smith (2002)
Shading	After 5 weeks, reduced growth, net primary productivity and respiration. Altered community structure after bleaching and death in several coral species	Rogers (1979)
Turbidity	High turbidity (28–30 NTU) increased mucus production, depressed P:R ratio to below 1.0, possibly due to increased respiration	Telesnicki and Goldberg (1995)
Shading (plus 1–16 mg l^{-1} SPM)	After 4 weeks exposure: reduced calcification, reduced tissue biomass, reduced lipids in 2 species. In 1 species, feeding on 16 mg l^{-1} SPM annulled shading effects	Anthony and Fabricius (2000)
(d) Sedimentation		
Low sedimentation	Increased respiration, reduced net photosynthesis; Species-specific rejection efficiency	Abdel-Salam et al. (1988)
Sedimentation	Coral cover and coral species diversity increase with distance from the sediment source Partial or total burial of colonies, bleaching and surface colonisation by filamentous blue-green algae	Acevedo and Morelock (1988)
Sedimentation	Low or brief sedimentation: reduced photosynthetic yield; high or prolonged sedimentation: loss of zooxanthellae, partial mortality, but species-specific tolerances	Philipp and Fabricius (2003)
Sedimentation (30 mg cm^{-2})	Species-specific rejection efficiency	Hodgson (1990b)
Sedimentation (50–1000 mg cm^{-2} of four particle sizes, and 200 mg cm^{-2})	Species-specific rejection efficiency: rejection rates positively correlated with calice size, and faster for medium-fine (63–250 μm) than for coarse (500–1000 μm) sediment. Bleaching and partial mortality within 48 h in some species, but clearance times generally <2 days	Stafford-Smith and Ormond (1992) and Stafford-Smith (1993)
Sedimentation (up to 14 mg cm^{-2} d^{-1})	Passive sediment removal more successful for fine grain sizes, tall polyps, and convex colonies, active removal independent of colony morphology	Lasker (1980)
Heavy sedimentation (>10 mg cm^{-2} d^{-1} and >10 mg l^{-1})	Reduction in coral species richness, live coral cover, coral growth rates, calcification, net productivity of corals, and rates of reef accretion; increased proportion of branching forms. Species-specific capabilities for particle rejection and for surviving lower light levels	Rogers (1990)
High sedimentation	Reduced linear extension: growth inversely related to sediment resuspension	Cortes and Risk (1985) and Dodge et al. (1974)
Sedimentation	Loss of zooxanthellae, reduced calcification	Bak (1978)
Sedimentation	Reduced mean colony sizes (through stunted growth and/or reduced life expectancy)	Van Woesik and Done (1997)

(*continued on next page*)

Table 2 (*continued*)

Parameter	Response	Source
Sedimentation	Increased mean colony sizes (through reduced recruitment)	Wesseling et al. (2001), Cortes and Risk (1985) and Tomascik and Sander (1985)
Terrestrial runoff and sedimentation	Partial mortality: High proportion of injured or algae infested corals, and/or high soft coral cover, and/or high proportion of rocky substrate suitable for, but unoccupied by, living corals	van Katwijk et al. (1993)
Sedimentation	Partial mortality: colony lesion densities increase with sedimentation, wave exposure, colony size, and intensity of human reef exploitation. Colony size, live coral cover and *Acropora* cover decrease with intensity of human reef exploitation	Wesseling et al. (2001)
Sedimentation	Reduced coral cover	Loya (1976), Cortes and Risk (1985), Acevedo and Morelock (1988), Brown et al. (1990), Chansang et al. (1981) and Morelock et al. (1983)
Sedimentation	Changed coral community structure and life forms, reduced species richness	Loya (1976), Morelock et al. (1983), Pastorok and Bilyard (1985), Acevedo and Morelock (1988), Rogers (1990), Brown et al. (1990), Edinger et al. (1998) and West and Van Woesik, 2001

nitrogen (DIN, as nitrate or ammonium) and phosphate (DIP) on coral calcification, tissue growth and zooxanthellae. Table 2a, and detailed reviews by Dubinsky and Stambler (1996) and Szmant (2002) show that most experiments were conducted at environmentally unrealistically high levels, and that significant inconsistencies exist across studies that are as yet unresolved. Many studies found that high levels of DIN and DIP both reduce calcification up to 50%, while other studies found no change in growth rates, or reported slightly increased rates of calcification and linear extension but reduced skeletal densities (Table 2a). Effects of DIN on tissue growth and composition vary across studies, with some reporting reduced lipids (Koop et al., 2001), and others finding enhanced zooxanthellae protein but unaltered host protein (Marubini, 1996). Increased DIP appears to have little effect on tissue growth. Most studies found that increased DIN increases zooxanthellae density, increases the contents of nitrogen and chlorophyll *a* per zooxanthellae, and increases photosynthetic rates. In contrast, high levels of DIP did not affect zooxanthella densities. In experimental studies, colony survival was generally unaffected by DIN and DIP, while coral mortality increased, for unknown reasons, in one species after a 1-years field exposure to high daily pulses of both DIN and DIP (Koop et al., 2001); however, such high and frequent nutrient pulses are unlikely to be encountered in nature for sustained periods except near sewage outfall sites.

Zooxanthellae are typically nitrogen-limited at high irradiance when ample photosynthetically fixed carbon is available (C/N ratios are up to 30), whereas they may not be nitrogen-limited at lower irradiance (C/N ratios about 10; Falkowski et al., 1984; Dubinsky and Jokiel, 1994). Zooxanthellae densities increase in response to enhanced DIN availability because this nutrient is preferentially used for zooxanthellae growth rather than the growth of host tissue (in contrast to nutrients derived from zooplankton feeding which increase both tissue and zooxanthellae growth; Dubinsky and Jokiel, 1994). Reduced calcification at elevated DIN has been explained as follows: zooxanthellae populations increase after release of N limitation, these cells have preferential access to the available CO_2 which they use for photosynthesis, hence less CO_2 is available for calcification and CO_2 becomes a limiting factor (Marubini and Atkinson, 1999; Marubini and Thake, 1999). Evidence for this hypothesis is provided by data that show that DIN causes no growth reduction in the presence of high levels of bicarbonate (Marubini and Thake, 1999). Reduced calcification at higher DIP availability seems to be caused by another, as yet not fully understood mechanism (Marubini and Davies, 1996). Hypotheses focus on the reduced chemical $CaCO_3$ crystal formation in the presence of phosphate (Simkiss, 1964), or experimental artifacts based on lowered pH from using unbuffered PO_4. Possibly due to the presence of two different mechanisms, simultaneous increases of DIN and DIP generally do not result in interactive effects on calcification rates (Table 2a, Marubini and Davies, 1996).

In the field, both DIN and DIP are quickly taken up by phytoplankton and bacteria and benthic food webs. Hence elevated nutrients are available in their dissolved inorganic form only for short periods of time

over relatively limited areas. Severe direct effects of dissolved inorganic nutrients on corals appear restricted to heavily polluted, poorly-flushed locations such as semi-enclosed lagoons and bays, where they are linked to reduced reef calcification, coral cover and biodiversity (Table 1). Away from the coast, regions that regularly experience the upwelling of cool waters (i.e., rich in dissolved inorganic nutrients but no sedimentation or light reduction) have also been used to assess the effects of DIN and DIP on calcification. Coral calcification can be up to 50% reduced in upwelling regions, which has been attributed to elevated nutrients as well as to cool temperatures (Kinsey and Davies, 1979; Wellington and Glynn, 1983). Reef formation is noticeably restricted in places where upwelling is a common occurrence, such as along western tropical and subtropical land masses (Birkeland, 1987; Achituv and Dubinksy, 1990). This has lead to the conclusion that reduced calcification from exposure to periodic or chronically elevated dissolved inorganic nutrients can substantially alter coral populations and communities (Kinsey and Davies, 1979; Hallock, 1988; Wilson et al., 2003); however cool temperatures may to a large part explain such low calcification (e.g., calcification declines by 50% with every 3° temperature in massive *Porites*; Lough and Barnes, 2000).

In summary, the available information suggests that short-term exposure to high levels of unprocessed DIN and DIP does not kill or greatly harm individual coral colonies, however chronically increased levels of dissolved inorganic nutrients may alter reef metabolism and reef calcification sufficiently to cause noticeable changes in coral communities. Existing data indicate (Fig. 1) that: (a) there is strong evidence that zooxanthellae numbers, chlorophyll per unit surface area, and photosynthetic rates increase with increasing DIN (but not DIP), affecting the transfer of energy, CO_2 and nutrients between zooxanthellae and host; (b) there is little evidence that dissolved inorganic nutrients alter tissue thickness, lipids or coral protein per unit surface area; and (c) while some studies found increased or unaltered skeletal growth (measured as linear skeletal extension, skeletal density and/or calcification), many controlled experimental studies found a reduction in growth at elevated levels of DIN and/or DIP. Combining the few existing physiological data with environmental data leads to the suggestion that coral growth (calcification) declines gradually with increasing dissolved inorganic nutrient availability (Fig. 2a), but levels of dissolved inorganic nutrients will often not greatly increase along pollution gradients. In reality, response curves are likely to be more complex, for the following reasons: (1) there are complex interactions between the growth of tissue, zooxanthellae and calcification, (2) nutrient limitation occurs predominantly at high irradiance where carbon is available in overabundance, hence nutrient addition may be only of conse-

	DIN	DIP	POM	Light reduction	Sedimentation
Calcification	↓	↓	↑	⬇	⬇
Tissue thickness	–	–	↑	↓	⬇
Zooxanthellae density	⬆	–	↑	↑	↓
Photosynthesis	↑	↑	↑	⬇	⬇
Adult colony survival	–	–	↑	↓	⬇

Fig. 1. Synthesis of documented direct effects (Tables 1 and 2) of the four main parameters of terrestrial runoff on the growth and survival in adult corals, based on published studies or known biological properties and processes. The arrows indicate the relative strength and direction of the response (arrows pointing up or down = increasing or decreasing, thick arrow = strong, medium = moderate, thin = weak effect); a dash indicates that a response is unlikely; empty cells indicate that insufficient data are available.

quence in highlight environments; (3) other limitations such as that of CO_2 co-occur; and (4) nutrient uptake rates are partly mass transfer limited, hence not only a function of concentrations but also of water currents (Hearn et al., 2001). All these factors are insufficiently considered in most experimental studies, and may contribute to explaining the inconsistencies between results.

2.1.2. Particulate organic matter

Particulate organic matter (POM) greatly contributes to nutrient availability in many coastal regions, because a majority of nutrients are discharged to the marine environment in particulate form, and much of the dissolved inorganic nutrients can be taken up and converted into particulate form within hours to days (Furnas, 2003). Suspended particulate matter in areas of high sediment resuspension can have a nutrient content of >5%, either contained in the bacteria, phytoplankton, zooplankton and detritus, or absorbed to the surfaces of fine inorganic particles; the nutrient content is even higher offshore where less inert material is suspended from the seafloor. POM can be used by a range of benthic organisms including corals (Lewis, 1976; Anthony, 1999). However the ability to utilize POM varies widely between coral species, and a number of species are naturally restricted to clear water habitats (Veron, 2000). Depending on species, feeding saturation may occur at low to moderately high levels of POM: some species become mixotrophic at high turbidity, while others remain mostly phototrophic and gain a small proportion of their energy demand from particle feeding (Anthony and Fabricius, 2000). Rates of POM intake furthermore depend on water current speeds, with intake rates being generally higher at moderate to fast flow than in sheltered locations.

Moderate loads of POM have been linked to increases in tissue thickness in some species (Tables 1 and 2b). Linear skeletal extension may double, while
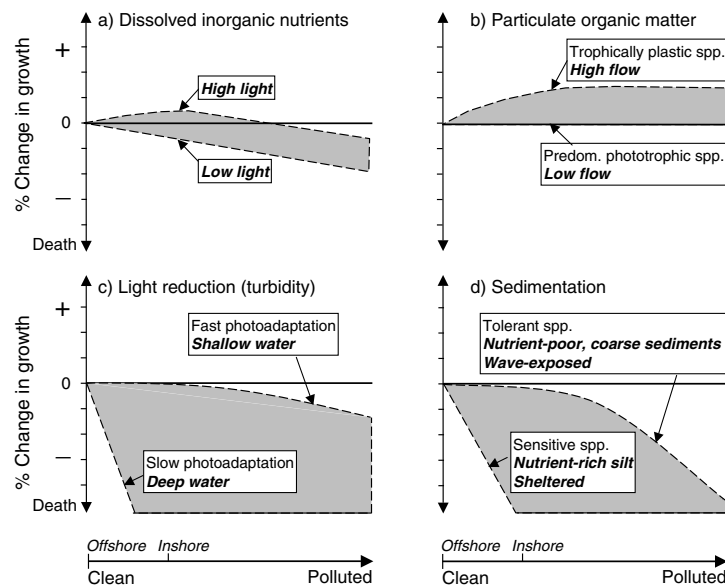
Fig. 2. Schematic representation of direct effects of terrestrial runoff on coral growth (measured as change in calcification and/or linear extension, i.e., addition of skeletal biomass) and survival along environmental gradients. Plotted are changes in coral growth in response to (a) uptake of dissolved inorganic nutrients, (b) feeding on suspended particulate organic matter, (c) light reduction from turbidity, hence reduction in gross photosynthesis, and (d) disturbance by sedimentation. The x-axis represents a hypothetical water quality gradient from offshore water quality to polluted conditions, also indicting the relative positions of offshore and inshore conditions unaltered by human activities. The y-axis scale represents relative units of changes in growth, with severe long-term reduction in growth effectively representing 'colony death'. Grey shading is used to approximate typical response envelopes due to species-specific differences (normal font) and local environmental conditions (bold italic font).

skeletal density may be up to 20% reduced in response to POM feeding, with varying effects on overall calcification rates. In fragile branching species, increased linear skeletal extension may be partly offset by greater breakage due to reduced skeletal density. Zooxanthellae densities appear to increase to a lesser extent in response to organic enrichment than in response to dissolved inorganic nutrients, possibly because POM promotes the growth both of host and zooxanthellae, in contrast to dissolved inorganic nutrients that are preferentially used for zooxanthellae rather than host tissue growth (Dubinsky and Jokiel, 1994).

In the field, coral calcification and growth appears to change in a modal fashion along eutrophication gradients: in areas of intermediate turbidity where particulate and dissolved nutrient loads were high, corals had higher concentration of photosynthetic pigments, calcification, gross photosynthesis and respiration compared to a cleaner site (Tomascik and Sander, 1985; Marubini, 1996). At the most eutrophic site, pigment concentration was even higher than at the intermediate site, however light reduction from turbidity annulated the growth advantages from POM feeding, consequently calcification, gross photosynthesis and respiration was lower at the most eutrophic site than at the intermediate site (Marubini, 1996). Photosynthetic pigment concentrations in corals have therefore been suggested as the most linear and hence most useful early-warning indicator for nutrification (Marubini, 1996).

In summary, the limited existing data suggest that moderate concentrations of POM can provide substantial energy and growth benefits for some, but not all coral species, especially at high water flow and high irradiance (Fig. 2b). Overall, of the four parameters of terrestrial runoff considered, POM is the one parameter that can enhance growth in some species, at moderate levels compensating for growth reduction from the other three parameters. At higher levels of POM, feeding saturation prevents additional energy gains, while losses from the associated light reduction, dissolved inorganic nutrients and sedimentation outweigh the benefits of POM feeding.

2.1.3. Light reduction

The availability of light decreases directly as a function of particle concentration and water depth, but also depends on the nature of the suspended particles (Te, 1997). Fine clays and organic particles are easily suspended from the sea floor, reducing light for prolonged periods while undergoing cycles of deposition and resuspension. Increased nutrient runoff into semi-enclosed seas accelerates phytoplankton production to the point that it also increases turbidity and reduces light penetration (Abal and Dennison, 1996). In areas of nutrient-enrichment, light for benthic organisms can be additionally severely reduced by dense stands of large frondose macroalgae (see below), and to a minor extent by particles settling on colony surfaces.

Shading temporarily reduces photosynthesis by zooxanthellae, leading to lower carbon gain, slower calcification and thinner tissues (Table 2c; Rogers, 1979; Telesnicki and Goldberg, 1995; Anthony and Hoegh-Guldberg, 2003). Within 5–10 days, many corals can adjust to somewhat lower light by increasing the size and amount of chloroplasts in zooxanthellae (not altering zooxanthellae densities per unit area), a process known as photoacclimation. However, light exposure on inshore reefs fluctuates through a fivefold range on a time scale of days to weeks as a result of tides, resuspension and clouds (Anthony and Hoegh-Guldberg, 2003). Under such variable conditions, photoacclimation does not significantly enhance gross productivity, because delays in upward- and downward-regulation of photosynthesis in response to altered light are symmetrical and compensate for each other over longer periods (Anthony and Hoegh-Guldberg, 2003). Therefore, the maximum depth for photocompensation (the depth range within which corals can survive or maintain active reef growth) diminishes as a direct function of turbidity from >40 m to <4 m depth (Birkeland, 1987; Yentsch et al., 2002).

In the field, the effects of light reduction on species richness are strongly depth-dependent, as light requirements greatly vary between species. Few species can tolerate the low light levels at deep depths or at high levels of turbidity. On the other hand, in high-irradiance conditions many slower-growing species are out-competed by fast-growing phototrophic species, hence species richness is often highest at intermediate light levels (Cornell and Karlson, 2000).

Historic data on water clarity in coastal marine systems are sparse. Indeed, only few records of changes in water clarity exist, and these are from places where research stations are located, or in areas of extreme pollution. Reduced visibility has been linked to phytoplankton blooms around a sewage outfall site in Kaneohe Bay, Hawaii (Hunter and Evans, 1995), and around floating fish farms in the Northern Red Sea (Loya, 2004). Some researchers argue that resuspension, governed by water depth and wave height, is the best predictor of turbidity over a sediment-covered seafloor, and nearshore water clarity therefore would not substantially increase due to increased sediment discharges from the land (Larcombe and Woolfe, 1999). In contrast, other researchers point out that biological processes such as water column productivity can also reduce water clarity, and that nepheloid layers can form and reduce water clarity offshore at regional scales, such as described off a mud-enriched coastline along the central Great Barrier Reef (Wolanski et al., 2003). Given the strong link between turbidity, light reduction and lower depth limits for coral reefs, more research is needed to understand conditions leading to long-term changes in water clarity in tropical coastal systems.

In summary, the effects of shading from turbidity are minimal in shallow water and progressively increase with increasing depth, but effects greatly vary between species (Fig. 2c). The main symptoms in the field are more compressed depth distribution zones, low biodiversity at deeper depths, and an overall more shallow lower depth limit for reef growth.

2.1.4. Sedimentation

Enhanced levels of sedimentation from coastal erosion have severely degraded many coastal reefs around the world (Table 2d, Rogers, 1990). Most sediments are imported into coastal marine systems via rivers, with >95% of the larger sediment grain fractions being deposited within a few kilometres of the river mouth, while fine grains may be transported over longer distances. Near the source, benthic communities are easily smothered by sedimentation (e.g., Golbuu et al., 2003), as high sedimentation rates (accumulating to >100 mg dry weight cm^{-2} deposits) can kill exposed coral tissue within a period of a few days (Riegl and Branch, 1995). Lower (<100 mg cm^{-2}) sedimentation levels reduce photosynthetic yields in corals (Philipp and Fabricius, 2003), and the removal of settled particles increases metabolic costs (Telesnicki and Goldberg, 1995). In coral colonies, sedimentation stress increases linearly with the duration and amount of sedimentation: for example, a certain amount of sediment deposited on the coral for one time unit exerts the same measurable photophysiological stress as twice the amount deposited for half the time (Philipp and Fabricius, 2003).

Coral damage appears to not only depend on the amount and duration of sedimentation, but also strongly depends on the sediment type. For example, tissue damage under a layer of sediment increases with increasing organic content and bacterial activity, and with decreasing grain sizes (Hodgson, 1990b; Weber et al., 2004). Low-level sedimentation ($\sim$12 mg cm^{-2}) when combined with transparent exopolymer particles (polysaccharides possibly exuded by bacteria and diatoms, called 'marine snow') kills newly settled coral recruits, whereas the same amount of sediment without the addition of marine snow does not reduce their short-term survival (Fabricius et al., 2003). Marine snow aggregates are found in high concentrations in coastal and inshore areas of the central Great Barrier Reef. These and similar data demonstrate the critical (but as yet poorly understood) interactions between sediment quality and quantity on coral damage (Fabricius and Wolanski, 2000). They also show that short exposure to sediments (few days) can cause long-term effects in populations, by removing cohorts of young corals and thus retarding reef recovery after a disturbance.

In the field, sedimentation is greatest on sheltered, wave-protected lagoons, bays or deeper reef slopes,

whereas sediment deposition is minimal in wave-exposed shallow-water areas. Sedimentation has been linked to profound changes in coral population structures, such as altered size frequencies, declining mean colony sizes, altered growth forms, and reduced growth and survival (Table 2d; Rogers, 1990). However, sedimentation tolerances greatly vary among coral species. Large colonies or those with branching growth forms or thick tissues are more tolerant of sedimentation, whereas small colonies or species with thin tissues and flat surfaces are often highly sensitive (Rogers, 1990). Some species with thick tissues can remove particles from their surfaces by tissue extension, mucus production or ciliary movement (such as found in *Fungia*) and are therefore quite sediment tolerant (Stafford-Smith and Ormond, 1992). As tolerance of sedimentation varies widely among species, a reduction in biodiversity is a common outcome of sedimentation stress, with fewer sensitive species and persistence of more tolerant species (such as massive *Porites*) in the coral communities (Table 2d).

In summary, sedimentation effects greatly vary between coral species, but also between sediment types and between environmental conditions (Fig. 2d). Only few species can persist in wave-protected regions where silt-sized, nutrient-enriched sediments are deposited. In contrast, more wave-exposed areas, or areas with nutrient-poor or coarse-grained sediments will support a wider range of species even at moderate levels of sedimentation.

2.2. Reproduction and recruitment

In most cases where terrestrial runoff causes reef degradation, disturbances other than eutrophication were the proximate causes of coral mortality, and runoff effects only became obvious when hard corals failed to reestablish after such disturbances (see Tables 1 and 3 for references). This indicates that coral reproduction and/or recruitment are affected by terrestrial runoff. Indeed, sedimentation and eutrophication have commonly been related to decreased juvenile densities on reefs (for references see Table 3). This section presents a brief literature overview to resolve how the four main parameters of terrestrial runoff affect the six main pre- and post-settlement processes, namely (1) gamete production, (2) egg fertilisation, (3) embryo development and larval survival, (4) larval settlement and metamorphosis, (5) recruit survival, and (6) juvenile growth and survival.

The limited available experimental data suggest that the three main pre-settlement stages of coral reproduction (gamete production, egg fertilization, and larval development and survival), as well as larval settlement rates, are sensitive to dissolved inorganic nutrients (Table 3). In acroporid corals, fecundity, egg sizes, egg fertilisation rates and embryo development are all reduced, and the occurrence of irregular embryos increased, at slightly elevated levels of dissolved inorganic nutrients (from $1 \mu M$ NH_4 and $0.1 \mu M$ PO_4, i.e., at <10% of concentrations that detrimentally affect adult corals; Ward and Harrison, 2000; Harrison and Ward, 2001). Furthermore, spat densities were reduced at elevated levels of nitrogen (Ward and Harrison, 1997). Other observed effects include failed planulation in the brooding coral *Pocillopora damicornis*, and reduced egg sizes in *Montipora* that releases zooxanthellate eggs, after four months of exposure to elevated ammonium levels (Cox and Ward, 2002). The underlying mechanisms for such surprisingly high levels of sensitivity are presently not understood.

Laboratory experiments show that POM can inhibit egg fertilization rates, larval development, larval survival, settlement and metamorphosis (Gilmour, 1999). It is unknown to what extent juveniles (like adult colonies, see above) benefit from feeding on POM. Light affects both reproduction and recruitment, as coral fecundity decreases in low-light conditions, and coral larvae use light quantity and quality to choose their settlement site. At low light levels, corals preferentially settle on upper surfaces, where the risk of sedimentation damage is high, rather than on vertical of downward facing surfaces (Birkeland et al., 1981). At highly turbid conditions, coral recruits may undergo reverse metamorphosis, indicating conditions are unsuitable for continued development and growth (Te, 1992). Light reduction from turbidity is therefore likely to result in compressed depth zonations. Finally, sedimentation also strongly inhibits successful coral reproduction, especially coral settlement and recruit and juvenile survival. Sedimentation mortality thresholds for coral recruits are an order of magnitude lower than those for larger colonies (loads of tens rather than hundreds of $mg \, cm^{-2}$; Fabricius et al., 2003). Few coral larvae settle on sediment-covered surfaces, and survival on such surfaces is minimal. At moderate to high rates of sedimentation, successful larval settlement is restricted to downward-facing surfaces where growth and survival are negatively affected by low light.

In summary, existing data suggest that coral reproduction and recruitment are far more sensitive to changes in water quality than adult corals, and are highly dependent on clean water and low sedimentation. Each of the four water quality parameters affect different stages of coral recruitment, and each of the effects is a negative one (Fig. 3): dissolved inorganic nutrients inhibits its fecundity, fertilization, embryo and larval development, and possibly larval settlement; suspended particulate matter reduces pre-settlement survival; shading alters larval settlement, and sedimentation inhibits settlement and increases post-settlement mortality. Cer-

Table 3
Summary of reported effects of water quality on coral reproduction and early life stages in corals (see also Fig. 3)

Agent	Response	Source
$\geqslant 1\,\mu M$ NH$_4$ and/or $\geqslant 1\,\mu M$ PO$_4$	Reduced egg fertilisation rates in *Acropora*, increased rate of abnormally formed embryos	Harrison and Ward (2001)
NH$_4$ (11–36 $\mu M\,m^{-3}$) and/or PO$_4$ (2–5 $\mu M\,m^{-3}$)	Reduced spat densities on tiles in NH$_4$ enriched, but not in PO$_4$ enriched treatments	Ward and Harrison (1997)
NH$_4$ (11–36 $\mu M\,m^{-3}$) and/or PO$_4$ (2–5 $\mu M\,m^{-3}$)	Smaller and fewer eggs per polyp, reduced egg fertilization, increased proportion of irregular embryos	Ward and Harrison (2000)
20 μM NH$_4$ for 4 months	Failed planulation in *Pocillopora damicornis*. Reduced egg size, but no difference in fecundity and fertilisation in *Montipora* with zooxanthellate eggs	Cox and Ward (2002)
Increased nutrients from floating fish farms	Reduced coral planulation	Loya et al. (2004)
Eutrophication gradient	Reduced gametogenesis, larval development, larval settlement, recruit and juvenile density and diversity, increased juvenile mortality	Tomascik and Sander (1987a), Tomascik (1991), Hunte and Wittenberg (1992) and Wittenberg and Hunte (1992)
Suspended sediment (50 and 100 mg l^{-1})	Reduced fertilisation, uninhibited post-fertilisation embryonic development, reduced larval survival and larval settlement	Gilmour (1999)
Turbidity by SPM (0, 10, 100, 1000 mg l^{-1})	Unaltered settlement rates, but increased rates of reversed metamorphosis after settlement ("polyp bail-out") at 100 and 1000 mg l^{-1}	Te (1992)
Turbidity, sedimentation	Reduced fecundity	Kojis and Quinn (1984)
Shading	Reduced fecundity	Carlon (2002)
Shading	Species-specific effects on settlement and metamorphosis	Mundy and Babcock (1998) and Babcock and Mundy (1996)
Sedimentation	Reduced larval settlement on upper surfaces, especially when sediments are trapped by thick turf algae	Hodgson (1990a), Babcock and Davies (1991), Te (1992), Babcock and Mundy (1996), Babcock and Smith (2002) and Birrell et al. (in press)
Sedimentation (1–11.7 mg cm^{-2} d^{-1})	Reduced recruit survival	Babcock and Smith (2002)
Muddy marine sediments (14 mg cm^{-2}), with and without enrichment with marine snow	After 48 h, reduced recruit survival in sediments enriched with marine snow	Fabricius et al. (2003)
Sedimentation	Increased juvenile mortality (abrasion, smothering, competition with algae)	Birkeland (1977), Sato (1985), Sammarco (1991) and Wittenberg and Hunte (1992)
Eutrophication, sedimentation	Increased mean colony sizes (interpreted as sign of low recruitment rates)	Cortes and Risk (1985) and Tomascik and Sander (1985)
Terrestrial runoff, heavy sedimentation (> 10 mg cm^{-2} d^{-1} and > 10 mg l^{-1})	Reduced coral recruitment	Pastorok and Bilyard (1985), Rogers (1990) and Richmond (1997)
Water from creek runoff (28 ppt salinity)	Reduced fertilisation (−86%), reduced larval development (up to −50%)	Richmond and Walton Smith (1993)
Gradient in exposure to terrestrial runoff	Reduced recruit and juvenile density	Smith et al. (in press)

tainly more experimental studies are needed to verify and complement the data synthesis of Fig. 3.

3. Effects of terrestrial runoff on benthic organisms that affect corals and coral communities

Abundances of a large number of invertebrates and algae in coral reef communities change along environmental gradients influenced by terrestrial runoff. This section focuses on the responses of those organism groups that profoundly affect health and abundance of corals; hence changes in their abundances in response to terrestrial runoff induce secondary or indirect effects on corals. The six main groups of organisms are those that (1) facilitate coral settlement (especially crustose coralline algae), (2) alter the structural strength of the reef substratum (internal bioeroders), (3) compete for space with corals (macroalgae), (4) do not contribute to reef calcification (heterotrophic filter feeders and octocorals), (5) infect corals with diseases, and (6) predate on corals (the crown-of thorns starfish *Acanthaster planci*).

3.1. Organisms that determine coral settlement

Substratum availability, and especially the presence of certain species of crustose coralline algae (CCA) and the absence of sediment layers are essential for coral

	Dissolved inorg. nutr.	POM	Light reduction	Sedimentation
Fecundity	↓		↓	↓
Fertilization	▼	↓	—	—
Embryo develop./ larval surv.	▼	↓	—	—
Settlement / metamorphosis	↓	↓	▼	▼
Recruit survival			↓	▼
Juvenile growth / survival			↓	↓

Fig. 3. Synthesis of documented direct effects (Table 3) of the four main parameters of terrestrial runoff on the six main processes associated with coral reproduction and recruitment (Table 3). Symbols as in Fig. 1.

settlement (Harrington et al., in press a). Few experimental data exist to assess the effects of terrestrial runoff on substratum availability and suitability for coral settlement. Some experiments and field data suggest that sedimentation may be a major factor influencing CCA abundances. CCA cover on reefs is negatively related to sedimentation (Kendrick, 1991), with cover decreasing from >30% in some low sedimentation habitats to 1% at high sedimentation on the Great Barrier Reef (Fabricius and De'ath, 2001b). Laboratory experiments suggest that some coral reef associated CCA survive burial under coarse inorganic sediments for days to weeks, but their survival is compromised if sediments are fine-grained (<0.63 m) or organically enriched (Harrington et al., in press b). The responses of CCA to sediments is complicated by their interaction with turf algae that efficiently trap sediments (Purcell, 2000), and by this means not only smother and replace CCA (Steneck, 1997) but also make the surrounding substratum less suitable for coral settlement (Birrell et al., in press). Light also affects CCA abundances, however responses are species-specific, with high-irradiance species being replaced by low-light species as light availability decreases. Laboratory studies show that elevated levels of orthophosphate can reduce calcification in tropical CCA (Brown et al., 1977; Björk et al., 1995), but field experiments found no responses by either CCA or turf algae to enrichment with dissolved inorganic nutrients (Koop et al., 2001).

3.2. Organisms that determine structural strength of the substratum

By far the largest proportion of filter feeders lives below the reef surface. Some types, especially sponges, bryozoans, ascidians, molluscs and some polychaetes, colonise existing cracks and crevices of the substratum. Others actively bore into or chemically erode the inorganic reef substratum and the calcium carbonate skeletons of live corals. These are internal macrobioeroders

that can reach densities of thousands of individuals m^{-2} reef area, weakening the structure of coral reefs and affecting their susceptibility to storm damage (Rose and Risk, 1985). The main groups are sponges such as the boring sponge Cliona spp., and bivalves such as the date mussel Lithophaga spp., the latter known to redissolve up to 40% of skeletons of living coral by direct boring and by changing alkalinity around the bore holes (Loya, 1991). The boring activity of these filter feeders is complemented by internal microboring green and blue-green microalgae. Several studies have documented increased abundances of internal macro- and microbioeroders in response to enhanced nutrient availability (Rose and Risk, 1985; Hallock and Schlager, 1986; Hallock, 1988; Cuet et al., 1988; Holmes, 2000; Chazottes et al., 2002). For example, abundances of the boring sponge Cliona delitrix increased fivefold in an area exposed to untreated fecal sewage (Rose and Risk, 1985). Similarly, erosion by boring microalgae and other microbes is enhanced 10-fold by fertiliser application (Carriero-Silva et al., in press). While certain borers are detrimentally affected by sedimentation (Hutchings et al., in press), abundances of most internal macrobioeroders are highest in the more productive inshore environments than offshore (Sammarco and Risk, 1990; Edinger and Risk, 1996). Of greatest concern is that increased bioerosion in areas of nutrient enrichment, combined with reduced coral growth, skeletal densities and recruitment rates, can lead to conditions where reef erosion exceeds calcium carbonate accretion (Montaggioni et al., 1993; Edinger et al., 2000; Pari et al., 2002; Carriero-Silva et al., in press).

3.3. Organisms in competitive interaction with corals: macroalgae

Hard corals are competitive in low-nutrient environments because of efficient internal recycling of nutrients and energy between host and zooxanthellae, and because they occupy almost all available trophic levels simultaneously: they are efficient in photosynthesis, they take up dissolved inorganic and organic nutrients, feed on primary producers such as large phytoplankton, capture and prey upon herbivorous and predatory zooplankton, and also feed on decompositional material such as detritus (Lewis, 1976; Rosenfeld et al., 1999). Additionally, corals show considerable trophic plasticity in response to light and food availability. Such remarkable ability to gain energy at most trophic levels simultaneously allows hard corals to grow in nutrient-poor as well as quite productive environments. This trophic flexibility contrasts with the more specialised feeding strategies of other major benthic groups on coral reefs, the most important ones being macroalgae and heterotrophic filter feeders.

Macroalgal communities are an integral and often diverse component of inshore reef systems. However at certain environmental conditions, some macroalgal species can form dense mats that overgrow or damage large areas of coral by trapping sediment, restricting gas exchange, and creating anoxic conditions when mats age and collapse. For example, mats of the ephemeral green filamentous *Enteromorpha* sp. can smother adult corals by depleting oxygen at night. A 50% local increase in nutrients in the northern-most part of the Red Sea (Eilat, Gulf of Aqaba) has led to such blooms, reducing coral cover by 50% and reef ecosystem calcification by a factor of 3–4 since 1990 (Loya, 2004). Other, fleshy perennial species such as *Sargassum* spp. seasonally grow to form up to 2 m tall forests. Such forests shade corals underneath and their fronds can cause some tissue abrasion in coral. Rather than directly smothering adult corals, they tend to establish after corals are killed by other disturbance, however once established, they can become a major factor retarding coral recovery (Schaffelke et al., in press). Both types of macroalgae (low ephemeral mats and fleshy perennial stands) inhibit coral recruitment by space occupancy, allelopathy, silt trapping or shading (Sammarco, 1980; Connell et al., 1997; Hughes and Tanner, 2000; Szmant, 2002; Schaffelke et al., in press).

Macroalgae cover their carbon demand by photosynthesis, and their nutrient demand by uptake of dissolved inorganic nutrients, plus in some species by decomposing particulate organic matter deposited on their fronds (Schaffelke, 1999b). In the absence of grazing control, the growth and productivity of certain groups of macroalgae is nutrient limited and increases with slight increases in dissolved inorganic nutrients and POM (Schaffelke, 1999a, Schaffelke et al., in press). High standing biomass of fleshy, silt-trapping macroalgae has been reported around many point nutrient sources (Table 1), such as Kaneohe Bay (Smith et al., 1981), Brazil (Costa Jr et al., 2000) or the Bahamas (Lapointe et al., 2004). On inshore reefs of the central and northern Great Barrier Reef, total macroalgal cover (especially red and green algae) increases by up to 50% from reefs in water with lowest nutrient and particle loads to those in least clean water (van Woesik et al., 1999; Fabricius and De'ath, 2004; Fabricius et al., in press). Time series data of sites where macroalgal cover expanded with increasing nutrients from coastal runoff on Reunion Island (Cuet et al., 1988), and where macroalgal cover decreased after sewage diversion in Kaneohe Bay (Smith et al., 1981), add evidence for a causal link between increasing macroalgal abundances with increasing nutrient availability. The prevalence of macroalgae on eastern sides of large land masses from which most rivers originate (Birkeland, 1987), the increase of both macroalgal biomass and nutrients with latitude (Johannes et al., 1983), and the high abundances of macroalgae found in areas of nutrient upwelling (Birkeland, 1988), add further strong evidence to the conclusion that nutrients can limit macroalgal biomass, and that they can have a negative effects on reef development. However, interactions between macroalgae and nutrients are complicated by the fact that macroalgal biomass is co-limited by grazing (McCook, 1997; Hughes et al., 1999), and in turbid or deeper water by light availability. The link between nutrients and macroalgal productivity is further complicated by the fact that nutrient uptake is mass transfer limited and increases with water flow (such as in wave zones) as well as with nutrient concentrations.

3.4. Surface occupying organisms that do not calcify: heterotrophic filter feeders and octocorals

Filter feeders (predominantly sponges, bivalves, ascidians, bryozoans and barnacles) that occupy the reef surface also increase in densities in response to nutrient enrichment (Birkeland, 1977; Smith et al., 1981; Costa Jr et al., 2000). Most actively pumping benthic filter feeders are asymbiotic, feeding on a narrow size range of plankton particles, and are often unable to obtain a positive carbon balance in oligotrophic waters (Birkeland, 1988). Again, heterotrophic filter feeders contribute to the biodiversity of coral reefs, and indeed only few examples exist of filter feeders (in particular some sponges; Aerts and Van Soest, 1997; Aronson et al., 2002) directly competing with corals for space, replacing corals and preventing further reef growth. Such takeover seems restricted to areas of low light, high phytoplankton concentrations and organic enrichment (Smith et al., 1981; Brock and Smith, 1983). Other filter feeders are sensitive to sedimentation and therefore disadvantaged by terrestrial runoff. Unlike macroalgae that directly compete with corals for well-lit habitats, surface-inhabiting heterotrophic filter feeders are generally low in profile, and tend to monopolise space only in poorly lit, highly productive environments that are per se marginal or unsuitable for corals. It therefore seems that, with few locally restricted exceptions involving one or few fast-growing species, the decline of corals and the spread of filter feeders are largely independent symptoms of high nutrient loads in the water, driven by organic enrichment rather than by competition between the two disparate groups.

Octocorals are also suspension feeders, however most of the more abundant genera with larger colonies tend to be zooxanthellate and therefore depend on light. There are some reports of zooxanthellate soft corals monopolizing space in productive waters (Fabricius and Dommisse, 2000) or after hard coral disturbance (Nishihira, 1981), but this is probably not a widespread phenomenon (Fabricius, 1998). Exceptions are found in some species of the families Alcyoniidae (especially the genus *Sinularia*), Briareidae and Clavulariidae that can

locally establish space dominance at moderate concentrations of suspended particulate matter (Fabricius, 1998; Fabricius and Dommisse, 2000), but their success in space competition with hard corals tends to be restricted to high-irradiance, high-current and wave-protected inshore reefs. Indeed, octocorals appear to be overall more strongly affected by declining water quality than hard corals are (Fabricius et al., in press): octocoral species richness declines by up to 60% along a gradient of increasing turbidity, mostly due to the disappearance of zooxanthellate octocorals (Fabricius and De'ath, 2001a). Some octocorals are also more sensitive to sedimentation than hard corals (Riegl and Branch, 1995).

3.5. Organisms that cause diseases in corals

Bacteria, cyanobacteria, fungi and protists cause diseases in coral reef organisms, and some of these are now major factors threatening coral and octocoral populations in the Caribbean (Linton et al., 2002). Slow-release fertiliser experiments have demonstrated that infection rates and the spread of certain coral and octocoral diseases are accelerated by experimentally enhancing concentrations of inorganic nutrients (Bruno et al., 2003). On regional scales, disease prevalence has been attributed to increasing seawater temperatures as well as to sedimentation, pathogens transported via airborne dust from expanding deserts, eutrophication and pollution (Sutherland et al., 2004). Overall, more data are needed to test for the potential links between water quality and disease prevalence and virulence in coral reef organisms.

3.6. Organisms that predate on corals

Another indirect, and particularly severe effect of water quality on the status of the wider coral reef ecosystem is the apparent link between frequencies of population outbreaks of the coral eating crown-of-thorns starfish *Acanthaster planci*, and terrestrial runoff. A strong spatial and temporal relationship exists between drought-breaking floods around high continental Indo-Pacific islands and outbreaks of *A. planci* (Birkeland, 1982). Experimental studies document faster development and enhanced survival of the planktotrophic larvae of *A. planci* when concentrations of large phytoplankton are sufficiently high (Lucas, 1982; Okaji et al., 1997). Large phytoplankton groups tend to be nutrient-limited and bloom in response to nutrification events. New research further strengthens the evidence that higher outbreak frequencies of *A. planci* are linked to terrestrial runoff, while acknowledging that the removal of predators of *A. planci* can further enhance the likelihood of outbreaks (Brodie et al., in press; De'ath et al., unpublished data). The offsprings of the

	Dissolved inorg. nutr.	POM*	Light reduction	Sedimentation
Crustose coralline algae	↓			⬇
Bioeroders	↑	⬆		↓
Macroalgae	⬆	↑	⬇	↓
Heterotrophic filter feeders		⬆	⬆	↓
Coral diseases	↑			↑
Coral predators		⬆		

* including phytoplankton

Fig. 4. Synthesis of effects of the four main parameters of terrestrial runoff on the five main groups of organisms that affect coral cover. High abundances crustose coralline algae as settlement substrata promote coral populations, whereas high abundances of the other groups are assumed to negatively affect coral populations. Symbols as in Fig. 1.

primary *A. planci* outbreak that formed in a region with high phytoplankton concentrations are moved by currents to more remote offshore reefs, hence new *A. planci* outbreaks can form even in areas that are far away from sources of terrestrial runoff.

In summary, the different groups of organisms that interact with corals are inhibited or promoted in diverse ways by the four water quality variables (Fig. 4). Dissolved inorganic nutrients affect at least four of the six groups, especially macroalgae. However, dissolved inorganic nutrients are also converted to organically enriched suspended particulate matter, and hence in this way, promote the growth of filter feeding bioeroders, larvae of *A. planci* and heterotrophic filter feeders. Sedimentation strongly inhibits some crustose coralline algae, but can also interfere with certain bioeroders and space competitors. Overall, two of these indirect effects, namely increased abundances of macroalgae and increased frequencies of outbreaks of *A. planci*, arguably affect adult corals more than do the direct effects of nutrient enrichment.

4. Reef properties related to resistance, resilience and risk

Inshore reefs vary considerably in their resistance against detrimental effects from terrestrial runoff and their resilience after exposure. Understanding properties of reefs or regions that contribute to their resistance and resilience could underpin management decisions, e.g., by prioritizing protection of reefs that have the greatest chance of withstanding degradation by terrestrial runoff. This section provides an assessment of the physical, hydrodynamic, spatial and biological properties that may contribute to protecting coral reefs from deterioration at local and regional scales (Table 4). This list of risk factors is preliminary and qualita-

Table 4
Spatial, physical and hydrodynamic, and biological properties of coral reefs, affecting reef resistance and resilience to degradation by exposure to poor water quality from terrestrial runoff

Most affected reef areas	Mechanism	Least affected reef areas
(a) *Spatial, physical and hydrodynamic properties*		
Short distance and/or downstream location relative to discharge source	More frequent exposure to less diluted discharges	Far away or upstream of source of discharge
Shallow surrounding seafloor on wide continental shelf	Resuspension, retention	Deep or precipitating surrounding seafloor
Small (< 2 m) tidal range; or very large (> 4 m) tidal range	Retention of pollutants and sedimentation, esp. in bays at small tides; or chronic resuspension/turbidity and low capacity for photoacclimation at very large tidal ranges	Intermediate tidal range (2–4 m)
Low current area	Retention of pollutants, sedimentation, slow dilution	Current-swept front reef, flank or channels
Embayment, lagoon	Small water volume hence low dilution	Large, open water body
No waves; or high wave exposure	Retention of pollutants, sedimentation; or storm damage and bioerosion due to low skeletal densities in corals	Moderate wave exposure
Deeper reef slope	Low light, slow growth rates, high sediment deposition	Reef crest, upper reef slope
(b) *Biological properties*		
Overfished area	Reduced macroalgal grazers and predators of *A. planci*	Healthy abundances of herbivores and predators (fish, molluscs)
Region prone to frequent or severe disturbances	Removal of adult populations, slow recovery	Region with low disturbance regime
Poor connectivity to larval pools	Low recruitment, slow recovery	High connectivity to larval pools
Region of low biodiversity	Low species redundancy, less functional replacement	Region of high biodiversity

tive, based on previously discussed ideas as well as commonalities that emerged by comparing the better-described regions (Tables 1–3); a formal risk analysis is needed to confirm the contributions of the properties identified.

An important factor that has been previously identified to determine the risk of degradation is the level of exposure (concentration and duration) to terrestrial runoff of a reef system. This exposure is spatially determined by the downstream distance between a reef and the major sources of discharge, the mean annual pollutant load from the source, and dilution processes (West and Van Woesik, 2001; Bourke et al., 2002; Devlin et al., 2003). Exposure is also determined by the rate of retention of pollutants in the ecosystem: any mechanism that promotes retention will enhance exposure and hence the risk of degradation. Retention and removal depend on hydrodynamic processes (flushing rates, dilution), hydrology (e.g., accumulation and slow discharge via groundwater) as well as biological processes (e.g., absorption and storage of pollutant spikes in tissues, altering the organisms' physiology throughout a whole growing season).

At regional scales, tides are important factors determining rates of pollutant removal. Estuarine areas with <2 m tidal amplitudes are more vulnerable to eutrophi-

cation than those with large tides (Monbet, 1992). However, extreme tidal ranges also inhibit reef growth by causing continuous sediment resuspension and chronic turbidity (Kleypas, 1996). A shallow and wide continental shelf is also likely to enhance retention and hence susceptibility of reefs to degradation. This is because material undergoes cycles of deposition and resuspension from a shallow sea floor, whereas the same material is rapidly removed from reefs surrounded by deep water. For example, the shallow and wide northeast Australian continental shelf may play an important role in determining the level of susceptibility of the Great Barrier Reef to terrestrial runoff. A large proportion of the imported material remains in its inshore system for prolonged periods of time due to wave-driven currents and the Coriolis force, and the fine particle fraction (which carries most of the nutrients) is repeatedly resuspended from the shallow sea floor. Possibly as a consequence, although nutrient enrichment on the Great Barrier Reef is less severe than in many other regions, reef communities clearly change along water quality gradients (van Woesik et al., 1999; Fabricius et al., in press; Fabricius and De'ath, 2004).

At local scales, current-swept reef fronts, flanks and channels are likely to experience relatively low levels of retention, as pollutants are rapidly carried away

and diluted. In contrast, poorly flushed bays and lagoons with small water volumes are most likely to be damaged by terrestrial runoff (e.g., Kaneohe Bay, Smith et al., 1981). Upper reef slopes and crests are also less affected by turbidity and sedimentation than deeper areas (Fig. 2). This is because light becomes limiting for corals at greater depths, and sediment deposition is normally greater below the reach of surface waves than on reef crests (except in sheltered bays). Locations with moderate wave action also facilitate coral growth, as waves prevent sediment retention, but strong wave action may result in coral breakage in nutrient-rich areas where coral skeletal densities are weak. Current-swept areas and well-lit reef crests with moderate wave action are therefore likely to be the locations with best coral growth and fastest recovery from disturbance. For example, reef development on the most turbid inshore reefs of the Great Barrier Reef is naturally restricted to sheltered bays, whereas exposed headlands and depositional back reef areas do not support reef accretion. However, current flow, waves and light also facilitate macroalgal growth, as nutrient uptake is flow-dependent, and areas with high light and wave-enhanced nutrient fluxes are also the zones where competition with macroalgae is likely to be most intense.

Biological properties of reefs can also enhance the resistance and resilience of coral reefs. In particular, healthy populations of herbivores help controlling algal or prey populations, hence regions that have high grazer abundances are less likely to respond to deteriorating water quality with macroalgal dominance (McCook, 1999). Importantly, regions that are prone to severe or frequent disturbances (e.g., from coral bleaching, storms, cold water upwelling, or outbreaks of crown-of-thorns starfish) are also likely to be more prone to degradation than less frequently disturbed regions. This is because poor water quality often does not directly kill the adult coral populations (see above), but retards coral recruitment and hence the speed of recovery from such unrelated disturbances. Consequently, connectivity due to lateral transport by currents will contribute to enhancing resilience, as reefs that are well connected to upstream larval sources will recover more quickly from disturbance than reefs that are poorly connected. The role of biodiversity in supporting resistance and resilience is comparatively less understood and needs further research. It appears plausible that regions of high biodiversity have more functional redundancy, and structural changes in diverse regions may be prevented by species replacement when some species disappear in response to changing water quality. In contrast, regions of lower biodiversity may not have suitable species to replace the loss of sensitive species, and are more likely to undergo structural and functional change in their communities (Bellwood et al., 2004). At present it is unknown whether marginal reefs at high latitudes, with their higher macroalgal biomass, lower coral biodiversity and low calcification rates differ in their resistance and resilience to degradation by poor water quality to those at low latitudes.

In summary, reefs that are surrounded by a shallow sea floor, reefs in poorly flushed bays or lagoons, deeper reef slopes, and frequently disturbed reefs are likely to experience changes even at low levels of pollution, in particular when populations of herbivores are low. In contrast, well-flushed shallow reef crests surrounded by deep sea floors or in areas of moderate tides are likely to have the highest level of resistance and resilience, especially when inhabited by healthy populations of herbivores that protect against overgrowth by sediment-trapping macroalgae.

5. Conclusions

Models of the global scale of pollution around coral reefs estimate that 22% of all coral reefs worldwide are classified as at high (12%) or medium (10%) threat from inland pollution and soil erosion (Bryant et al., 1998). The percentage of reefs at risk is highest in countries with widespread land clearing, such as Taiwan and Vietnam with 50% of their reefs at risk from terrestrial runoff, or the Philippines with 35% (Bourke et al., 2002). The models also classify 30% of reefs as threatened from coastal development (proximity to cities, mines and resorts), and 12% at threat from marine pollution (distance to ports, oil tanks, oil wells and shipping areas; Bryant et al., 1998). On a global scale, pollution is therefore rated as a threat to coral reefs similar in severity and scale to coral bleaching, overfishing and destructive fishing (Spalding et al., 2001). On local scales, it can be the single most significant pressure on coastal and inshore coral reefs (Table 1).

This literature review indicates that four fundamentally different processes have to be distinguished when assessing the effects of terrestrial runoff on coral reefs:

1. Dissolved inorganic nutrients can reduce coral calcification and fertilization rates, and increase macroalgal abundances (Figs. 2a, 3 and 4). In the field however, dissolved inorganic nutrients disappear so quickly that their main role may that of curbing organic enrichment of benthos, sediments and suspended POM, except in areas of upwelling and near sewage outfalls.
2. Enrichment with POM enhances feeding rates and growth in some corals, providing a growth advantage that can partly or fully compensate for light reduction, especially in high-flow environments (Fig. 2b). However, while some corals can benefit from POM, heterotrophic filter feeders will benefit even more than corals do, hence the competitive advantage

shifts from corals that can grow at extremely low food concentrations to simpler, more heterotrophic communities. A promotion of the growth and survival of filter feeding larvae of *A. planci* has also profound negative consequences for coral populations (Fig. 4).

3. Turbidity-related light limitation reduces gross photosynthesis (Fig. 2c). Light limitation increases with depth and under macroalgae, but will not occur in shallow water, even in very turbid environments. The effects of light limitation are more severe for phototrophic than mixotrophic species, while heterotrophic species such as filter feeders may be promoted. Light limitation also greatly reduces coral recruitment (Fig. 3).

4. Sedimentation represents a severe disturbance for coral reefs. It reduces growth and survival in a wide range of coral species, although responses differ substantially between species and also between different sediment types (Fig. 2d). Smothering by sedimentation or sediment-trapping macroalgae is the main factor affecting recruitment and the survival of early life stages in corals: settlement rates are near-zero on sediment-covered surfaces, and sedimentation tolerance in coral recruits is at least one order of magnitude lower than for adult corals (Fig. 3). Some of the bioeroding and space-competing groups of organisms are also sensitive to sedimentation by fine silt, and so are crustose coralline algae, with negative consequences for coral recruitment (Fig. 4).

The type and severity of response to terrestrial runoff at any particular location depends on whether changes occurred predominantly in sedimentation, turbidity, POM or dissolved inorganic nutrients, and also depend on the physical, hydrodynamic, spatial and biological properties of a location. In most places, reduced recruitment success in corals, together with the promotion of macroalgae and *A. planci*, arguably represent the most significant direct effect of terrestrial runoff on coral reefs. In severe conditions, the overall outcome is reduced reef calcification, shallower photosynthetic compensation points, changed coral community structure, and greatly reduced species richness. Hence reef ecosystems increasingly simplify with increasing exposure to terrestrial runoff, compromising their ability to maintain essential ecosystem functions at the presently increasing frequencies of human-induced disturbances.

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Global inputs of biological nitrogen fixation in agricultural systems

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Abstract Biological dinitrogen (N_2) fixation is a natural process of significant importance in world agriculture. The demand for accurate determinations of global inputs of biologically-fixed nitrogen (N) is strong and will continue to be fuelled by the need to understand and effectively manage the global N cycle. In this paper we review and update long-standing and more recent estimates of biological N_2 fixation for the different agricultural systems, including the extensive, uncultivated tropical savannas used for grazing. Our methodology was to combine data on the areas and yields of legumes and cereals from the Food and Agriculture Organization (FAO) database on world agricultural production (FAOSTAT) with published and unpublished data on N_2 fixation. As the FAO lists grain legumes only, and not forage, fodder and green manure legumes, other literature was accessed to obtain approximate estimates in these cases. Below-ground plant N was factored into the estimations. The most important N_2-fixing agents in agricultural systems are the symbiotic associations between crop and forage/fodder legumes and rhizobia. Annual inputs of fixed N are calculated to be 2.95 Tg for the pulses and 18.5 Tg for the oilseed legumes. Soybean (*Glycine max*) is the dominant crop legume, representing 50% of the global crop legume area and 68% of global production. We calculate soybean to fix 16.4 Tg N annually, representing 77% of the N fixed by the crop legumes. Annual N_2 fixation by soybean in the U.S., Brazil and Argentina is calculated at 5.7, 4.6 and 3.4 Tg, respectively. Accurately estimating global N_2 fixation for the symbioses of the forage and fodder legumes is challenging because statistics on the areas and productivity of these legumes are almost impossible to obtain. The uncertainty increases as we move to the other agricultural-production systems—rice (*Oryza sativa*), sugar cane (*Saccharum* spp.), cereal and oilseed (non-legume) crop lands and extensive, grazed savannas. Nonetheless, the estimates of annual N_2 fixation inputs are 12–25 Tg (pasture and fodder legumes), 5 Tg (rice), 0.5 Tg (sugar cane), <4 Tg (non-legume crop lands) and <14 Tg (extensive savannas). Aggregating these individual estimates provides an overall estimate of 50–70 Tg N fixed biologically in agricultural systems. The uncertainty of this range

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would be reduced with the publication of more accurate statistics on areas and productivity of forage and fodder legumes and the publication of many more estimates of N_2 fixation, particularly in the cereal, oilseed and non-legume crop lands and extensive tropical savannas used for grazing.

Introduction

Just over 25 years ago, Bob Burris wrote a paper entitled "The global nitrogen budget—science or séance?" in which he discussed the challenges of scaling up plot measurements of dinitrogen (N_2) fixation and other nitrogen (N) flows to calculate global N budgets (Burris 1980). With tongue in cheek, he suggested that potential authors could use a variety of methods to fill in the values in the N cycle, from gazing at crystal balls, consulting sages to cranking out computer-generated random numbers. He did acknowledge, however, that the common method was to consult the literature, choose the data that seem to make sense, then construct the budget accordingly.

Delwiche (1970) and Burns and Hardy (1975) had previously estimated annual, global biological N_2 fixation at 100 and 175 million tonnes (Tg) N,

respectively. The latter estimate was revised downwards at an international conference in Sweden soon afterwards to 122 Tg N, principally by downgrading inputs of fixed N in forests and natural grasslands. Burris (1980) accepted this amended figure of 122 Tg N fixed annually and noted that it seemed to be compatible with the published values for the global carbon (C) cycle. The global N_2 fixation estimates of Delwiche (1970), Burns and Hardy (1975) and Burris (1980) have been widely quoted ever since. Note that these estimates cover both agricultural and natural systems, including marine, and were largely derived using acetylene (C_2H_2) reduction, N difference and N balance methodologies. The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in Fig. 1.

New figures for global N_2 fixation have been published more recently (e.g. Galloway et al. 1995; Smil 1999) and are also widely quoted (Vitousek et al. 1997; Boyer et al. 2004; Galloway et al. 2004; Mosier et al. 2004). Galloway et al. (1995) and Smil (1999) estimated global N_2 fixation for cultivated agricultural systems, i.e. excluding the extensive tropical savannas, at 43 Tg (range 32–53 Tg) and 33 Tg (range 25–41 Tg) annually. Cleveland et al. (1999) estimated terrestrial global N_2 fixation by considering 23 biome types covering the whole planet, but did not consider the extent of agricultural activity in these biomes, or the presence of cultivated legumes capable of large per ha inputs of N_2 fixation.

Fig. 1 Biological N_2-fixing agents in agricultural and terrestrial natural systems
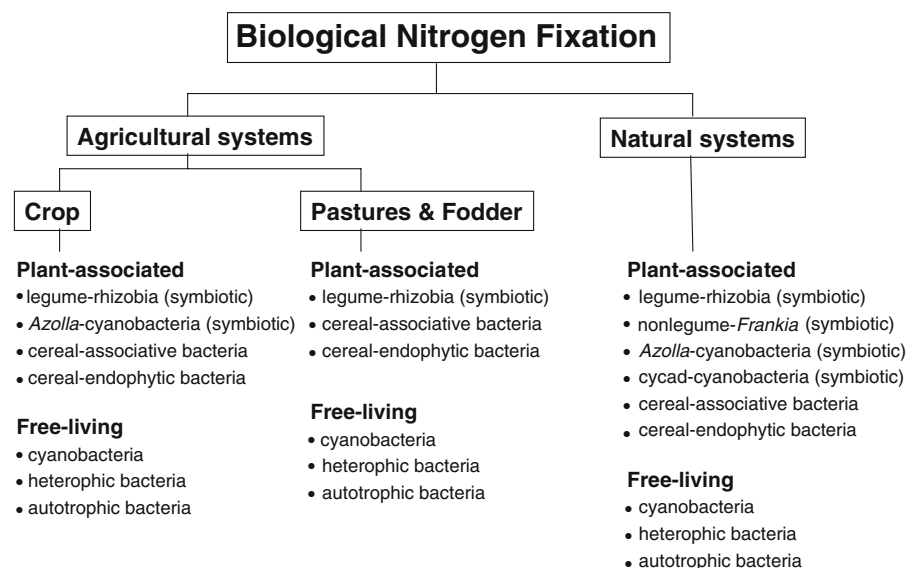
Galloway et al. (2004) covered all aspects of the N cycle and incorporated estimates of N_2 fixation in cultivated agricultural systems (32 Tg N/year) using data from earlier papers (Galloway et al. 1995; Smil 1999).

In this review we reconsidered N_2 fixation inputs into agricultural systems. As in past reviews (e.g. Smil 1999), we included cultivated land used for agriculture, but also included uncultivated agricultural lands, such as the tropical savannas used for grazing. Our strategy was to combine data on the areas and yields of legumes and cereals from the Food and Agriculture Organization (FAO) database on world agricultural production (FAOSTAT) with published and unpublished data on N_2 fixation. As the FAO lists grain legumes only, and not forage, fodder and green manure legumes, other literature was accessed to obtain approximate estimates in these cases.

The difficulties and potential errors in calculating N_2 fixation at global scales are magnified substantially when moving from agricultural systems to the natural systems. The agents of N_2 fixation are essentially the same as in agricultural systems, although the species may be different. The main problems are the uncertainty in estimating N_2 fixation intensity per unit area, the likely bias of those estimates, and the difficulty in scaling up because of uncertainties in spatial coverage of the putative N_2-fixing species. Galloway et al. (2004) stated: "In a recent compilation of rates of natural biological nitrogen fixation (BNF) by Cleveland et al. (1999), symbiotic BNF rates for several biome types are based on one-to-few published rates of symbiotic BNF at the plot scale within each particular biome. For example, based on a few estimates of symbiotic BNF available for tropical rain forests, estimated BNF in these systems represents ~24% of total natural terrestrial BNF globally on an annual basis (Cleveland et al. 1999). While the relative richness of potential N_2-fixing legumes in tropical forests suggests that symbiotic BNF in these systems is relatively high (Crews 1999), the paucity of actual BNF rate estimates in these systems suggest caution when attempting to extrapolate plot scale estimates of BNF and highlights the difficulties to attempting to estimate natural BNF at the global scale." Because of the uncertainties, we have not attempted in this review to quantify global N_2 fixation in natural systems.

Measurement of N_2 fixation

Notwithstanding the difficulties and errors, the demand for accurate determinations of global inputs of biologically-fixed N is strong and will continue to be fuelled by the need to understand and effectively manage the global N cycle. There are five basic methodologies available to quantify biological N_2 fixation:

1. The enzyme nitrogenase, universally responsible for biological N_2 fixation, is also capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4). Both gases can be readily detected and quantified using gas chromatography (Schollhorn and Burris 1967; Hardy et al. 1968). Thus, the C_2H_2 reduction assay is a sensitive measure of nitrogenase activity at a point in time and can be very useful for detecting N_2 fixation activity of, for example, bacterial cultures or plant residues that may be harbouring N_2-fixing bacteria. However, in enclosing the particular agent in a gas-tight vessel to evaluate ethylene (C_2H_4) evolution, physical disturbance of the N_2-fixing species is almost inevitable and this results in a decline in activity (Minchin et al. 1986; Boddey 1987). Even the partial substitution of N_2 by C_2H_2 is sufficient to reduce N_2-fixing activity (Minchin et al. 1983). Scaling up point-source C_2H_2 reduction values to account for spatial and temporal variations and converting them to amounts of N fixed is difficult, if not impossible, and is not recommended.

   Hydrogen is an obligate product of N_2 reduction and its measurement can also be used to assay nitrogenase activity (Hunt and Layzell 1993; Dong et al. 2001). However, the method has never been applied as a routine field assay owing to practical difficulties.

2. The total N-balance method is based on the principal that the plant/soil system will accumulate N over time if there is an input of N_2 fixation. However, measures of N_2 fixation may be underestimated because of N losses from the system during the period of study through ammonia volatilisation, denitrification, leaching etc, or confounded by other external inputs of N unrelated to N_2 fixation (e.g. N dissolved in rainfall, N in dust, gaseous N etc). Hence N balance requires measurements of as many potential N inputs and outputs as possible. The time-frame is generally

several years because of the need to measure incremental changes in the N content of the soil against large background amounts (Peoples and Herridge 1990; Giller and Merckx 2003). Clearly the methodology is technically challenging, requiring substantial inputs of labour for long periods. Additionally, errors in quantifying the N fluxes, and inaccuracies in sampling and analysing soil for changes in total N and bulk density, can introduce substantial uncertainties into the final estimates of N_2 fixation (Chalk 1998). The N balance method was more commonly used some time ago (e.g. Vallis 1972; Wetselaar et al. 1973), but in recent years has been largely replaced by ^{15}N and ureide methods, described below.

3. A simple variation of N balance for quantifying N_2 fixation is N difference. With this method, total N accumulated by N_2-fixing plants is compared with that of neighbouring non N_2-fixing plants, with the difference between the two assumed to be due to N_2 fixation. The main assumption is that the N_2-fixing plants assimilate the same amount of soil mineral N as the neighbouring non N_2-fixing plants. In soils of limited N supply, this method can be used with considerable success, especially if the N_2-fixing plants derive large amounts of N from N_2 fixation. It may be less useful in moderate-to-high N soils because differences between N_2-fixing and non N_2-fixing plants in root morphology and rooting depth can result in different capacities to exploit soil N (Herridge et al. 1995; Chalk 1998). It is also of limited value for on-farm surveys where appropriate non N_2-fixing plants may not be present. Good examples of the application of this technique were published in the 1960–1970s (Weber 1966; Bell and Nutman 1971). As with N balance, this method has been largely replaced by ^{15}N and ureide methods.

4. The heavy isotope of nitrogen, ^{15}N, was first used to evaluate N_2 fixation by bacteria in the 1940s (Burris et al. 1942), but the availability of materials enriched with ^{15}N and mass spectrometers to analyse the samples severely restricted its general application for many years. That situation started to change in the 1970s, facilitating more widespread use of ^{15}N-based methodologies during the 1980s and beyond. Experimental protocols involved: (i) labelling N_2 in the atmosphere surrounding the N_2-fixing plants ($^{15}N_2$

incorporation—Warembourg et al. 1982) followed by measurement of incorporation of ^{15}N by the plants, and (ii) growing the plants in ^{15}N-enriched soil or other growth medium (^{15}N isotope dilution—McAuliffe et al. 1958; Chalk 1985) and calculating the extent of dilution of ^{15}N in the plants by atmospheric (fixed) ^{14}N. A later variation of ^{15}N isotope dilution utilised the natural ^{15}N enrichment of soils, thereby avoiding the need to add ^{15}N-enriched materials (natural ^{15}N abundance—Shearer and Kohl 1986). The $^{15}N_2$ incorporation method is limited in application to short experimental periods in a laboratory or growth chamber. ^{15}N isotope dilution with artificial enrichment of soil was, until a few years ago, used widely to quantify N_2 fixation in agricultural systems (Chalk and Ladha 1999), although rarely on-farm in unreplicated, non-experimental studies. In recent years, natural ^{15}N abundance has gained prominence for work in both experimental plots and in farmers' fields, owing to the greater accessibility of scientists to high-precision, automated isotope-ratio mass spectrometers. Although natural ^{15}N abundance has been widely utilised in agricultural settings, there are a number of potential limitations that restrict its application in natural ecosystems (Boddey et al. 2000). In those systems, estimates of the percentage of plant N derived from N_2 fixation (%Ndfa) may not be possible owing to the large spatial variability, diversity and complexity of available-N pools in the soil with different ^{15}N signatures (e.g. Pate et al. 1993; Gehring and Vlek 2004).

5. The ureide method (McClure et al. 1980; Herridge and Peoples 1990) exploits the fact that many of the agronomically-important legumes of tropical origin (e.g. soybean [*Glycine max*], common bean [*Phaseolus vulgaris*], *Desmodium* spp.) export allantoin and allantoic acid (collectively known as ureides) as the products of N_2 fixation from their nodules to the shoots. In these legumes, the ratio of ureide N to total N in xylem sap or stem segments is highly correlated with %Ndfa. Although not applicable to all legumes, and to no other N_2-fixing associations, the technique has been widely used with both experimental and non-experimental (farmer) crops. The analytical procedures are simple with minimal requirements for sophisticated or expensive equipment.

The principles behind these methods and how to use them effectively have been described in varying degrees of detail in a substantial number of publications for nodulated legumes (e.g. Chalk 1985; Shearer and Kohl 1986; Witty and Minchin 1988; Witty et al. 1988; Peoples and Herridge 1990; Hardarson and Danso 1993; Danso et al. 1993; Vessey 1994; Unkovich and Pate 2000; Giller 2001; Peoples et al. 2002; Unkovich et al. 2008), and associative and free-living N_2-fixing agents (Boddey 1987; Chalk 1991; Boddey et al. 2001; Giller 2001; Giller and Merckx 2003; Unkovich et al. 2008). The N balance and N difference methods provide estimates of N_2 fixation on an area basis, i.e. kg N/ha. The ^{15}N and ureide methods, on the other hand, provide estimates of % Ndfa, i.e. the percentage of total N of the organism (bacteria, plant) that is derived from N_2 fixation. An amount of N_2 fixed per unit area or unit of production can only be calculated when %Ndfa is combined with an estimate of organism biomass and total N content. Although all methods have their unique limitations and sources of error, the N balance, N difference, ^{15}N (isotope dilution and natural abundance) and ureide methods arguably represent the best of what is currently available.

Reliability of current estimates of N_2 fixation in the different agricultural systems

The key ingredients for accurately estimating N_2 fixation at any scale—unit area (m^2 or ha), individual field, catchment, region, country, globe—are reliable values for %Ndfa and total N accumulation of the N_2-fixing agent for a specific period of time. Thus, global estimates of N_2 fixation of crop legumes in agricultural systems are likely to be sound because they draw on many hundreds of individual values of %Ndfa and the annual area and production statistics of the FAO, published as FAOSTAT (Table 1). FAOSTAT is the web-based statistical database of the FAO (http://faostat.fao.org) covering many aspects of world agriculture, including crops in the section Production/Crops. Estimates of N_2 fixation of forage and fodder legumes will be less reliable because global areas of land with forage and fodder legumes are difficult to assemble as are estimates of %Ndfa of legumes in those lands.

The most reliable information on the other N_2-fixing agents in agricultural systems—the azolla/cyanobacteria association, free-living cyanobacteria and other autotrophic bacteria, and the numerous genera of heterotrophic bacteria that utilise either C-rich exudates of living plants or degrading crop residues as energy sources—are the areas in which they potentially exist. For example, the FAOSTAT database can provide figures for the global area and production of rice (*Oryza sativa*) that can be combined with published estimates of N_2 fixation of free-living cyanobacteria and the azolla–cyanobacteria association to calculate potential N_2 fixation in this system (Smil 1999). Similarly, FAOSTAT can also provide accurate data on areas and production of sugarcane (*Saccharum* sp.) for calculating potential N_2 fixation of the endophytic and associative bacteria in this particular system. To calculate actual, rather than potential, N_2 fixation is far more difficult because of the uncertainty in determining the occurrence and activity of the N_2-fixing agents across the global reach of these systems (Table 1).

Table 1 Assessments of the reliability of estimating %Ndfa and total N of the different N_2-fixing agents in agricultural systems (the more +++ the better)

N_2-fixing agent	Agricultural system	Reliability in estimating %Ndfa	Reliability in estimating total N of the N_2-fixing agent globally
Legume–rhizobia	Legume cropping	+++++	+++++
Legume–rhizobia	Pasture/fodder	+++++	+++
Azolla–cyanobacteria, cyanobacteria	Rice	++++	+++
Endophytic, associative and free-living bacteria	Sugar cane	++	++
Endophytic, associative and free-living bacteria	Other cropping lands	+	+
Endophytic, associative and free-living bacteria	Extensive tropical savannas used for grazing	+	+

Below-ground N—the underestimated component of N₂-fixing plants

The majority of published values for legume N_2 fixation were based on shoots only. Fixed N contained in attached and detached roots and nodules, and rhizodeposition was essentially ignored (e.g. Evans and Herridge 1987; Danso et al. 1993; Unkovich et al. 1997; Smil 1999; Carlsson and Huss-Danell 2003; Russelle and Birr 2004). In other reports, a factor was used to account for below-ground N (BGN), usually based on a published or experimentally-determined value derived from the physical recovery of roots (e.g. Herridge et al. 1995; Evans et al. 2001). We are now starting to see a change, however, with acknowledgement that published values for legume N_2 fixation are low because they do not account for the large proportion of below-ground N contained in non-recovered roots, detached nodules, and products of root and nodule necrosis (Carlsson and Huss-Danell 2003; Crews and Peoples 2005; McNeill and Fillery 2008). For example, Walley et al. (2007) assumed root N was 14% of total plant N and rhizodeposited N an additional 10% when calculating N_2 fixation of the pulse legumes in the Northern Great Plains of North America. This change in thinking has been brought about by advances in methodologies for estimating BGN.

In the past, the most simple and commonly-used method for determining BGN was to physically remove roots from the soil. Values for BGN as a percentage of total plant N were usually <15% (Sheldrake and Narayanan 1979; Bergersen et al. 1989; Danso et al. 1993; Toomsan et al. 1995), although higher values (24–40%) were sometimes reported (Chapman and Myers 1987; Dalal et al. 1997).

Russell and Fillery (1996a, b), McNeill et al. (1997, 1998) and others used ^{15}N as a tracer to quantify BGN of nodulated legumes. The experimental protocol involved in-situ ^{15}N shoot-labelling of plants during early vegetative growth, followed by quantification at the end of the growth cycle of ^{15}N in shoot and root biomass and in the root-zone soil. The approach capitalised on earlier ^{15}N tracer studies with both legumes and non-legumes (Janzen and Bruinsma 1989; Zebarth et al. 1991). In the decade since the initial publications by Russell and Fillery (1996a, b), the technique has been applied to a number of species. Thus, published values for BGN as a percentage of the total plant N are 22–68% for the

pulse and oilseed legumes, soybean, faba bean (*Vicia faba*), chickpea (*Cicer arietinum*), mungbean (*Vigna radiata*), narrow-leafed lupin (*Lupinus angustifolius*), pea (*Pisum sativum*) and pigeonpea (*Cajanus cajan*), and 34–68% for the pasture/fodder legumes, subterranean clover (*Trifolium subterraneum*), serradella (*Ornithopus compressus*), white clover (*Trifolium repens*) and alfalfa (*Medicago sativa*) (Zebarth et al. 1991; Russell and Fillery 1996b; McNeill et al. 1997; Jørgensen and Ledgard 1997; Rochester et al. 1998; Khan et al. 2002, 2003; Yasmin et al. 2006; Mahieu et al. 2007; McNeill and Fillery 2008).

Clearly, there is no single value for BGN, with the variation in published estimates reflecting effects of species, soil and climate on the partitioning of N within the plant. To account for BGN when calculating N_2 fixation, we used a multiplication factor of 2.0 for the pasture/fodder legumes and chickpea (assumes 50% of plant N is below-ground), 1.5 for soybean (assumes 33% BGN) and 1.4 for the remainder of the pulse and oilseed legumes (assumes 30% BGN). Although these factors are approximations, we would argue that the errors associated with their use are far less than the errors associated with ignoring BGN or using values for physically-recovered roots. It is also worth noting that reported BGN values for non-legumes, such as wheat and barley, are similar to those of the legumes. For example, Khan et al. (2003) estimated BGN of field-grown barley (*Hordeum vulgare*) at 30%.

Legumes–rhizobia

The most important N_2-fixing agents in agricultural systems are the symbiotic associations between crop and forage/fodder legumes and rhizobia. Smil (1999) suggested that we are still not able to make reliable, average estimates of legume N_2 fixation. Theoretically, that might be correct although the reasons are more to do with the large variations in N_2 fixing intensity than limitations in methodology. In practice, there are now sufficient estimates of N_2 fixation in the literature to calculate reasonably accurate average values.

Crop legumes–rhizobia

The %Ndfa values for the crop legume symbioses in Table 2 were sourced from Peoples et al. (2008) in which data from a number of reviews and experimental

Table 2 Average values for %Ndfa for the major crop legumes in experiments and farmers' fields

Legume	Experiments[a]		Farmers' fields[b]
	%Ndfa range	%Ndfa average	%Ndfa average
Common bean	0–73	40	36
Chickpea, lentil, pea, cowpea, mungbean, pigeonpea etc	8–97	63	65
Soybean, groundnut	0–95	68	58
Fababean, lupin	29–97	75	68

[a] Collated from Peoples et al. (2008) in which data from a number of reviews and experimental papers were summarised with additional information on N_2 fixation of common bean from Rennie and Kemp (1982a, b) and Hardarson et al. (1993)

[b] Sourced from Peoples et al. (2008), comprising >800 determinations

papers were summarised (Peoples and Craswell 1992; Herridge and Danso 1995; Peoples et al. 1995; Wani et al. 1995; Jensen 1997; Unkovich et al. 1997; Schulz et al. 1999; Unkovich and Pate 2000; Giller 2001; Rochester et al. 2001; Turpin et al. 2002; Aslam et al. 2003; Shah et al. 2003). Additional information on N_2 fixation of common bean was sourced from Rennie and Kemp (1982a, b) and Hardarson et al. (1993). We grouped the legumes according to their ability to fix N in experiments. Common bean has the lowest capacity for N_2 fixation and is in a group by itself, with an average Ndfa of 40%. The next group includes most of the winter and summer pulses, with an average Ndfa of 63%. The third group includes soybean and groundnut (*Arachis hypogaea*), with Ndfa of 68% and the final group includes faba bean and lupin (*Lupinus* spp.) with Ndfa of 75%. The ranges of values within the four groups are large and reflect variations in legume growth, set by genetic, agronomic, environmental and experimental factors, the availability of soil mineral N and numbers and effectiveness of rhizobia in the vicinity of the growing root system. The groupings are reasonably consistent with those described by Hardarson and Atkins (2003) for food legumes involved in FAO/International Atomic Energy Agency co-ordinated research programs across a number of countries and with those of Walley et al. (2007) for the pulse legumes in the Northern Great Plains of North America.

Average %Ndfa values for legumes growing in >800 farmers' fields in Europe, Africa, Asia, South America and Australia are shown in the final column, Table 2. Values were taken from Peoples et al. (2008) using data sourced from Rupela et al. (1997), Rochester et al. (1998), Schwenke et al. (1998), Maskey et al. (2001), Peoples et al. (2001), Hiep et al. (2002), Hoa et al. (2002) and Herridge et al. (2005). The %Ndfa values

for the farmers' fields are in reasonable agreement with the experimental data and support three of the four groupings of the crop legumes. The %Ndfa values for soybean in farmers' fields are lower than those in the experiments, principally reflecting the regions in which these particular crops were grown. Only 21 of the 133 estimates were from Brazil and none were from Argentina. The two countries together grow >40% of the world's soybean with relatively high %Ndfa values (Alves et al. 2003; Hungria et al. 2005) (see also Table 3).

To differentiate %Ndfa for the different legumes at smaller scales, i.e. field, catchment, region, according to local soil and plant-growth conditions and then aggregate those estimates to generate country and global values would be extremely difficult and may not improve accuracy. Having said that, %Ndfa of soybean needs to be differentiated for the principal soybean-producing countries as this crop is responsible for most of the N fixed by legumes, and there are considerable differences in soil type, climate and plant-cultural practices amongst those countries (Table 3).

In the U.S., soils used for soybean production tend to be fertile, with moderate-high concentrations of clay, organic matter and plant-available N (e.g. Russelle and Birr 2004). As a result, reported Ndfa values mostly range between 40% and 80% (van Kessel and Hartley 2000; Peoples et al. 2008; Salvagiotti et al. 2008), with an overall average value of 60%.

The average Ndfa value for soybean in Brazil is calculated at 80%, reflecting the widespread use of rhizobial inoculants, the high N demand of the crops (about 300 kg N/ha) coupled with low inputs of fertiliser N, and the high proportion of the crops that are no-tilled (Hungria and Vargas 2000; Hungria et al. 2005, 2006; Alves et al. 2003; FAOSTAT). Alves et al. (2003) and

Table 3 Estimates of amounts of N fixed annually by soybean in the major soybean-producing countries, using FAO statistical data for 2005 (FAOSTAT), estimates of country-specific %Ndfa, and estimates of harvest index, %N shoots and below-ground N as % of total crop N

Country	Area (Mha)	Grain yield (Tg)	Shoot DM (Tg)[a]	Shoot N (Tg)[b]	Crop N (Tg)[c]	%Ndfa	Crop N fixed (Tg)
U.S.	30.0	85.0	212.6	6.38	9.56	60	5.74
Brazil	22.9	51.2	128.0	3.84	5.76	80	4.61
Argentina	14.0	38.3	95.8	2.87	4.31	80	3.44
China	9.6	16.8	42.0	1.26	1.88	50	0.95
Soybean	**93.4**	**214.8**	**537.1**	**16.12**	**24.17**	**68**	**16.44**

[a] Using harvest index (grain dry matter as a proportion of total above-ground dry matter) value of 0.4 (Jefing et al. 1992; Herridge and Holland 1992; Guafa et al. 1993; Herridge and Peoples 2002; Shutsrirung et al. 2002; Gan et al. 2002, 2003; Salvagiotti et al. 2008)

[b] Using %N shoots of 3.0% (Herridge et al. 1990; Herridge and Holland 1992; Herridge and Peoples 2002; Shutsrirung et al. 2002; Gan et al. 2002, 2003; Salvagiotti et al. 2008)

[c] Multiplying shoot N by 1.5 (Rochester et al. 1998)

others (see review by van Kessel and Hartley 2000) reported consistent increases in nodulation and N$_2$ fixation of no-tilled soybean compared with crops grown under cultivation. The increases under no till were thought to be due principally to reduced levels of nitrate coupled with improved moisture conditions in the soil. Thus, Alves et al. (2003) reported that Brazilian soybean derived 70–85% of crop N from N$_2$ fixation, equivalent to 70–250 kg N/ha. In the case of high-yielding crops, i.e. >4.0 t/ha, as much as 350–400 kg N/ha may be fixed. Similarly, Hungria et al. (2005) reported Ndfa values of 69–94% for inoculated soybean in Brazil.

There are very few reports quantifying N$_2$ fixation of soybean in Argentina. Published Ndfa values are 30–70% (Garcia 2004) and 40–50% (Gutiérrez-Boem et al. 2004; Di Ciocco et al. 2004), but these estimates were from experimental sites and not farmer's fields. However, Argentinian soybean farmers, like the Brazilian farmers, commonly use inoculants and no-tillage production systems with negligible fertiliser N (Garcia 2004; Hungria et al. 2005; Peloni 2006; FAOSTAT). Garcia (2004) also noted that most of the soils used for soybean production in Argentina have nutrient deficiencies, including N. Taken together, these reports suggest that the high N demand crops would need to fix a large proportion of their N requirements. We therefore assume the same average Ndfa value for soybean in Argentina as for soybean in Brazil, i.e. 80%.

Chinese farmers reportedly apply fertiliser N to soybean and rely on the naturalised soil rhizobia to nodulate the crops rather than use inoculants (Gan et al. 2002; Ruiz Sainz et al. 2005). P.W. Singleton (personal communication) estimated that about

0.54 Tg fertiliser N was applied to 10.5 Mha soybean and groundnut in 1994. The fertiliser N inputs plus residual mineral N in the soil from previous crops would depress N$_2$ fixation activity substantially. Thus, we estimate the average Ndfa value for China at 50% (Ruiz Sainz et al. 2005).

The total amount of N$_2$ fixed by soybean for each of the four major soybean-producing countries can now be estimated by combining the %Ndfa values with production statistics from FAOSTAT. First, the total amount of soybean N is calculated by dividing the FAOSTAT crop production data (Column 3, Table 3) by an average harvest index value (0.4) to determine shoot dry matter (DM) (Column 4). Shoot N (Column 5) and crop N (Column 6) are then calculated using 3% for the N concentration of shoots and a multiplication factor of 1.5 to account for below-ground N (Rochester et al. 1998). Crop N fixed (final column) is then determined as crop N × %Ndfa. Thus, estimates of total crop N fixed by soybean range between 0.95 Tg annually for China to 3.4 Tg for Argentina, 4.6 Tg for Brazil and 5.7 Tg for the U.S.

We used the same series of calculations to estimate global N$_2$ fixation of the major pulse and oilseed legumes (Table 4). The final column contains the calculated values for annual crop N fixed for each species plus total values for the pulse legumes (2.95 Tg), oilseed legumes (18.5 Tg) and all crop legumes (21.5 Tg).

In a previous publication we calculated global N$_2$ fixation by the pulse and oilseed legumes by using estimates of average amounts of N fixed per unit shoot biomass (Peoples et al. 2008). This approach was based on the observation that amounts of N$_2$

Table 4 Estimates of amounts of N fixed annually by the major pulse and oilseed (crop) legumes, using FAO statistical data for 2005 (FAOSTAT), values for average %Ndfa from Table 2 and estimates of values for harvest index, %N shoots and below-ground N as % of total crop N

Legume	Area (Mha)	Grain yield (Tg)	Shoot DM (Tg)[a]	Shoot N (Tg)[b]	Crop N (Tg)[c]	%Ndfa	Crop N fixed (Tg)
Common bean	25.1	18.1	51.7	1.03	1.45	40	0.58
Cowpea	9.2	4.6	13.3	0.27	0.37	63	0.23
Chickpea	10.4	8.4	23.9	0.48	0.96	63	0.60
Pea	6.6	11.3	32.3	0.65	0.90	63	0.57
Lentil	4.1	4.1	11.8	0.24	0.33	63	0.21
Fababean	2.7	4.3	12.4	0.27	0.38	75	0.29
Other pulses	11.4	9.4	26.8	0.54	0.75	63	0.47
Total pulses	**69.7**	**60.2**	**171.9**	**3.48**	**5.14**	**57**	**2.95**
Groundnut	23.4	37.6	93.9	2.16	3.03	68	2.06
Soybean	93.4	214.8	537.1	16.11	24.17	68	16.44
Total oilseeds	**116.7**	**252.4**	**707.8**	**18.27**	**27.20**	**68**	**18.50**
Total crop legumes	**186.4**	**312.6**	**879.7**	**21.75**	**32.34**	**66**	**21.45**

[a] Using harvest index (grain dry matter as a proportion of total above-ground dry matter) values of 0.4 for groundnut and soybean and 0.35 for the remainder (see references in footnote Table 3; also Schwenke et al. 1998; Evans et al. 2001; Hiep et al. 2002; Hoa et al. 2002; MJ Unkovich, personal communication)

[b] Using %N shoots of 3.0% for soybean, 2.3% for groundnut, 2.2% for fababean and 2.0% for the remainder (see references in footnote Table 3; also Schwenke et al. 1998; Evans et al. 2001; Hiep et al. 2002; Hoa et al. 2002)

[c] Multiplying shoot N by 2.0 (chickpea), 1.5 (soybean) and 1.4 (remainder) to account for below-ground N.

fixed by legumes in any agroecosystem were primarily regulated by plant growth and DM production. The provisos were that effective rhizobia were present in the soil and concentrations of soil mineral N were not excessive. Data collected from both experimental trials and farmers' crops indicated that crop legumes generally fix 15–25 kg shoot N for every Mg shoot DM accumulated, with averages of 20 kg shoot N/Mg shoot DM (Fig. 2; see also Evans et al. 2001; Maskey et al. 2001; Peoples et al. 2001). Fixed N associated with the nodulated roots increased the value to 30 kg total crop N/Mg shoot DM. Common bean, chickpea and soybean were identified as the exceptions, with values for common bean of 15 kg total crop N fixed/Mg shoot DM, and for chickpea and soybean of 40 kg crop N fixed/Mg shoot DM. We used these values to calculate global N_2 fixation of 4 and 18 Tg N (total 22 Tg N) annually by the pulses and oilseed legumes, respectively, using FAOSTAT production statistics for 2000–2004.

Smil (1999) used yet another approach to calculate average annual values for global N_2 fixation by the crop legumes. Ranges of values (minimum, mean, maximum) for crop N fixed for each species were estimated on an area basis (kg N/ha), then applied to the global areas of the legumes from FAOSTAT.

Comparisons of the Smil (1999) estimates of legume N_2 fixation (area basis, kg N/ha) and estimates using the data in Table 3 are shown in Table 5.

There is generally good agreement between the Smil (1999) values for crop N_2 fixed (kg/ha) and our values calculated from Table 4, except for soybean and pea (Table 5). The difference in the case of soybean can be explained by the recent expansion of production in Argentina and Brazil where the use of fertiliser N is low, inoculation is widespread and the N demands of the predominantly no-tilled crops are large because of relatively high grain yields (2.73 Mg/ha for Argentina and 2.23 Mg/ha for Brazil, FAOSTAT for 2005). The long-standing notion that soybean fix, on average, about 50% of their N needs would appear to be no longer valid.

Smil (1999) estimated crop legumes to fix a total of 10 Tg N annually, compared with our estimate of 21.5 Tg annually. As mentioned above, the discrepancy results mainly from the different values of %Ndfa for pea and soybean, our inclusion of estimates of below-ground fixed N associated with, or released from, roots and nodules, and the use of updated FAOSTAT statistics, i.e. 2005 data used for calculations in Tables 3 and 4 compared with mid 1990s data used by Smil (1999).

Fig. 2 Examples of the relationship between amounts of N$_2$ fixed (as kg N/ha in shoots) and shoot dry matter (Mg/ha) for crop legumes growing in different geographic regions. Data includes both rainfed and irrigated cool-season (*open circles*) and warm-season legumes (*closed triangles*). The *dashed lines* indicate 15 and 25 kg N fixed per Mg dry matter. Relationship modified from Peoples et al. (2008) who used published and unpublished data collated from studies undertaken in the Middle East and Asia (Syria, Nepal, Pakistan, Thailand), Oceania (Australia), South America (Brazil), North America (Canada and USA), and Europe (Austria, Denmark and France)
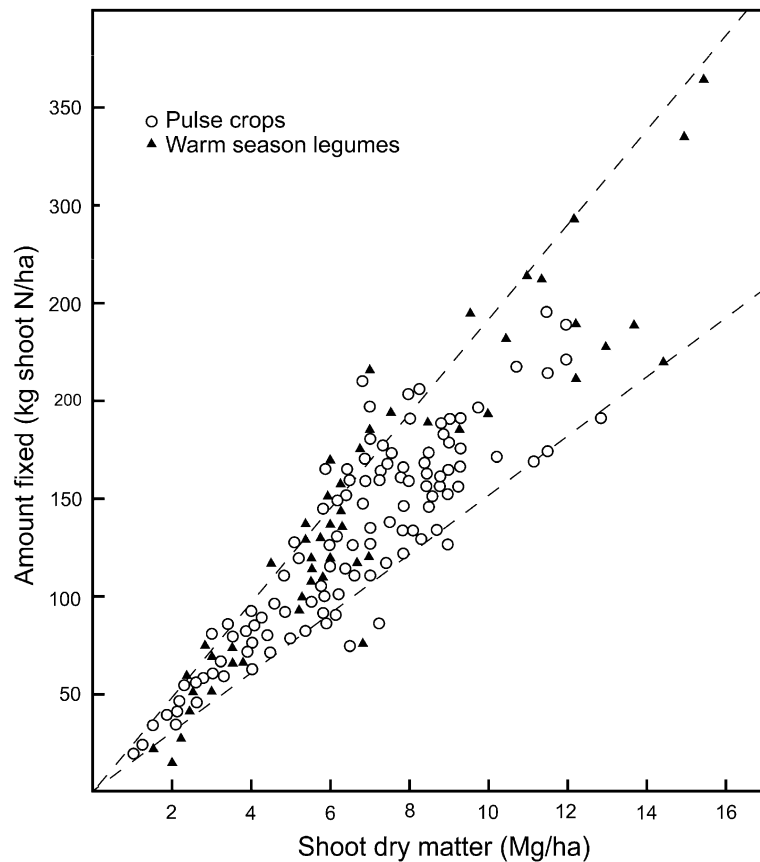
Forage/fodder legumes–rhizobia

Accurately estimating global N$_2$ fixation for the symbioses of the forage and fodder legumes is challenging because statistics on the areas and productivity of these legumes are almost impossible to obtain. Smil (1999) reported 100–120 Mha of land in fodder and forage legumes and green manure crops. He assumed average annual N$_2$ fixation rates of 200 kg N/ha for alfalfa, 150 kg N/ha for the clovers (*Trifolium* spp.), 100 kg N/ha for other forages and 50 kg N/ha for legume–grass pastures. Thus, total N$_2$

Table 5 Comparing estimates of N$_2$ fixation/unit area (kg/ha) by Smil (1999) with estimates calculated from legume global areas (Table 4, column 2) and crop N fixed (Table 4, column 8)

Legume	Smil (1999) ranges of values (kg N/ha/year)			Calculated from Table 4 (kg N/ha/year)
	Minimum	Mean	Maximum	
Common bean	30	40	50	23
Chickpea	40	50	60	58
Pea	30	40	50	86
Lentil	30	40	50	51
Fababean	80	100	120	107
Other pulses	40	60	80	41
Groundnut	60	80	100	88
Soybean	60	80	100	176

fixation for the forage and fodder legumes was calculated at 12 Tg annually (average of about 110 kg N/ha/year) (Table 6).

A substantial body of work in Australia and northern Europe shows that forage/fodder legumes have an average Ndfa value of about 70% and 25 kg N is fixed in the shoots for every Mg shoot biomass produced (Peoples and Baldock 2001; Carlsson and Huss-Danell 2003). It should be noted that Peoples and Baldock (2001) reported wide variations for this value, ranging 8–53 kg shoot N fixed/Mg shoot biomass. Such variation would have been caused by differences in soil nitrate levels and pasture vigour, as well as species differences in foliage-N content, experimental treatment and error. Assuming 50% of forage legume nitrogen is below-ground (McNeill et al. 1997; Peoples and Baldock 2001), the overall average for N_2 fixation by forage legumes becomes 50 kg N fixed/Mg shoot biomass.

Smil (1999) estimated global shoot productivity of the forages at 500 Tg from the 100–120 Mha, equivalent to 4.2–5.0 Mg/ha. Global N fixed by the forage and fodder legumes can be calculated by combing the overall annual production of 500 Tg with the rate of N_2 fixation per unit of forage (50 kg N fixed/Mg shoot biomass). Thus, a value of 25 Tg N/annually is obtained, a value about double that of Smil (1999).

The same value of 25 Tg N can be calculated if the following figures and assumptions are used: globally 110 Mha legumes with an average Ndfa of 70%, average shoot DM production of 4.5 Mg/ha, shoot N concentration of 3.6% and below-ground N of 50%. Thus, average annual N_2 fixation is calculated at 227 kg/ha and global N_2 fixation at 25 Tg.

So, what is a realistic figure for N_2 fixation by the forage and fodder legumes in agricultural systems? The Smil (1999) figure of 12 Tg annually may be low because it does not reasonably account for below-ground N, but without reliable data on global forage and fodder legume areas and production statistics for those areas, it is impossible to provide an alternative. The real figure may lie somewhere between 12 and 25 Tg annually (Table 6).

Table 6 Summary of estimates of N fixed annually in agricultural systems by rhizobia in symbiosis with crop, pasture and fodder legumes, numerous genera of bacteria associated with non-leguminous species and free-living bacteria

Agent	Agricultural system	Area[a] (Mha)	Rate of N_2 fixation (kg N/ha/year)	Crop N fixed (Tg/year)	Comments on validity of global N_2 fixation estimates
Legume–rhizobia	Crop (pulse and oilseed) legumes	186	115	21	May be a robust estimate and substantially higher than the Smil (1999) estimate of 10 Tg fixed
Legume–rhizobia	Pasture and fodder legumes	110	110–227	12–25	Difficult to accurately assess because of uncertainty in legume areas and production
Azolla– cyanobacteria, cyanobacteria	Rice	150	33	5	Smil (1999) estimate of 5 Tg N/year reasonable, although primarily based on C_2H_2 reduction technique
Endophytic, associative & free-living bacteria	Sugar cane	20	25	0.5	Large variations in apparent N_2 fixation, using natural ^{15}N abundance, make estimations difficult and speculative
Endophytic, associative & free-living bacteria	Crop lands other than used for legumes and rice	800	<5	<4	N_2 fixation likely to be <5 kg N/ha/year and total of <4 Tg N/year, but not sufficient data to provide more robust values
Endophytic, associative & free-living bacteria	Extensive, tropical savannas primarily used for grazing	1,390	<10	<14	Cleveland et al. (1999) estimate of 42 Tg N/year likely to be high. Not sufficient data to provide more robust values

[a] Data on land areas of the different agricultural systems are for 2005 taken from FAOSTAT, Smil (1999) and Cleveland et al. (1999)

Azolla–cyanobacteria and free-living cyanobacteria in rice paddies

Smil (1999) estimated N_2 fixation by free-living cyanobacteria and cyanobacteria in symbiosis with the water fern *Azolla* at 4–6 Tg annually. Estimates were based on rates of N_2 fixation of 20–30 kg N/ha by cyanobacteria during the growing season and 50–90 kg N/ha by the cyanobacteria–*Azolla* symbiosis. Giller (2001) was more conservative, referring to average rates by free-living cyanobacteria of 12 kg N/ha/cropping season in a study of 190 rice fields in the Philippines and 27 kg N/ha/cropping season in a review of published estimates. Giller (2001), however, cautioned that the vast majority of the estimates were based on acetylene reduction assays and likely to be inaccurate.

Apparent N_2 fixation rates of the cyanobacteria–*Azolla* symbiosis are impressive, e.g. daily accumulation rates of Azolla N of 0.4–3.6 kg N/ha with a mean of 2 kg N/ha and total growing season accumulation of 25–170 kg N/ha (mean of 40 kg N/ha) (Giller 2001). It is probable that N_2 fixation contributes at least 80% of the Azolla N.

It would be reasonable to assume that most of the world's rice paddies contain free-living cyanobacteria, but that the cyanobacteria–*Azolla* symbiosis is present in only about 2% (3 Mha) of the paddies (Giller 2001). Thus, the average estimates of N_2 fixation in rice paddies of about 30 kg N/ha/year and a total of 5 Tg N/year appear reasonable (Table 6).

Endophytic, associative and free-living bacteria in sugar cane systems

Smil (1999) reported that the world's 20 Mha of sugar cane fix, on average, 100 kg N/ha, based principally on research in Brazil (e.g. Boddey et al. 1995). The evidence for substantial inputs of fixed N in Brazilian sugar cane grown in large pots is strong (Lima et al. 1987; Urquiaga et al. 1992) and is supported by the isolation of a large and diverse number of N_2-fixing bacteria from inside and outside of the cane roots (see Boddey et al. 2003). Data on N_2 fixation of field-grown plants using ^{15}N natural abundance, however, is more equivocal (Yoneyama et al. 1997; Biggs et al. 2002, Hoefsloot et al. 2005). In the Yoneyama et al.

(1997) study of 50 Brazilian sugar cane crops, the overall average $\delta^{15}N$ value for the cane was +5.32‰ (range +2.0‰ to +11.0‰), compared with +6.13‰ (range −0.4‰ to +12.9‰) for the reference samples. An aggregated estimate of Ndfa, using those average values, is just 13%.

Boddey et al. (2001) reported a second study to quantify N_2 fixation in 11 commercial crops of sugar cane in Brazil, also using ^{15}N natural abundance. Their data provide a stronger case for consistent and substantial N_2 fixation. They reported an overall average $\delta^{15}N$ value for the cane of +6.38‰ (range +3.3‰ to +13.2‰), compared with +9.10‰ (range +5.4‰ to +26.5‰) for the reference samples. An aggregated estimate of Ndfa, using those average values, is 30%. The authors concluded that N_2 fixation appeared to supply between zero and 60% of the N in the sugar cane crops in the study. They also acknowledged that the complex interactions between plant genotype, the suite of N_2-fixing (and other) bacteria associated with the plant and the environmental and edaphic conditions need to be defined before agronomically-significant inputs of fixed N can be guaranteed.

Given the large variations in apparent N_2 fixation of sugar cane in the field studies in Brazil (Yoneyama et al. 1997; Boddey et al. 2001, 2003), Japan and the Philippines (Yoneyama et al. 1997), Australia (Biggs et al. 2002) and South Africa (Hoefsloot et al. 2005), it is impossible to estimate global N_2 fixation with confidence. The proposition of Smil (1999) that the world's 20 Mha of sugar cane fix, on average, 100 kg N/ha/year is not supported by the literature. It is also unlikely that Brazil's 7 Mha of sugar cane sustain N_2 fixation at such high rates—a more realistic value for Brazil would be 40 kg N/ha, calculated using average Ndfa of 20% and total crop N of 200 kg/ha. Reasonable, but speculative, values for the remaining 14 Mha might be an average of 20 kg N/ha fixed, assuming Ndfa of 10% (Table 6).

Endophytic, associative and free-living bacteria in crop lands not used for legumes and rice

Smil (1999) suggested the plant-associated and free-living bacteria in the 800 Mha of cropping lands used primarily for the cultivation of cereals and oilseeds fixed N at an average, annual rate of 5 kg/ha and a

global, annual rate of 4 Tg N (Table 6). These values are very speculative but, with current knowledge, it is impossible to offer alternatives. A number of reviews of plant-associated N_2 fixation have clearly highlighted the many methodological problems and inconsistencies in the published studies (Boddey 1987; Chalk 1991; Giller 2001; Kennedy and Islam 2001; Giller and Merckx 2003). One of the key problems is distinguishing between inputs of N by free-living and associative agents and other external sources of N contributing to agricultural soils, e.g. N in rainfall and dry deposition. Such inputs can represent 3–50 kg N/ha/year (Goulding et al. 1998; Giller and Merckx 2003; McNeill and Unkovich 2007).

Roper and Ladha (1995) concluded that the free-living, heterotrophic bacteria may fix significant amounts of N in agricultural systems, using crop residues as an energy source. However, they did not speculate as to what the average rate of N_2 fixation might be. More recently, Gupta et al. (2006) suggested N_2 fixation rates of 1–25 kg N/ha/year for dryland cereal systems in southern Australia. Other reviews present similar ranges, or suggest a maximum value that is unlikely to be exceeded. For example, Giller (2001) concluded that N_2 fixation by free-living bacteria would rarely exceed 5 kg N/ha/year. However, none of these publications offer a rate figure that could be applied globally, although Kennedy and Islam (2001) were optimistic that perceived problems in manipulating the associative systems to the point that they could be utilised more effectively would be overcome with additional research.

Endophytic, associative and free-living bacteria in extensive, tropical savannas primarily used for grazing

Cleveland et al. (1999) estimated that the 1,390 Mha of tropical savannas fixed, on average, 30 kg N/ha/year for a total of 42 Tg annually. Using just seven sets of data, the authors calculated legume (symbiotic) N_2 fixation in the range 3–90 kg N/ha/year and free-living (non symbiotic) N_2 fixation at 3–30 kg N/ha/year. All N_2 fixation estimates appear to be based on short-term C_2H_2 reduction assays and were scaled up substantially from the unit measurements, sometimes against the advice of the original authors (e.g. Stewart et al. 1978). Thus, the estimates of 30 kg N fixed/ha/year and 42 Tg

fixed annually are questionable and are likely to be far too high (Table 6).

The savannas do produce substantial quantities of C-rich plant residues that are a potential energy source for N_2-fixing bacteria. As well, a large proportion of the savannas are now used for grazing and, in countries like Brazil, Venezuela and Colombia, have been oversown with improved species of grasses, such as *Brachiaria* spp., *Panicum maximum,* and *Andropogon gayanus.* There may be about 200 Mha tropical savannas that contain improved grass species (RM Boddey, personal communication). Reis et al. (2001), using natural ^{15}N abundance, reported Ndfa values of 25–40% for genotypes of *P. purpureum* and 2–26% for five species of *Brachiaria,* and N_2 fixation values >100 kg N/ha. Although these data suggest a large potential for N_2 fixation by bacteria associated with some of the tropical grasses, there are still questions as to whether the apparent ^{15}N isotope dilution is due to N_2 fixation, or to other effects, or to a combination of both. Thus, the occurrence and intensity of N_2 fixation in this system by the cyanobacteria, endophytic and associative bacteria and heterotrophic free-living bacteria are essentially unknown. A notional rate of <10 kg N/ha/year would seem reasonable (Table 6).

Conclusions

The major inputs of N into terrestrial ecosystems are through the biological and industrial fixation of atmospheric N_2 to ammonia (NH_3), with more modest inputs via wet (rain) and dry (dust) deposition of particulate N, NH_3, NH_4^+ and nitrate (McNeill and Unkovich 2007). Others (e.g. Vitousek et al. 1997) have argued that improving the efficiency with which fertiliser N, the major product of industrial N_2 fixation, is used in world agriculture is vital to the long-term sustainability of the planet. That would appear to be a reasonable goal, given the often low efficiency of fertiliser-N use with gaseous losses contributing to global warming, and leaching and erosion losses to the degradation of water courses and storages (Vitousek et al. 1997; McNeill and Unkovich 2007). An equally important goal could be the more effective exploitation and utilisation of biologically-fixed N in agricultural systems. It would, at the least, compliment fertiliser-N use and may ease the long-term pressure for expanded

production. It is also possible that particular systems within the global food production framework could become more reliant on biologically fixed N, rather than fertiliser N, for N inputs.

In this context, soybean cropping in Brazil and Argentina provide impressive case histories. Soybean areas in the two countries essentially doubled during the 5 years between 2000 and 2005 to 37 million ha and, with that expansion, N_2 fixation inputs increased to 8 Tg annually (Table 3). On the other hand, the use of fertiliser N in the agricultural systems of Brazil and Argentina is relatively low with inputs of little more than 2 Tg annually (FAOSTAT). Clearly, a number of factors contributed to the situation in South America, including positive market signals, the availability for redevelopment of large tracts of land for this purpose, appropriate soils and climate etc. The expansion of soybean cropping was also underpinned by effective breeding, agronomy research and extension (Alves 2003; Hungria et al. 2005). A major contributing factor, however, was an understanding of the role that N_2 fixation could play in underpinning legume productivity and how highly efficient rhizobia–soybean symbioses might be achieved in commercial practice (e.g. Dobereiner et al. 1978; Hungria et al. 2005). Reproducing the success of the South American soybean for the other agricultural systems covered in this review requires a substantial investment in fundamental research to optimise the various N_2-fixing systems and have them applied. The levels of N_2 fixation activity of those systems also need to be quantified using appropriate methodologies, otherwise management becomes essentially impossible.

In this review, we calculated N_2 fixation by the crop legume–rhizobia symbioses with some degree of confidence (21 Tg N annually) and by the forage and fodder legume–rhizobia symbioses with less confidence (12–25 Tg annually) (Table 6). These estimates are higher than those published previously, partly because of the recent expansion of highly-productive soybean cropping in South America, but also because we accounted for below-ground fixed N. The estimated 5 Tg N fixed annually in rice paddies and 0.5 Tg N fixed by bacteria associated with sugar cane may also be reasonable. Amounts of N_2 fixed by the other symbiotic, associative and free-living bacteria in the cereal, oilseed and other crop lands and extensive tropical savannas used for grazing are very difficult, if not impossible, to estimate. It is suggested that

together these systems fix <18 Tg N annually, but that is speculative. Taken together, 50–70 Tg N may be fixed annually by biological agents in agricultural systems. The uncertainty of this range would be reduced with the publication of more accurate statistics on areas and productivity of forage and fodder legumes and the publication of many more estimates of N_2 fixation, particularly in the cereal, oilseed and non-legume crop lands and extensive tropical savannas used for grazing.

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Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs

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Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs

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ABSTRACT: Agriculturally important grasses such as sugar cane (*Saccharum* sp.), rice (*Oryza sativa*), wheat (*Triticum aestivum*) sorghum (*Sorghum bicolor*), maize (*Zea mays*), *Panicum maximum*, *Brachiaria* spp., and *Pennisetum purpureum* contain numerous diazotrophic bacteria, such as, *Acetobacter diazotrophicus*, *Herbaspirillum* spp., *Azospirillum* spp. These bacteria do not usually cause disease symptoms in the plants with which they are associated and the more numerous of them, for example, *Herbaspirillum* spp. and *A. diazotrophicus*, are obligate or facultative endophytes that do not survive well (or at all) in native soil; these are thought to be spread from plant generation to plant generation via seeds, vegetative propagation, dead plant material, and possibly by insect sap feeders. By contrast, *Azospirillum* spp. are not wholly endophytic but are root-associated, soil-dwelling bacteria that are also often found within plants, probably entering host plants via seeds or via wounds/cracks at lateral root junctions. Endophytic diazotrophs have been isolated from a number of grasses in which significant biological N_2 fixation (BNF) has been demonstrated, particularly Brazilian sugar cane varieties, but also in rice, maize, and sorghum. However, although the endophytic diazotrophs are held to be the causative agents of the observed BNF, direct evidence for this is lacking. Therefore, in this review we examine probable sites of bacterial multiplication and/or BNF within endophyte-containing grasses and discuss these in terms of potential benefits (or not) to both host plants and bacteria. In particular, we examine how potentially large numbers of bacteria, especially *Herbaspirillum* spp., *A. diazotrophicus*, and *Azospirillum* spp., can exist extracellularly within non-specialized (for symbiotic purposes) regions such as xylem vessels and intercellular spaces. The processes of infection and colonization of various grasses (particularly sugar cane) by diazotrophic endophytes are also described, and these are compared with those of important (nondiazotrophic) endophytic sugar cane pathogens such as *Clavibacter xyli* subsp. *xyli* and *Xanthomonas albilineans*.

KEY WORDS: *Acetobacter diazotrophicus*, *Azospirillum* spp., biological N_2 fixation, endophytic diazotrophs, gramineae, *Herbaspirillum* spp., infection, sugar cane.

I. INTRODUCTION

It has long been a goal of many biological N_2 fixation (BNF) researchers to transfer the ability to fix N_2 into crops that are not normally regarded as having this ability (Kennedy and Tchan, 1992; Spencer et al., 1994; de Bruijn et al., 1995; Triplett, 1996). In particular, attention has focussed on grasses such as rice, wheat, maize, sorghum, and sugar cane, which currently have much of their N needs supplied by costly mineral fertilizers (Döbereiner et al., 1995a; Triplett, 1996). Recent attempts have involved the induction of paranodules on the roots and the subsequent colonization of the paranodules and/or roots by rhizobia, usually tropical in origin (Cocking et al., 1995), or other rhizosphere diazotrophs, such as, *Azospirillum brasilense* (Kennedy and Tchan, 1992; Christiansen-Weniger and Vanderleyden, 1994). For further details of paranodules see the review by Christiansen-Weniger in this issue. However, there has been relatively little practical success with this approach as the techniques involved (application of auxins, cell wall-degrading enzymes and/or genetical modification of the bacteria) are not readily applied to the field (de Bruijn et al., 1995). An alternative, and possibly more practical, approach is to exploit naturally occurring associations between (mainly tropical) grasses and diazotrophic endophytes.

Although endophytic bacteria have been widely reported within grasses (Kloepper et al., 1992; Kloepper and Beauchamp, 1992; Fisher et al., 1992; McInroy and Kloepper, 1995; Chanway, 1995), diazotrophic endophytes such as *Acetobacter diazotrophicus* and *Herbaspirillum* spp. are a relatively recent discovery and have so far been found only within a small range of tropical plants (Baldani et al., 1986a, 1996; Cavalcante and Döbereiner, 1988; Gillis et al., 1989; Reis et al., 1994). Indeed, *A. diazotrophicus* and *Herbaspirillum seropedicae* can only be isolated from the interior of various plants, particular-

ly grasses, and they do not survive long in the soil without the presence of host plants (Baldani et al., 1992; Reis et al., 1994; Olivares et al., 1996). Endophytic diazotrophs have been linked with the high N_2 fixation reported particularly in sugar cane where the bacteria are found in high numbers (Boddey et al., 1991, 1995a,b; Döbereiner et al., 1995a,b). Endophytic diazotrophs may have an advantage over root-associated diazotrophs, such as *Azospirillum* and *Azotobacter*, in that they colonize the interior rather than the surface of the plants, and hence are better placed to exploit carbon substrates supplied by the plant (Döbereiner et al., 1995b; McInroy and Kloepper, 1995; Boddey et al., 1995a,b; Sprent and James, 1995; Triplett, 1996). Moreover, as they are often located within underground roots and/or dense plant tissue, for example, stem nodes and xylem vessels, the bacteria are likely to be growing within a low pO_2 environment, which is necessary for the expression and operation of nitrogenase (Patriquin et al., 1983; Gallon, 1992; Baldani et al., 1997). However, as yet there has been no evidence linking diazotrophic endophytes *directly* with BNF in grasses. The aims of this review are to examine the processes by which these bacteria can infect and move within their host plants and then to assess the putative locations of diazotrophic endophytic bacteria with respect to the possibility that they are both fixing N_2 and transferring the fixed N products to the plant. We shall be concentrating mainly on *Herbaspirillum* spp. and *A. diazotrophicus*, as these are the diazotrophs that have been found in greatest numbers within sugar cane (Boddey, 1995; Boddey et al., 1995a,b). Other endophytic bacteria found within grasses, including common sugar cane pathogens, are discussed, but only when it is appropriate in the context of this review. For example, we do not discuss *Azoarcus* in any detail as it is being covered in another article in this issue (Reinhold-Hurek), and only *endophytic* examples of rhizosphere diazotrophs, such

as, *Azospirillum* and Klebsiella spp., are described.

II. DEFINITION OF "ENDOPHYTIC" BACTERIA

In this review we describe the known endophytic diazotrophs that colonize the internal tissues of sugar cane and other grasses. However, before we discuss the diazotrophic bacteria within these plants, it is necessary to put them into context with respect to the numerous other bacteria that inhabit sugar cane, and to also define what we mean by the term "endophytic". We have adopted the definition of "endophyte" given recently by Kloepper et al. (1992), who, on the basis that it is semantically incorrect and confusing, proposed to eliminate the term "endorhizosphere" when applied to bacteria living *within* plant tissues. Kloepper et al. (1992) suggested simply calling bacteria within tissues internal to the epidermis "endophytes" but, at the same time, also to describe their exact location within the plant. For example, in the case of roots, to describe whether the endophytes are found within the cortex, stele, or xylem. Moreover, although both fungal and bacterial endophytes have often been regarded by some authors as being exclusively *non*-pathogenic, that is, causing no disease symptoms on the plants that they infect (Misaghi and Donndelinger, 1990; Frommel et al., 1991; Fisher et al., 1992; Chanway, 1995), Kloepper et al. (1992) made no distinction between "pathogenic" and "non-pathogenic" endophytic bacteria. Therefore, with respect to this point, we have again adopted the definition of Kloepper et al. (1992) and have included in our review *all* bacteria that colonize the interior of plants, including active and latent pathogens. This is justified, as many of the bacteria found within apparently "symptomless" plants are known to be pathogenic in other locations (Cho et al., 1980;

Patriquin et al., 1983; Binns and Thomashow, 1988; Misaghi and Donndelinger, 1990; Fisher et al., 1992; Kloepper et al., 1992; Barbehenn and Purcell, 1993; McInroy and Kloepper, 1995; Purcell and Hopkins, 1996), including some diazotrophs, for example, "*Pseudomonas*" *rubrisubalbicans* (Hale and Wilkie, 1972a,b; Pimentel et al., 1991), *Erwinia* spp. (Fisher et al., 1992; Boddey et al., 1995a), *Agrobacterium* (Binns and Thomashow, 1988; McInroy and Kloepper, 1995), and even *Rhizobium* (Yang et al., 1992; Vasse et al., 1993; Perotto et al., 1994; Mellor and Collinge, 1995). Indeed, it is considered by some researchers that there is a fine dividing line between "symptomless" endophytes, pathogens, and symbionts (Patriquin et al., 1983; Djordjevic et al., 1987; Misaghi and Donndelinger, 1990; Kloepper et al., 1992; Sprent and James, 1995), with "quiescent" endophytic bacteria becoming "pathogenic" under certain conditions and/or within different host genotypes (Misaghi and Donndelinger, 1990). In sugar cane, as we will see later in the review, this latter point is particularly well illustrated by the important endophytic diazotroph, *Herbaspirillum* (Pimentel et al., 1991; Olivares et al., 1996, 1997 and see Section IV).

Recently, at least with respect to *diazorophic* endophytic bacteria, it has been proposed to divide them into two groups: *facultaive* and *obligate* (Baldani et al., 1997). Facultative endophytes are described as those bacteria that survive in the soil and/or on plant surfaces as well as being able to colonize the interior of some plants. Indeed, most endophytic *Azospirillum* strains are regarded as being facultative endophytes (Baldani et al., 1997). However, most of the bacteria that we describe in this review (i.e., *Herbaspirillum* spp., *A. diazotrophicus*, *Burkholderia* spp.) are "obligate" endophytes, as they survive poorly in the soil and appear to have a requirement for living within a host plant (Baldani et al., 1997). Nevertheless, during the course of the review it will become ap-

parent that such distinctions between facultative and obligate endophytes can become blurred; for example, *H. rubrisubalbicans* will clearly live for some time on leaf surfaces (Olivares et al., 1997).

III. NON-DIAZOTROPHIC BACTERIA WITHIN SUGAR CANE AND OTHER GRAMINACEOUS PLANTS

As well as the diazotrophs discussed in subsequent sections, sugar cane can contain many other N_2-fixing bacteria, such as species of *Bacillus*, *Beijerinckia*, *Azotobacter*, *Derxia*, *Enterobacter*, *Erwinia*, and *Klebsiella* (Graciolli and Ruschel, 1981; Rennie et al., 1982; Patriquin et al., 1983; Kennedy and Tchan, 1992; Boddey et al., 1995a), although none of these bacteria are considered to be present in sufficient numbers to be of benefit (or likely to be pathogenic) to the host plant. Moreover, as sugar cane is such a large plant, it is inevitable that, in addition to diazotrophs, it contains many other endophytes, some of which are potential pathogens. The most important of these are the xylem-dwelling bacteria, *Clavibacter xyli* subsp. *xyli* and *Xanthomonas albilineans*, the causes of ratoon stunting disease (RSD) and leaf scald disease, respectively.

C. xyli subsp. *xyli*, in common with its relative *C. xyli* subsp. *cynodontis*, is a coryneform, xylem-limited bacterium (XLB) that causes no external symptoms on the plants (mainly sugar cane) that it infects, other than stunted growth (Harrison and Davis, 1988; Gillaspie and Teakle, 1989; James, 1996). Indeed, under some circumstances, *C. xyli* may not cause any obvious harm (Barbehenn and Purcell, 1993; James, 1996; Purcell and Hopkins, 1996). *C. xyli* is regarded as an XLB, as it appears to be fastidious in its location within vascular tissue (Brlansky et al., 1982; Barbehenn and Purcell, 1993; Purcell and Hopkins, 1995; James, 1996), although Kao and Damann (1980) have reported that it will escape into the intercellular apoplast adja-cent to infected vessels. No commercial sugar cane cultivar is immune to infection with this bacterium (Harrison and Davis, 1988; Gillaspie and Teakle, 1989; James, 1996), and the Brazilian sugar cane collective, Copersucar, consider that all Brazilian cultivars are infected (J. I. Baldani, personal communication), although some cultivars are more resistant than others (Teakle et al., 1977; Harrison and Davis, 1988; Gillaspie and Teakle, 1989; Comstock et al., 1996; James, 1996). Resistance to RSD appears to be linked to the vascular anatomy of sugar cane stalks, that is, those cultivars that have profuse branching of vessels, and fewer vessels that pass directly through nodes, can restrict (but not prevent) intraxylar spread of the bacteria (Teakle et al., 1977; Harrison and Davis, 1988; Gillaspie and Teakle, 1989).

C. xyli subsp. *xyli* is commonly spread via vegetative propagation of sugar cane from infected seed pieces or "setts", and a frequent method of control is to heat the setts to 50°C for 2 to 3 h to kill the bacteria (Gillaspie and Teakle, 1989; Reis et al., 1994; James, 1996; Comstock et al., 1996). It is worth noting at this point that subsequent colonization of sugar cane plants by the endophytic diazotroph, *A. diazotrophicus*, which is also found within setts (see later), is unaffected by the heat treatment (Reis et al., 1994). Heat treating of setts is not an entirely effective method of controlling the transmission of *C. xyli* subsp. *xyli* as the bacteria can also infect the plants from contaminated soil and from cutting implements that have been contaminated by infected xylem sap (Gillaspie and Teakle, 1989; Barbehenn and Purcell, 1993; Comstock et al., 1996; James, 1996). Interestingly, even though *C. xyli* is readily isolated from sugar cane xylem sap (Harrison and Davis, 1988; Gillaspie and Teakle, 1989; Reis et al., 1994), unlike some other XLBs, the bacteria are not transmitted via xylem-sucking insects such as the "sharpshooter" leafhoppers (Purcell, 1989; Barbehenn and Purcell, 1993; Purcell and Hopkins, 1995), even though the latter can rapidly accumulate

vast numbers (> 300,000 cultivable cells per insect; Purcell, personal communication).

X. albilineans is a xylem-invading bacterium that causes symptoms on sugar cane leaves and stalks varying from a thin white streak to death of the entire plant (Ricaud and Ryan, 1989; Comstock, 1992; Rott et al., 1995). Like *C. xyli* subsp. *xyli*, it is transmitted via infected setts, and via mechanical cutting implements that have been contaminated by infected xylem sap (Ricaud and Ryan, 1989; Comstock, 1992; Rott et al., 1995). It is also transmitted aerially (Comstock, 1992), and possibly via leafhoppers and other insects (Ricaud and Ryan, 1989). Unlike *C. xyli* subsp. *xyli*, which is found mainly in the basal nodes (Harrison and Davis, 1988), *X. albilineans* is readily transmitted in the transpiration stream to the nodes further up the stalk (Comstock, 1992), suggesting that convolutions in vascular tissue in the stem nodes affect movement of this bacterium less than that of *C. xyli* subsp. *xyli* (Teakle et al., 1977; Harrison and Davis, 1988). Other xylem-dwelling *Xanthomonas* and *Pseudomonas* species also cause important diseases of sugar cane, such as, *X. campestris* pv. *vasculorum*, which causes "gumming disease" of the xylem (Ricaud and Autrey, 1989; Quobela and Claflin, 1992), and various *Pseudomonas* spp. which cause "red stripe" and "mottled stripe" diseases on sugar cane sorghum and maize (Hale and Wilkie, 1972a; Martin and Wismer, 1989). Indeed, in the next section on endophytic diazotrophs we shall be going into detail with respect to one of the latter species *P. rubrisubalbicans*, the causative agent of mottled stripe disease on sugar cane and a red stripe disease on sorghum (Hale and Wilkie, 1972b; Martin and Wismer, 1989; Pimentel et al., 1991). This species has now been shown to be an important endophytic diazotroph and has been reclassified as *Herbaspirillum rubrisubalbicans* (Baldani et al., 1996).

As the main endophytic pathogens of sugar cane and other grasses are xylem dwelling, if not actual XLBs (Purcell and Hopkins, 1995),

it is worth examining how they exist within the xylem. This is also relevant to the (usually) non-pathogenic endophytes of grasses, as many of these are also, but not exclusively, xylem dwellers, as we will detail later. Djordjevic et al. (1987) have suggested that xylem-inhabiting pathogens, for example, *Pseudomonas solanacearum* and *Erwinia amylovora*, are "advanced" as, in compatible interactions, they can colonize the entire host and multiply for a long time before they cause disease (Djordjevic et al., 1987). The disease is usually caused by the pathogen occluding vascular tissue with the sheer number of its cells and/or with enhanced production of released exopolysaccharide (EPS) (Gross and Cody, 1985; Bretschneider et al., 1989; Leigh and Coplin, 1992; Vasse et al., 1995). However, these bacteria tend to be recognized by the plant in early stages of infection and consequently elicit a generalized host defense response (Djordjevic et al., 1987). This response, which is not always successful, usually takes the form of the production of phenolic-containing gels and gums that surround and attack the bacteria (Wallis, 1977; VanderMolen et al., 1977; Kao and Damann, 1980; Bretschneider et al., 1989; Boher et al., 1995; Purcell and Hopkins, 1996). Examples of more highly advanced pathogens are *Pseudomonas syringae*, *X. campestris* pvs, and *Clavibacter* (see above) (Djordjevic et al., 1987). These bacteria are often asymptomatic for long periods; for example, *C. xyli* subsp. *xyli* (see above) tend to have very intimate contact with host cell walls and membranes, are highly host-specific, and are not readily recognized by the host (Djordjevic et al., 1987). Djordjevic et al. (1987) have suggested that, over evolutionary time, these bacteria may be approaching "full compatibility" with their hosts, that is, causing less and less damage. Indeed, advanced pathogens may be the precursors of symptomless endophytes and symbionts. For example, some "non-pathogenic", xylem-inhabiting endophytes, such as *H. rubrisubalbicans,* can also cause a host defense reac-

tion, depending on the bacterial species and the host genotype (Pimentel et al., 1991; Olivares et al., 1997; James et al., 1997, and see later). In general though, most symptomless bacterial endophytes of grasses appear not to accumulate within xylem vessels in sufficient numbers to elicit a host defense reaction, for example, *H. seropedicae* (James et al., 1997), *A. diazotrophicus* (James et al., 1994), and *A. brasilense* (Schloter et al., 1994).

Two factors that appear to be at odds with the colonization of xylem vessels are the low nutrient availability, particularly carbohydrates, and the theoretical possibility of cavitation of the infected vessels that are under tension within the transpiration stream (Raven, 1983; Purcell and Hopkins, 1996). In the latter case, although XLBs may eventually cause embolisms within infected vessels, they appear not to cause cavitation, even with very high bacterial populations (Purcell and Hopkins, 1996). Moreover, although the exact mechanisms have not been elucidated, it is also likely that XLBs can degrade pit membranes and move from vessel to vessel without necessarily causing embolisms or affecting transpiration rates (Kao and Damann, 1980; Purcell and Hopkins, 1996). Regarding the nutritive requirements of xylem-dwelling bacteria (i.e., those that are non-pathogenic or latently pathogenic), although xylem sap contains very low levels of organic compounds (relative to other plant tissues, for example, phloem sap: Hawker, 1965; Bull et al., 1972; Raven, 1983; Purcell, 1989; Welbaum et al. 1992; Purcell and Hopkins, 1996), it can contain relatively high concentrations (as a fraction of total organic compounds) of amino acids and amides, and its composition varies considerably (Raven, 1983; Purcell and Hopkins, 1996), with concentrations of most constituents being much higher at night (Raven, 1983). XLBs actually appear to be well adapted to the relatively low nutrient availability of the xylem. For example, it has been suggested (but not yet demonstrated) by some authors that XLBs may have "nutrient concentrating mechanisms", such as a polysaccharide "glycocalyx" that attaches the bacteria to vessel walls and that may absorb and concentrate ions within the sap (Purcell and Hopkins, 1996, and see references therein). The nutritive requirements of specific bacteria are dealt with in subsequent sections.

IV. DIAZOTROPHIC BACTERIA WITHIN SUGAR CANE AND OTHER GRAMINACEOUS PLANTS

A. *Herbaspirillum* spp.

Herbaspirillum seropedicae was originally isolated in Brazil from rhizosphere soil, washed roots and surface sterilized roots of maize, sorghum and rice by Baldani et al. (1986a), but could not be isolated from uncropped soil (Baldani et al., 1992). The bacteria are Gram-negative, curved rods with polar flagella and grow best on dicarboxylic acids, gluconate, glucose, and mannitol, fixing N_2 at a pH range of 5.3 to 8 (Baldani et al., 1986a, 1992; Ureta et al., 1995). They will tolerate, and fix N_2, in very high sucrose concentrations (up to 10%), even though they cannot metabolize this substrate (Baldani et al., 1992; Ureta et al., 1995). Indeed, unlike *A. diazotrophicus*, which can utilize sucrose for growth (see later), it is not clear at present what substrate(s) *Herbaspirillum* spp. exists on when endophytic within sugar cane (and other plants), where sucrose is likely to be the main C compound available to them. However, Boddey et al. (1995b) have suggested that fermentative organic acid production by *A. diazotrophicus* is a possible carbon and energy source for *Herbaspirillum* spp., and possibly *Azospirillum* spp. (Tarrand et al., 1978). *Herbaspirillum* spp. will grow and fix N_2 under relatively high pO_2s (3%) compared with *Azospirillum* spp. (2%) (Baldani et al., 1986a; Fu and Burris, 1989), although Vande Broek

et al. (1996), on the basis of *NifH* gene expression and acetylene reduction assay (ARA), recently classed *H. seropedicae* alongside *A. diazotrophicus* and *Azospirillum* spp. as being relatively non-tolerant to O_2. Unlike *A. diazotrophicus* (Gillis et al., 1989), *H. seropedicae* expresses nitrate reductase (Baldani et al., 1986a) and is able to grow, but not fix N_2, in the presence of fixed N (yeast extract, NO_3^-) (Baldani et al., 1986a, 1992), although nitrogenase activity is only partially inhibited by up to 20 mM ammonium (Fu and Burris, 1989).

H. seropedicae was originally thought to be a new *Azospirillum* species because of similar growth characteristics in the semi-solid, N-free, malate NFb medium devised for isolation of *Azospirillum* spp. (Tarrand et al., 1978), that is, growth and N_2 fixation with formation of a fine white pellicle beneath the surface (Baldani et al., 1986a, 1992). However, further analyses showed that it was in a completely new genus, *Herbaspirillum*, consisting (at that time) of just one species (Baldani et al., 1986). The similarity of *Herbaspirillum* and *Azospirillum* made further isolation and work on the former somewhat difficult, and therefore Baldani et al. (1992) devised a new semi-solid malate medium (JNFb medium) to more easily distinguish *Herbaspirillum* from *Azospirillum* spp. JNFb medium differs from NFb medium in having a lower pH (5.8), no vitamins, and a higher (threefold greater) phosphate concentration (Baldani et al., 1992). When the bacteria from JNFb medium are streaked onto NFb agar plates containing bromothymol blue and 50 mg/l of yeast extract, within 1 week they form characteristic (of *Herbaspirillum* spp.) smooth, white colonies with blue or green centers (Baldani et al., 1992, 1996; Olivares et al., 1996).

Using JNFb medium, Baldani et al. (1992) isolated *H. seropedicae* from washed and/or surface sterilized roots, and/or the rhizosphere, of various grasses, for example, Napier grass (*Pennisetum purpureum*), *Digitaria decumbens*, *Brachiaria decumbens*,

Melinus minutiflora, as well as stems and leaves of sugar cane. Until now, the bacterium has been reported in 13 members of the Gramineae, particularly within roots (Baldani et al., 1996; Olivares et al., 1996). Olivares et al. (1996) have also isolated *H. seropedicae* from stems and leaves of rice and maize, as well as from the stems of various varieties of sugar cane. However, in the case of sugar cane, contrary to the results of Baldani et al. (1992), Olivares et al. (1996) could not isolate *H. seropedicae* from the leaves, and Olivares et al. (1997) subsequently have shown that artificial inoculation of sugar cane leaves by *H. seropedicae* elicits a hypersensitive response (HR), suggesting that sugar cane leaves are not a compatible location for the bacteria. Recently, Olivares et al. (1996) have also confirmed the endophytic nature of *H. seropedicae*, which was originally suggested by Baldani et al. (1992). In a series of experiments, Olivares et al. (1996) inoculated soil from sugar cane fields with *Herbaspirillum* spp. and failed to reisolate the bacteria from the soil 30 d after inoculation. Interestingly, the bacteria could be isolated from sorghum plants that were planted into the soil 76 d after it was inoculated. Hence, Olivares et al. (1996) suggested that small numbers of the bacteria could remain viable, but unculturable, for prolonged periods within native soil, but also suggested that previous isolations from the rhizosphere of grasses (Baldani et al., 1986a, 1992) were likely to be due to pieces of host roots/root hairs in the soil examined.

In 1990, Gillis et al. reported that *H. seropedicae* was very closely related by phenotypical and genotypical characteristics to a mild pathogen of sugar cane and sorghum called "*Pseudomonas*" *rubrisubalbicans* (Hale and Wilkie, 1972a,b; Martin and Wismer, 1989; Pimentel et al., 1991; and see previous section), which also fixes N_2 (Pimentel et al., 1991; Baldani et al., 1992). After further analyses, "*Pseudomonas*" *rubrisubalbicans* has now been renamed *Herbaspirillum rubrisubalbi-*

cans on the basis of DNA–rRNA and DNA–DNA hybridizations (Baldani et al., 1996). Indeed, the genus *Herbaspirillum* now contains three species: *H. seropedicae*, *H. rubrisubalbicans*, and *Herbaspirillum* "species 3". However, unlike the other two species, "species 3" is non-diazotrophic and is mainly isolated from clinical material, such as wounds and feces, although a few strains have been isolated from sugar cane, sorghum, and maize (Baldani et al., 1996). As this latter species is non-diazotrophic, not commonly found in plants, and little is known about it, we will not dwell on it further. *H. rubrisubalbicans* was recently proven to be able to incorporate ^{15}N from labeled N_2 gas (Baldani et al., 1992) and is only the second confirmed diazotrophic plant pathogen, the first being *Agrobacterium tumefaciens* (Kanvinde and Sastry, 1990). *H. rubrisubalbicans* has physiological characteristics very similar to *H. seropedicae* and they differ only in the utilization (as sole carbon source) of *meso*-erythritol by *H. rubrisubalbicans* and *N*-acetylglucosamine by *H. seropedicae*, and by optimum growth temperatures (30°C, *H. rubrisubalbicans*; 34°C, *H. seropedicae*) (Baldani et al., 1996). They can also be distinguished using oligonucleotide probes (Baldani et al., 1996), which have been developed recently by Hartmann et al. (1995).

H. rubrisubalbicans also differs from *H. seropedicae* in the range of plants from which it has been isolated (Olivares et al., 1996; Baldani et al., 1996), as well as in its greater virulence as a plant pathogen (Pimentel et al., 1991; Olivares et al., 1997; James et al., 1997). So far, *H. rubrisubalbicans* (syn. *P. rubrisubalbicans*) has been isolated only from sugar cane (Pimentel et al., 1991; Olivares et al., 1996), sorghum (Hale and Wilkie, 1972b), rice, palm trees (Baldani et al., 1997), and the C4 grass *Miscanthus* (Eckert et al., 1997), but also infects Napier grass after artificial inoculation (Pimentel et al., 1991; Baldani, 1996). Both diazotrophic *Herbaspirillum* species will cause red stripe disease symptoms on leaves of some cultivars of sorghum, although in the case of *H. seropedicae* these symptoms are extremely mild (Pimentel et al., 1991; James et al., 1997). Only *H. rubrisubalbicans* will cause mottled stripe disease on sugar cane leaves (Pimentel et al., 1991; Olivares et al., 1997), although all Brazilian sugar cane cultivars are mottled stripe disease resistant, even though their symptomless leaves may still contain large numbers of the bacteria (Pimentel et al., 1991; Baldani et al., 1996; Olivares et al., 1996). Interestingly, it is mainly those varieties that are used in regions where high mineral N applications are grown that are disease susceptible (Baldani et al. 1996).

Using light and electron microscopy coupled with immunogold labeling, Olivares et al. (1997) compared a mottled stripe-disease susceptible variety of sugar cane (cv. B-4362 from Barbados) with one that is resistant (cv. SP 70-1143 from Brazil). Twenty days after inoculation, the leaves of cv. B-4362 exhibited classic mottled stripe symptoms, and these symptoms corresponded with the bacteria massively colonizing the xylem, intercellular spaces, and substomatal cavities (Figures 1 through 6), with some bacteria also being observed on the leaf surfaces. By contrast, cv. SP 70-1143 exhibited no symptoms except for some very small stripes down the leaf veins. These symptoms corresponded to colonization of the vascular tissue, where the bacteria were restricted to microcolonies encapsulated within polymeric material (Figure 3); the bacteria were not able to colonize the intercellular apoplast without provoking a host defense reaction. Interestingly, *H. rubrisubalbicans* seems to prefer the leaves of sugar cane, even though it will also colonize the stems and roots (Olivares et al., 1996). In contrast to *H. rubrisubalbicans*, *H. seropedicae* does not colonize the leaves of sugar cane (see above and Olivares et al., 1997), being found only in the roots and stems (Olivares et al., 1996), but it will colonize sorghum leaves
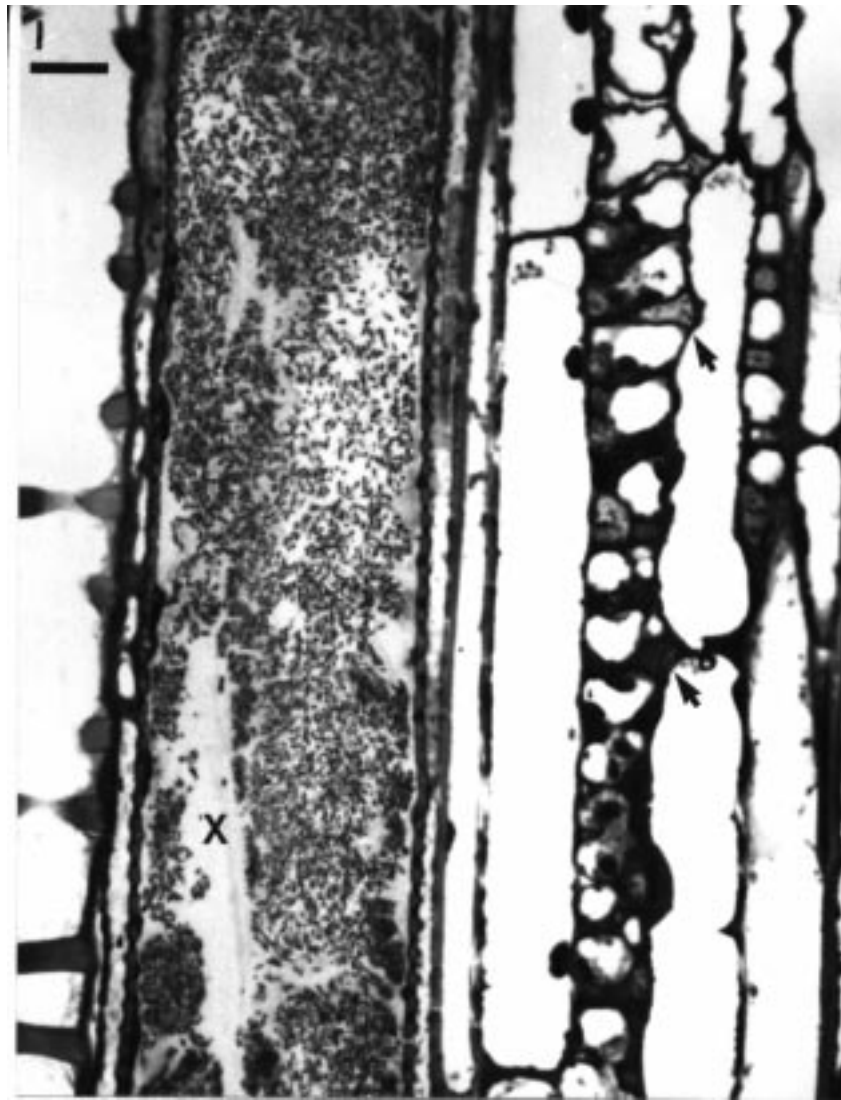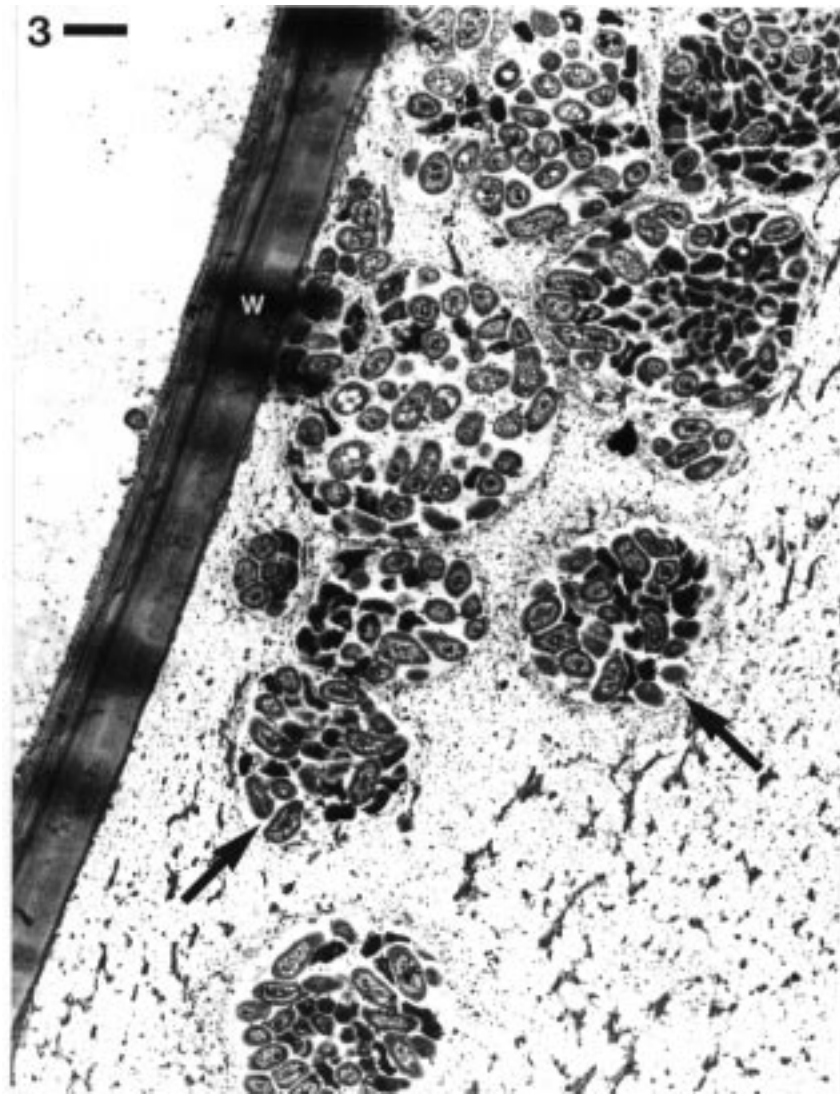
FIGURE 1. Light micrograph of a longitudinal section (LS) of a xylem vessel from the leaf of the mottled stripe disease-susceptible sugar cane cv. B–4362 20 d after inoculation (dai) with *Herbaspirillum rubrisubalbicans*. The vessel (X) and the adjacent intercellular apoplast (arrows) are both heavily colonized by the bacteria. (Bar = 10 μm.)

(James et al., 1997). In the case of sorghum leaves, James et al. (1997) have shown that both *H. seropedicae* and *H. rubrisubalbicans* are restricted to colonizing the xylem (Figures 7 and 8) and, unlike sugar cane infected with *H. rubrisubalbicans*, do not escape into the intercellular apoplast. Indeed, in sorghum both species could be regarded as XLBs (Purcell and Hopkins, 1996, and see earlier). This pattern of growth explains the "red stripe" symptoms in this species, as opposed to the "mottled stripe" on sugar cane leaves (Olivares et al., 1997). Both bacterial species form microcolonies in sorghum xylem, although, in contrast to *H. rubrisubalbicans*, *H. seropedicae* tends to be localized adjacent to the walls
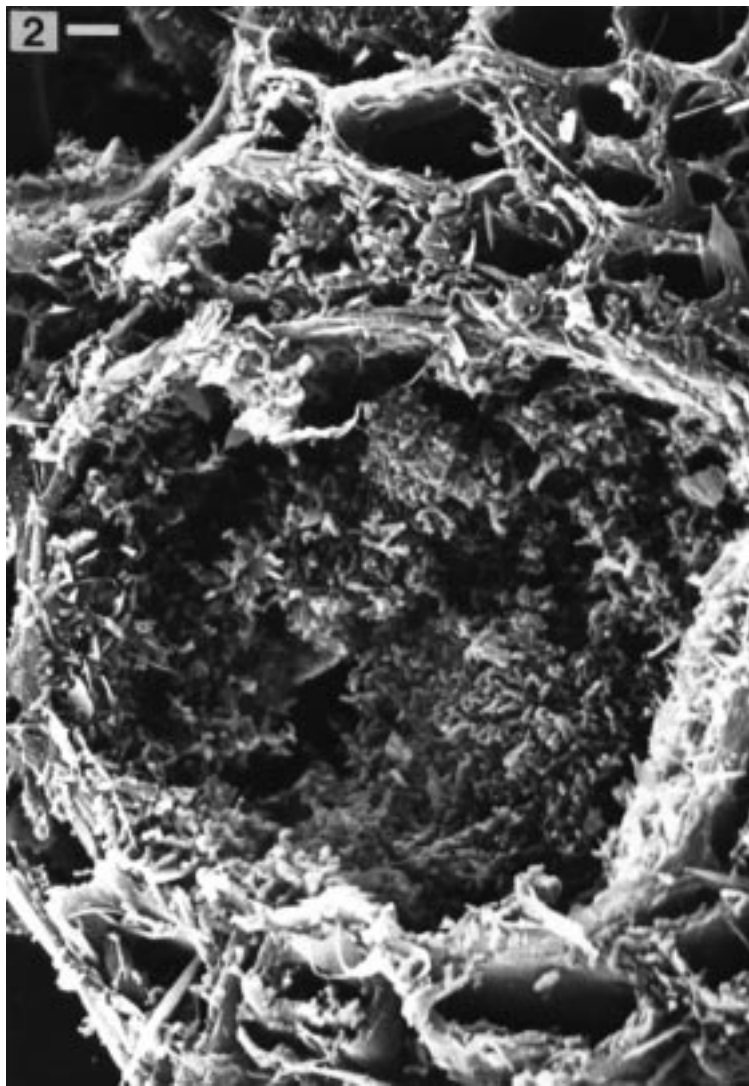
FIGURE 2. Scanning electron micrograph (SEM) of a transverse section (TS) of a xylem vessel from the leaf of sugar cane cv. B–4362 infected with *H. rubrisubalbicans*. 20 dai. (Bar = 1 μm.)

of the proto- and metaxylem rather than actually filling the lumens of these vessels. This restricted growth may explain why *H. seropedicae* does not generally form red stripe disease symptoms in sorghum (James et al., 1997). In both sugar cane and sorghum, *Herbaspirillum* released immunologically reactive material (Figures 6 and 8), which was probably largely exopolysaccharide (EPS) (James et al., 1994, 1997; Olivares et al., 1997).

As *Herbaspirillum* spp. are not pathogenic to Brazilian sugar cane cultivars but yet are found within them in substantial numbers, with these numbers increasing as the plants grow (da Silva et al., 1995), the obvious questions to be asked are, How do they infect the plants; Where do they live within them; and Do they give the plants any benefit? The same questions can also be asked with rice, as Boddey et al. (1995a) have re-

FIGURE 3. Transmission electron micrograph (TEM) of a longitudinal section (LS) of a xylem vessel from the leaf of the mottled stripe disease-resistant sugar cane cv. SP 70–1143 infected with *H. rubrisubalbicans*. 20 dai. Note that the bacteria are confined to microcolonies close to the walls of the vessel (arrows) and that they are surrounded by host-derived gums. W = xylem vessel wall. (Bar = 2 μm.)

ported (over a period of 108 d) an increase in, and correlation between, ARA and numbers of *Herbaspirillum* spp. in symptomless leaves, stems, and roots of the wetland variety IR 42. As with *A. diazotrophicus* and *C. xyli* subsp. *xyli* within sugar cane setts (Reis et al., 1994; James, 1996), Olivares et al. (1996)

recently demonstrated that *Herbaspirillum* spp. could enter host plants via vegetative propagation, the bacteria being found within micropropagated sugar cane plants grown from sterile apical meristems. Another possibility is infection via seed-borne bacteria (Olivares et al., 1996), a common route of bacterial
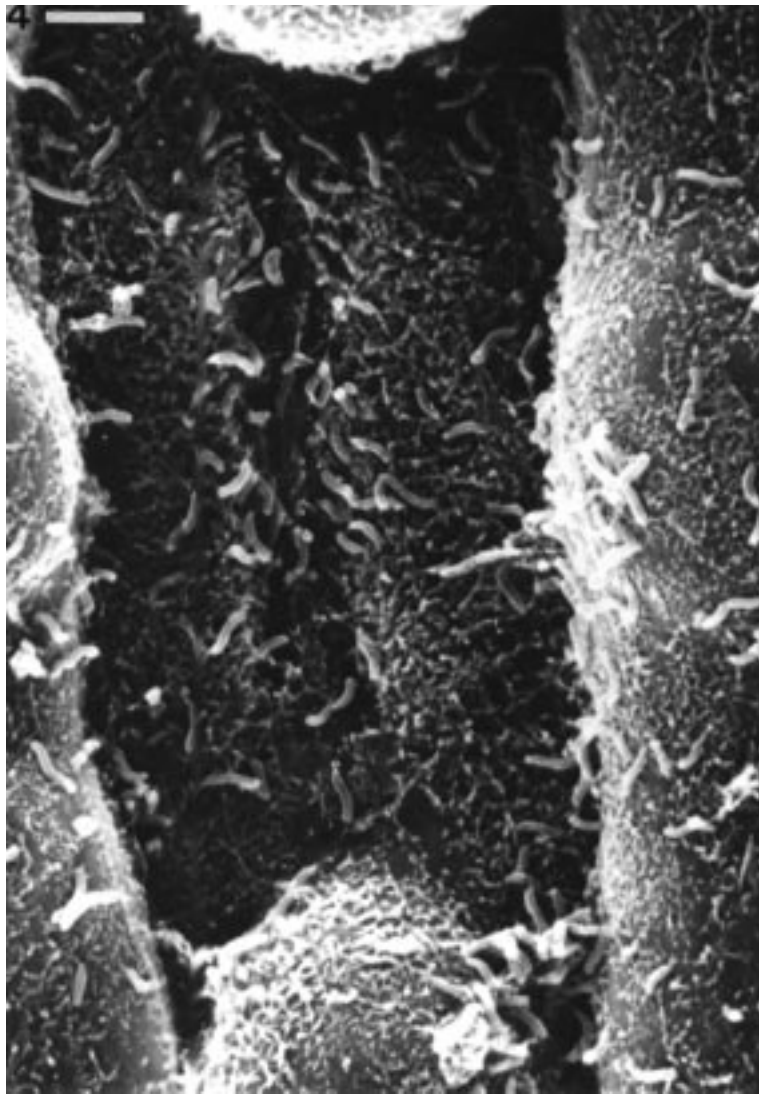
FIGURE 4. SEM of an *H. rubrisubalbicans*-infected stoma on the leaf of sugar cane cv. B–4362. 20 dai. (Bar = 1 μm.)

transmission (Mundt and Hinkle, 1976; Sundaram et al., 1988; McInroy and Kloepper, 1995). In support of the latter, Baldani et al. (1993) observed, using microscopy coupled with immunogold labeling, *H. seropedicae* and *Azospirillum brasilense* within cavities in surface-sterilized rice seeds and suggested that *H. seropedicae* are transferred from generation to generation via seeds. In further experiments

with surface-sterilized rice seeds in sterile tubes, Baldani et al. (1993) showed that *H. seropedicae* colonized the epidermis and cortex of the germinating roots, probably entering the intercellular spaces via loose epidermal cells. The bacteria even entered the cells of root tips, which was also observed within sugar cane roots infected with *H. seropedicae* (Olivares and James, unpublished; Figures 9 and 10). However, in both
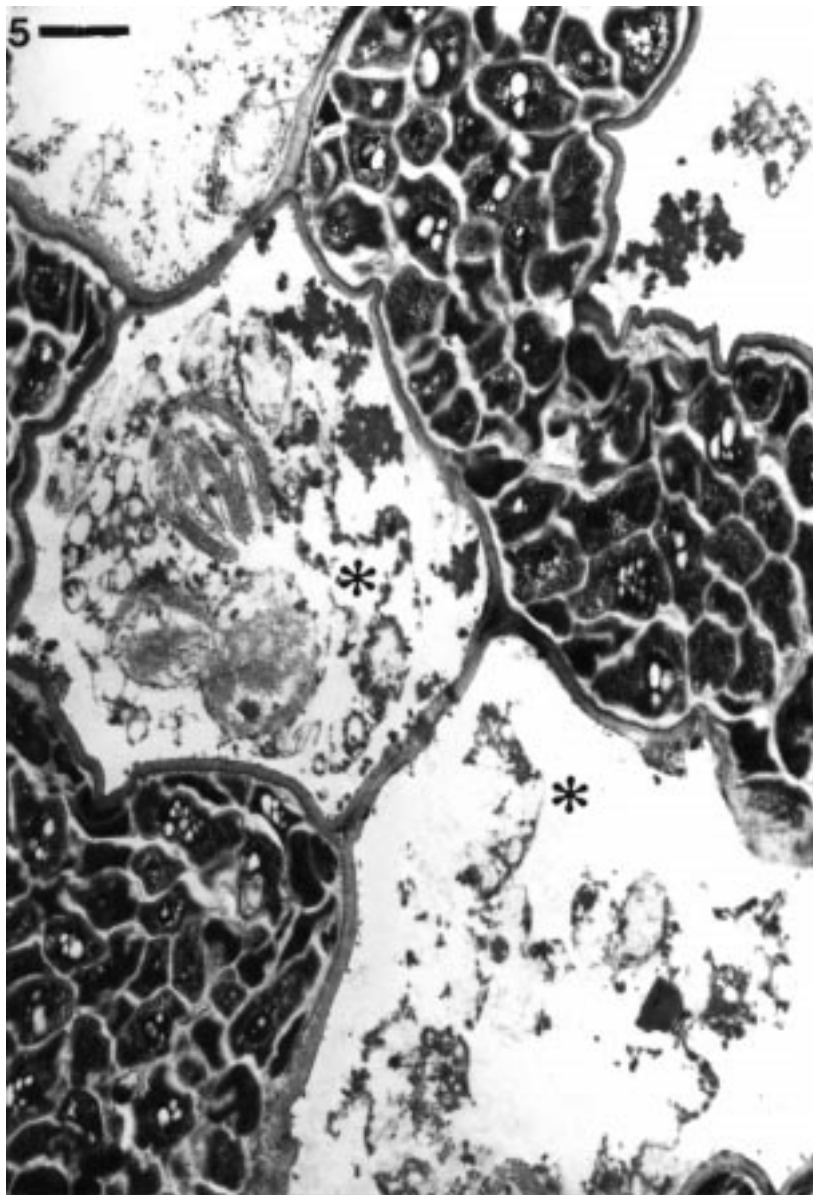
FIGURE 5. TEM of *H. rubrisubalbicans* within the intercellular spaces of a leaf of sugar cane cv. B–4362. 20 dai. This section was taken from a leaf in the mature phase of mottled stripe disease; note that the host cells are senescing (*). (Bar = 1 μm.)

rice and sugar cane, the intracellular bacteria appeared to be lysed by the host cells, or the host cells died, a situation similar to that observed with sugar cane root tips infected with *A. diazotrophicus* (James et al., 1994).

Host plants grown in cropped soil may also be infected from "viable" rhizosphere populations of *Herbaspirillum* spp. (Olivares et al., 1996), or from wind-borne *H. rubri-subalbicans* that have escaped from the inte-
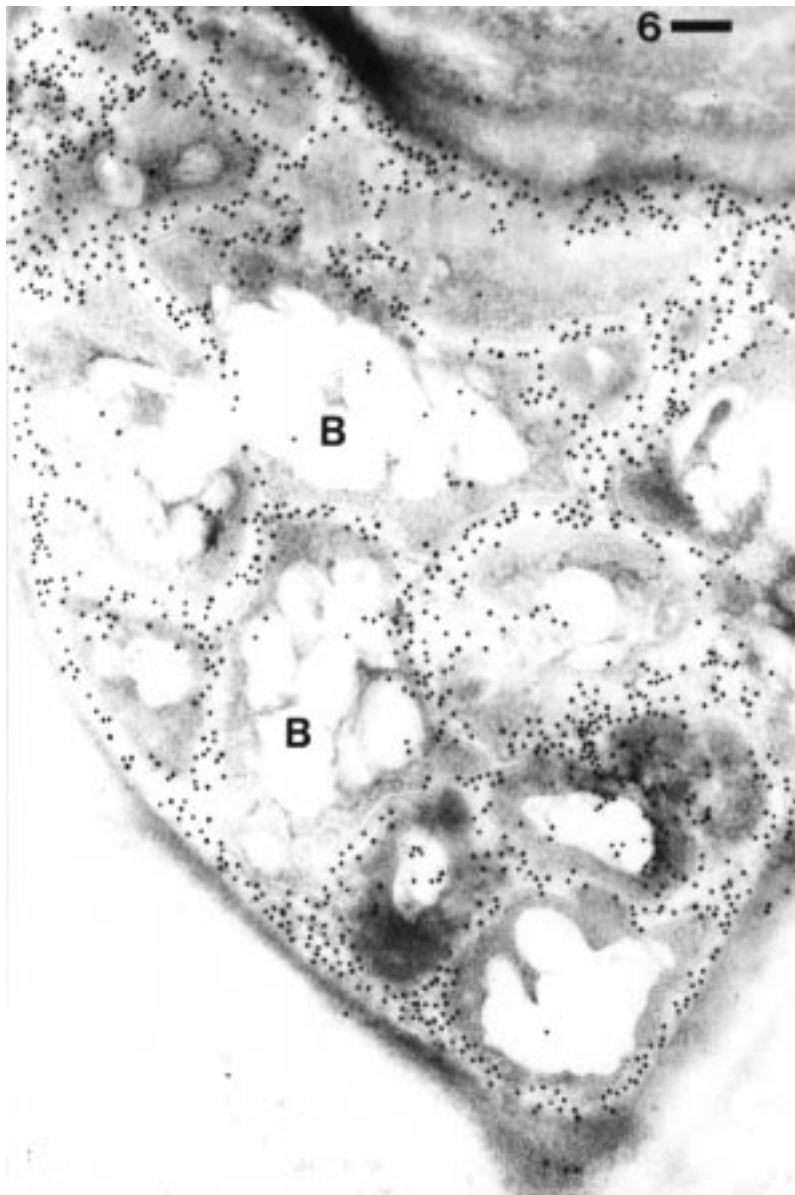
FIGURE 6. TEM of *H. rubrisubalbicans* within an intercellular space of a leaf of sugar cane cv. B-4362. 20 dai. This section was immunogold labeled with an antibody raised against *H. rubrisubalbicans* strain M4, and most of the labeling is of the bacterial surface and of material (mainly exopolysaccharide; EPS) released from the bacterial surface. B = bacteria. (Bar = 200 nm.)

rior of sugar cane leaves via stomata (disease-susceptible varieties only; Olivares et al., 1997). Soil-dwelling bacteria probably enter host plant roots via cracks in lateral root junctions, as has been observed with *A. diazotrophicus* and axenically grown sugar cane (James et al., 1994, Reis Jnr et al., 1995), as well as *Azospirillum* strains (see later). In sup-
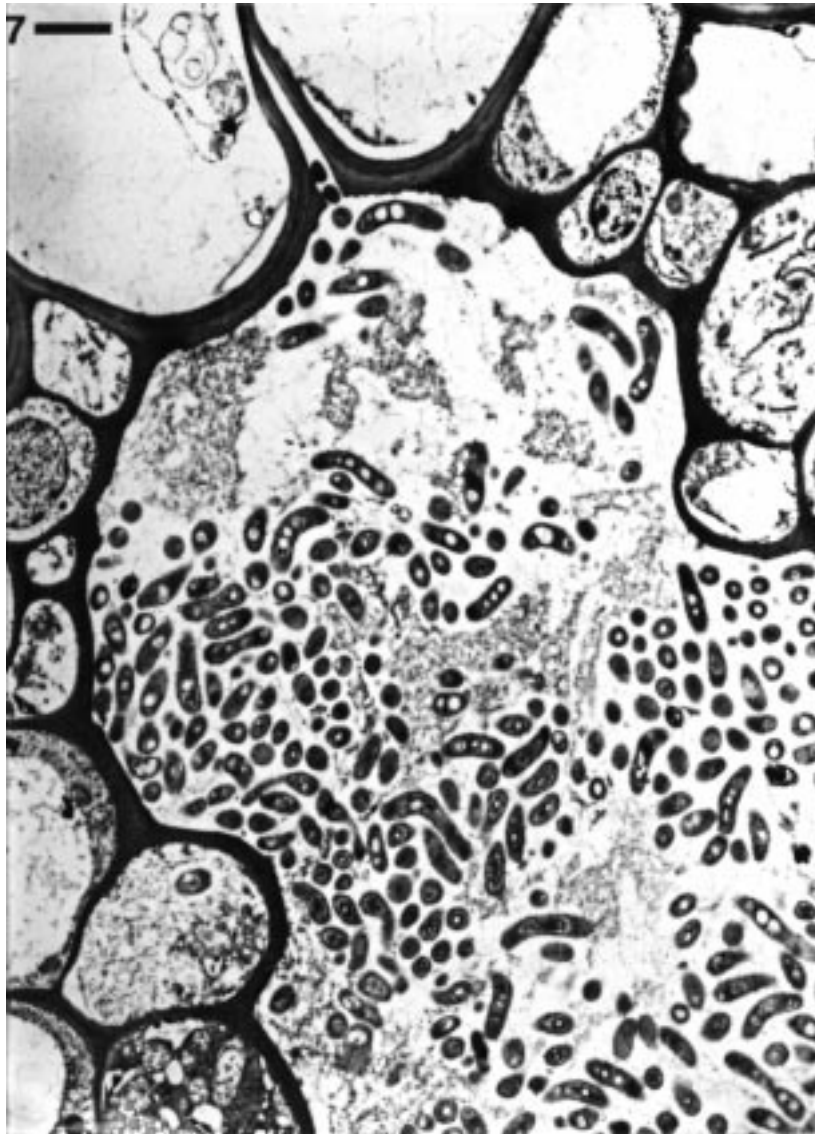
FIGURE 7. TEM of a TS of a protoxylem vessel and associated lacunae from a sorghum leaf colonized by *H. rubrisubalbicans*. 14 dai. (Bar = 2 μm.)

port of this, Olivares et al. (1995) and Olivares and James (unpublished) have shown that *Herbaspirillum* spp. colonize intercellular spaces and cells in the cortex of axenically grown sugar cane roots and also enter the xylem, at lateral root junctions (Figures 9 through 12); a similar process was shown recently by Vasse et al. (1995) in their excellent study of the infection of tomato (*Lycopersicon esculen-tum*) roots by *Pseudomonas solanacearum*. Entry into the vascular system at these points is made possible by endodermises being disrupted in the process of lateral root growth, thus providing a temporary apoplastic pathway between root cortex and stele (Patriquin and Döbereiner, 1978; Patriquin et al., 1983; Huang, 1986; Gagne et al., 1987; Kloepper et al., 1992; Vasse et al., 1995; Gough et al.,
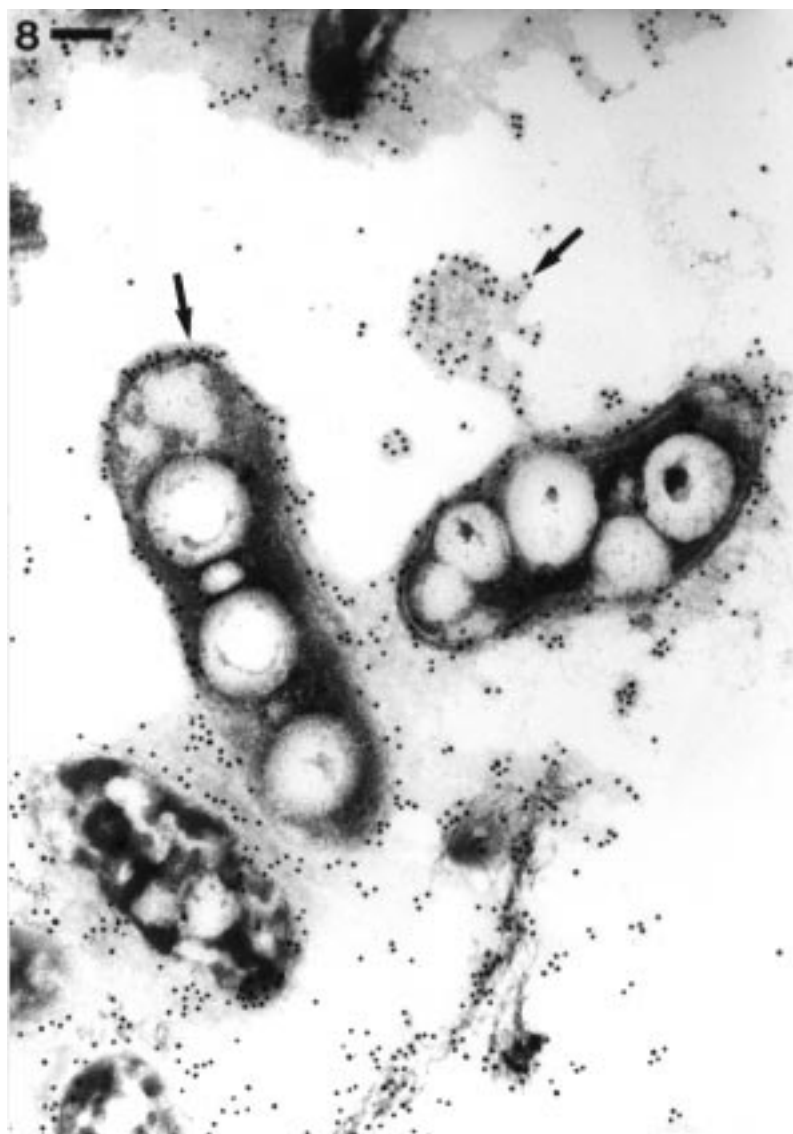
FIGURE 8. TEM of *H. seropedicae* within the protoxylem of a sorghum leaf. 14 dai. This section was immunogold labeled with an antibody raised against *H. seropedicae* strain Z67. As with *H. rubrisubalbicans* (Figure 6) and *A. diazotrophicus* (Figure 14), the antibody recognizes EPS-containing material on, and released from, the bacteria (arrows). (Bar = 200 nm.)

1997). Olivares et al. (1995) subsequently observed *Herbaspirillum* spp. in the sugar cane stem vascular system (Figure 13), and *H. seropedicae* could also be isolated from the aerial parts of axenically grown rice (Baldani et al., 1993). In addition, Pimentel et al. (1991), after artificially inoculating sorghum, sugar cane, and *Pennissetum purpureum*, showed that both diazotrophic *Herbaspirillum* spp. could be isolated from the 3rd to 5th leaves above the inoculated leaf up to 60 d after initial infection. Therefore, taken to-
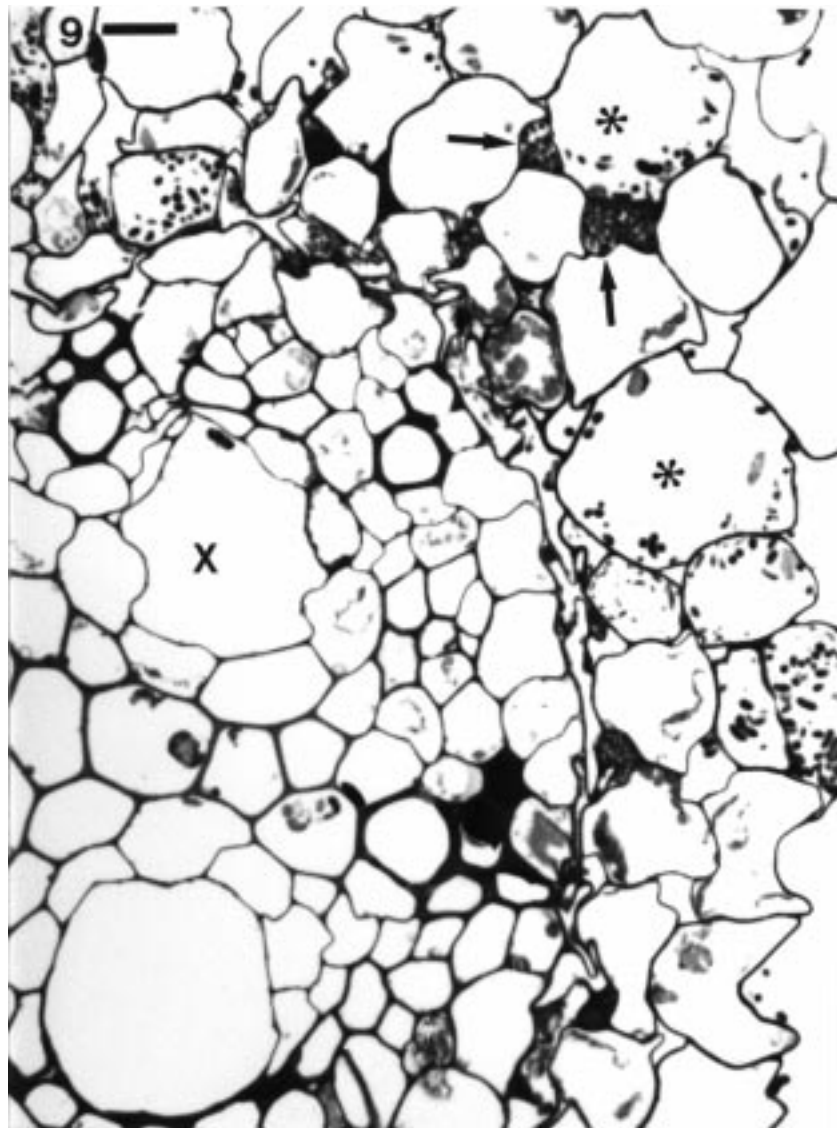
FIGURE 9. Light micrograph of a TS of a sugar cane root 4 dai with *H. seropedicae*. The axenically grown roots were inoculated by growing them in a semi-solid culture of the bacteria. This section was taken at a lateral root junction; note that the bacteria have colonized intercellular spaces (arrows), as well as cortical cells (*). X = xylem. (Bar = 10 μm.)

gether, these studies all suggest that *Herbaspirillum* spp. are translocated to the aerial parts of host plants in the transpiration stream, as shown previously with *Azoarcus* in rice and Kallar grass (Hurek et al., 1994), and with *A. diazotrophicus* in sugar cane (James et al., 1994; Fuentes-Ramirez et al., 1997).

The possible implications of this (at least in the case of rice) is that after germination, seed-borne *Herbaspirillum* spp. infect the interior of the developing plants and are translocated to the aerial, seed-bearing parts to infect the developing seeds of the next generation.

FIGURE 10. TEM of a sugar cane root cortical cell infected with *H. seropedicae*. 4 dai. This section was immunogold labeled with an antibody raised against *H. seropedicae* strain Z67. (Bar = 1 μm.

B. *Acetobacter Diazotrophicus*

This diazotroph, the only confirmed diazotroph within the genus *Acetobacter* (Gillis et al., 1989), is a relatively recent discovery, being found (using a low pH medium based on sugar cane juice; Cavalcante and Döbereiner, 1988) in high numbers mainly in the roots, stems, and leaves of sugar cane (Cavalcante and Döbereiner, 1988; Gillis et al., 1989; Li and MacRae, 1991, 1992; Reis et al., 1994), but also in a few other sugar/starch-rich plants, that is, *Pennissetum purpureum* and sweet potato (*Ipomoea batatas*) (Paula et al., 1991, 1992; Döbereiner et al., 1995b). In addition, recently Jimenez-Salgado et al. (1997) have
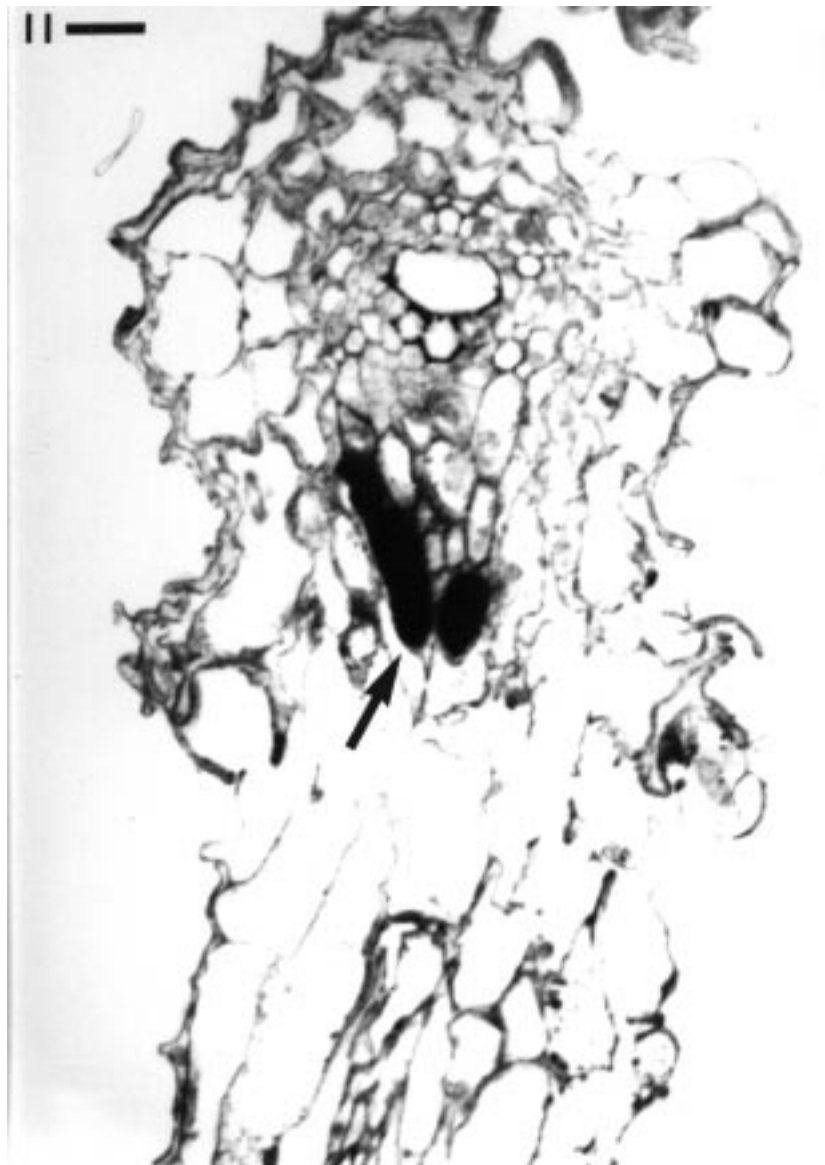
FIGURE 11. Light micrograph of a TS of a sugar cane root 2 dai with *H. seropedicae*. This section was immunogold labeled (followed by silver enhancement) with an antibody raised against *H. seropedicae* strain Z67. The bacteria are concentrated in the xylem vessels of the emerging lateral root (arrow). (Bar = 20 μm.)

reported the isolation of *A. diazotrophicus* from coffee (*Coffea arabica*), along with other N₂-fixing bacteria, that may belong to the genus *Acetobacter*. In Brazilian sugar cane fields, the bacterium was not found in the soil between rows of sugar cane plants, and it also does not surpive well when artificially in-

oculated into soil (Baldani et al., 1997). *A. diazotrophicus* also failed to be isolated from 11 weed species associated with Brazilian sugar cane fields, and it was also not found in six forage grass species, rice, sorghum roots, or maize (Boddey et al., 1991; Reis et al., 1994). The endophytic nature and specificity
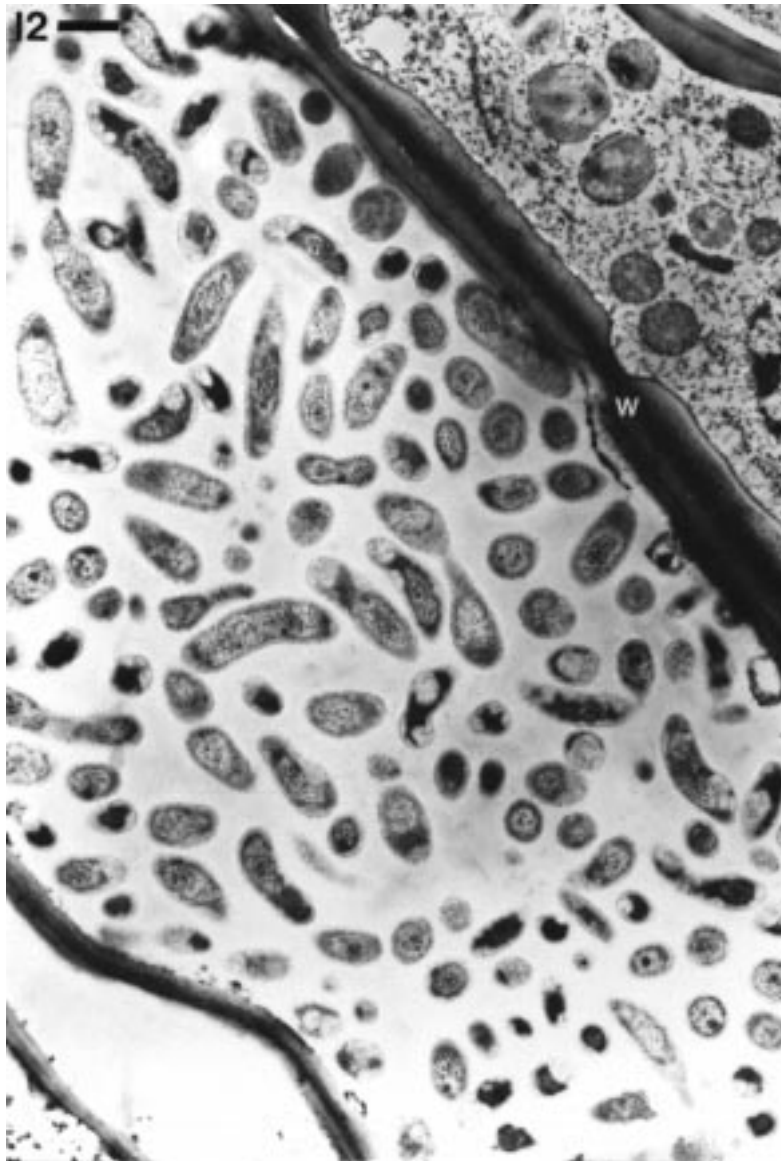
FIGURE 12. TEM of *H. seropedicae* within a xylem vessel in an emerging lateral root, 2 dai (see Figure 11). W = xylem vessel wall. (Bar = 1 μm.)

of *A. diazotrophicus* was confirmed in studies of 12 field-grown Australian sugar cane cultivars by Li and MacRae (1991, 1992), who also showed that the bacterium was absent from several other grasses. Interestingly, and in contrast to Brazilian data (Boddey et al., 1991; Reis et al., 1994), Li and MacRae (1991) also reported that *A. diazotrophicus* was present in low concentrations in the sugar cane rhizo-sphere, and speculated that the soil close to the host plant was enriched in sucrose. Caballero-Mellado and Martinez-Romero (1994) and Caballero-Mellado et al. (1995) have suggested that the narrow range of plant species containing the bacterium may explain the limited genetic diversity of *A. diazotrophicus*.

As with other plant-associated diazotrophs, such as *Azospirillum* and *Herbaspirillum*
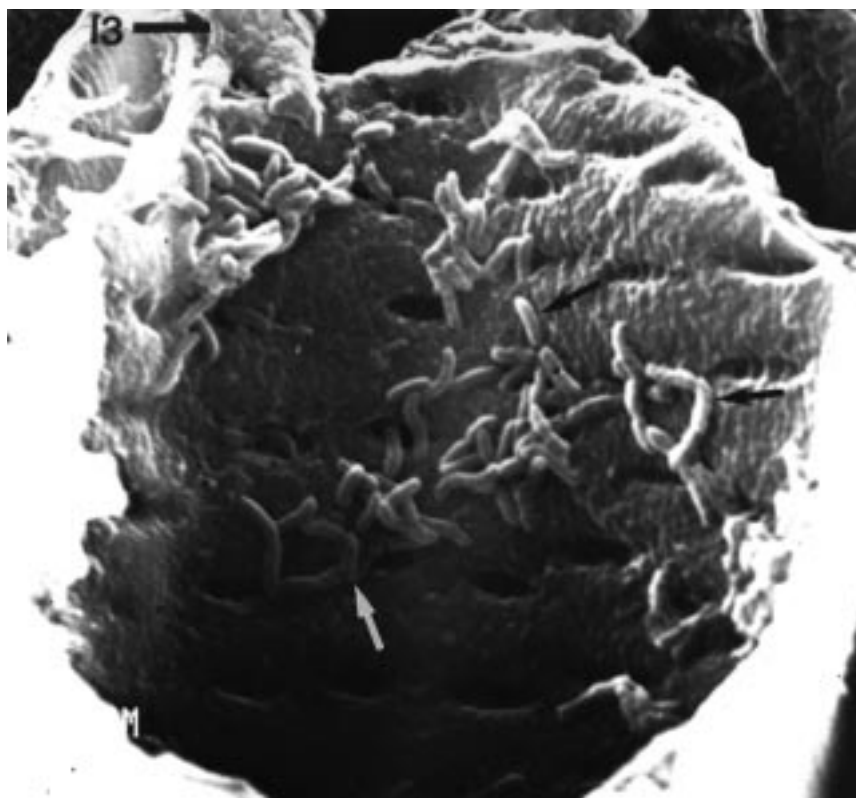
FIGURE 13. SEM of *H. seropedicae* within the xylem of a sugar cane stem 7 dai of the roots. Note that the bacteria are associated closely with the walls of the vessel. (Bar = 500 nm.)

(Tarrand et al., 1978; Baldani et al., 1992), *A. diazotrophicus* is best grown in a semi-solid medium (LGIP medium; Cavalcante and Döbereiner, 1988) to allow for bacterial movement and pellicle formation to achieve the microaerobic conditions necessary for N_2 fixation and growth (Döbereiner, 1992). Dong et al. (1994) have stated *A. diazotrophicus* is difficult to isolate and grows very slowly to first visibility, that is, 15 to 30 d. However, this is not the case if semi-solid media are used, where pellicle formation is visible after 5 d (Boddey et al., 1991). Semi-solid media are also best for counting the bacteria (Boddey et al., 1991; Li and MacRae, 1992; Reis et al., 1994), and hence the numbers given by Dong et al. (1994) for *A. diazotrophicus* with-

in the apoplastic fluid of the sugar cane cultivars Ja 60–5 and Media Luna (1.1×10^4 cell/ml) were probably a very large underestimate as they used a solid medium for the counts (Boddey et al., 1995b). Recently, Reis et al. (1994) have developed a more specific semi-solid medium for the improved isolation and enumeration of the bacterium; this is based on LGIP medium (Cavalcante and Döbereiner, 1988) and contains crystallized cane sugar (10%) supplemented with cane juice (0.5%), and a pH of 5.5.

The bacterium is a small, Gram-negative, aerobic rod showing pellicle formation in N-free semi-solid medium with 10% sucrose (Reis et al., 1994) and can grow in sucrose concentrations up to 30% (Cavalcante

and Döbereiner, 1988). Strong acid production results in a final pH of 3, or less, but growth and N_2 fixation can continue at this pH for several days (Stephan et al., 1991). The bacterium will also grow well over a range of pH values (Burris, 1994) on monosaccharides such as glucose, fructose, and galactose, and will also grow in glycerol, ethanol, and mannitol, but not on many other C compounds such as dicarboxylic acids or maltose (Cavalcante and Döbereiner, 1988; Gillis et al., 1989; Li and MacRae, 1991; Ureta et al., 1995). Indeed, despite its preference for living within sucrose-rich plants, recent results by Ureta et al. (1995) have shown that best growth of A. diazotrophicus actually occurs with high concentrations of (in descending order) gluconate, glucose, glycerol, and then sucrose. Moreover, recently Alvarez and Martinez-Drets (1995) have shown that A. diazotrophicus is actually unable to transport or respire sucrose. Alvarez and Martinez-Drets (1995) explained the ability of A. diazotrophicus to grow on sucrose as being due to extracellular saccharolytic enzyme activity actually providing the bacteria with glucose and fructose for growth. One of the key enzymes responsible for this saccharolytic activity is probably levansucrase, which acts on sucrose releasing fructo-oligosaccharides and levan, an EPS (Arrieta et al., 1996). This EPS is likely to be a constituent of the immunogenic extracellular material that James et al. (1994) observed surrounding the bacteria within xylem vessels (also see Figure 15). Therefore, its ability to grow on a range of C substrates means that A. diazotrophicus is not confined to living only in sucrose-rich environments such as the sugar cane stem intercellular apoplast (Hawker, 1965; Welbaum et al., 1992; Dong et al., 1994, 1997), and could explain how the bacteria survive in the fructose/glucose-rich honeydew exuded by the pink sugar cane mealy bug (*Saccharococcus sacchari*) (Ashbolt and Inkerman, 1990). In addition, Boddey et al. (1991) have shown that A. diazotrophicus will grow and fix N_2 well on su-

crose concentrations as low as 1%. This means that the bacteria could also grow and fix N_2 within the xylem, where sucrose (and other potential C substrates; Ureta et al., 1995) can be very low (0 to 9%; Hawker, 1965; Bull et al., 1972; Welbaum et al., 1992, and see earlier).

A. *diazotrophicus* contains no nitrate reductase and hence its nitrogenase activity is not affected by high levels of nitrate, 25 mM (Cavalcante and Döbereiner, 1988; Stephan et al., 1991; Boddey et al., 1991) or 80 mM (Li and MacRae, 1991). However, the bacterium will grow on ammonium as an N-source (Gillis et al., 1989), and nitrogenase activity is also only partially inhibited by ammonium and amino acids (cf. *H. seropedicae*; Fu and Burris, 1989), especially at high sucrose levels (Reis et al., 1990; Stephan et al., 1991; Boddey et al., 1991). Moreover, in the presence of 10% sucrose, ammonium assimilation by the bacterium is reduced by 65% compared with growth in 1% sucrose (Boddey et al., 1991). Taken together, Döbereiner et al. (1995b) have suggested that these characteristics may allow endophytic A. *diazotrophicus* to fix N_2 in parallel, and in complementation with, the plants assimilation of mineral N. A. *diazotrophicus* is relatively tolerant to O_2, at least compared with *Azospirillum*, and will continue to fix N_2 at a pO_2 of 4% in 10% sucrose (Reis, 1991; Boddey et al., 1991, 1995). However, in a recent study of *NifH* expression by several plant-associated diazotrophs, Vande Broek et al. (1996) classed A. *diazotrophicus* as being non-tolerant compared with *Azoarcus indigens*, and *Azorhizophilus paspali*, (formerly *Azotobacter paspali*; Thompson and Skerman, 1979).

The exact location of A. *diazotrophicus* within sugar cane plants has yet to be established satisfactorily. Li and MacRae (1992) and Reis et al. (1994) have isolated A. *diazotophicus* from several Australian and Brazilian sugar cane cultivars, the bacterium being found in the roots, stems, and aerial parts. Interestingly, the highest numbers in the Brazil-

ian cultivars (up to 8×10^6 g^{-1} fresh weight) were found within cane trash (Reis et al., 1994). A recent study of ontogenic variation in *A. diazotrophicus* numbers within roots, leaves, and stems of four Brazilian sugar cane varieties (CB 45–3, SP 70–1143, Krakatau, Chunnee) over a 15-month period showed no obvious pattern, except in cv. SP 70–1143, which showed an increase, thus suggesting that *A. diazotrophicus* populations are sensitive to sugar cane genotype (da Silva et al., 1995). The bacterium has also been isolated, in similar numbers to Brazilian cultivars, from sugar cane in Mexico, Cuba, and Argentina (Fuentes-Ramirez et al., 1993; Dong et al., 1994; Bellone et al., 1997), with highest numbers in the Mexican cultivars being in those that do not usually have mineral N added to them (Fuentes-Ramirez et al., 1993, 1997; Caballero-Mellado et al., 1995). Fuentes-Ramirez et al. (1993) have also shown that, in the cultivars that they studied, highest frequencies of the bacteria were found in the apical stem regions; this may correspond to mealy bug feeding areas (Ashbolt and Inkerman, 1990). Reis et al. (1994) have isolated *A. diazotrophicus* from xylem sap (cvs CB 45–3 and NA 56 79; Table 1, Caballero-Mellado et al., 1995) and suggested from this that the bacterium was translocated in the transpiration stream. This is a reasonable suggestion as the methods that Reis et al. (1994) used were those that are used routinely for isolating *C. xyli* subsp. *xyli* (see earlier). Indeed, Sprent and James (1995) have suggested previously that the xylem, despite the low sugar levels within it, is a suitable location for *A. diazotrophicus* and *Herbaspirillum* due to the low pO$_2$ (allowing for nitrogenase expression; Gallon, 1992) that is likely to pertain there (Clements, 1980; Patriquin et al., 1983, and see later).

Despite the studies of Reis et al. (1994) and Caballero-Mellado et al. (1995), Dong et al. (1994) concluded that *A. diazotrophicus* was present only in the intercellular apo-plast (i.e., the intercellular spaces) of sugar cane stems (cv. Ja 60–5), and was unlikely to enter the xylem, as it would cause a host defense reaction. However, using micropropagated sugar cane plantlets (cv. NA 56–79) grown in the presence of the bacteria, and immunogold labeling to confirm their identity, James et al. (1994) not only showed that *A. diazotrophicus* colonized the intercellular spaces of the root but also clearly showed the bacteria within xylem vessels at the base of the stem (also see Figures 14 and 15, and Döbereiner et al., 1995b). Indeed, the occurrence of *A. diazotrophicus* in stem xylem vessels (and the adjacent intercellular spaces) has been confirmed recently by Fuentes-Ramirez et al. (1997) who inoculated one-node setts with GUS-labeled bacteria prior to germination and subsequently microscopically examined the aerial tissues for the presence of the bacteria. *A. diazotrophicus* has also been observed within the xylem and intercellular apoplast of inoculated sugar cane plantlets by Sevilla et al. (1997) and Caballero-Mellado et al. (1997) observed it in the xylem of maize plants after seedlings were inoculated with the bacteria. Neither James et al. (1994) Sevilla et al. (1997), nor Fuentes-Ramirez et al. (1997) observed a host defense response by the plant in either the intercellular or xylem apoplast, and the bacteria did not appear to be sufficiently numerous to block the vessels (Figures 14 and 15). The results of James et al. (1994), Döbereiner et al. (1995b), Sevilla et al. (1997), and Fuentes-Ramirez et al. (1997) are supported by the fact that *A. diazotrophicus* can be isolated from the xylem sap of field-grown plants of the Brazilian cultivar NA 56–79 (Caballero-Mellado et al., 1995), and also by the earlier study of Patriquin et al. (1980) who reported unidentified N$_2$-fixing (tetrazolium-reducing) bacteria in the xylem of the same cultivar. The apparently conflicting reports of Dong et al. (1994, 1997) on the one hand, and the above studies on the other, could possibly be due to
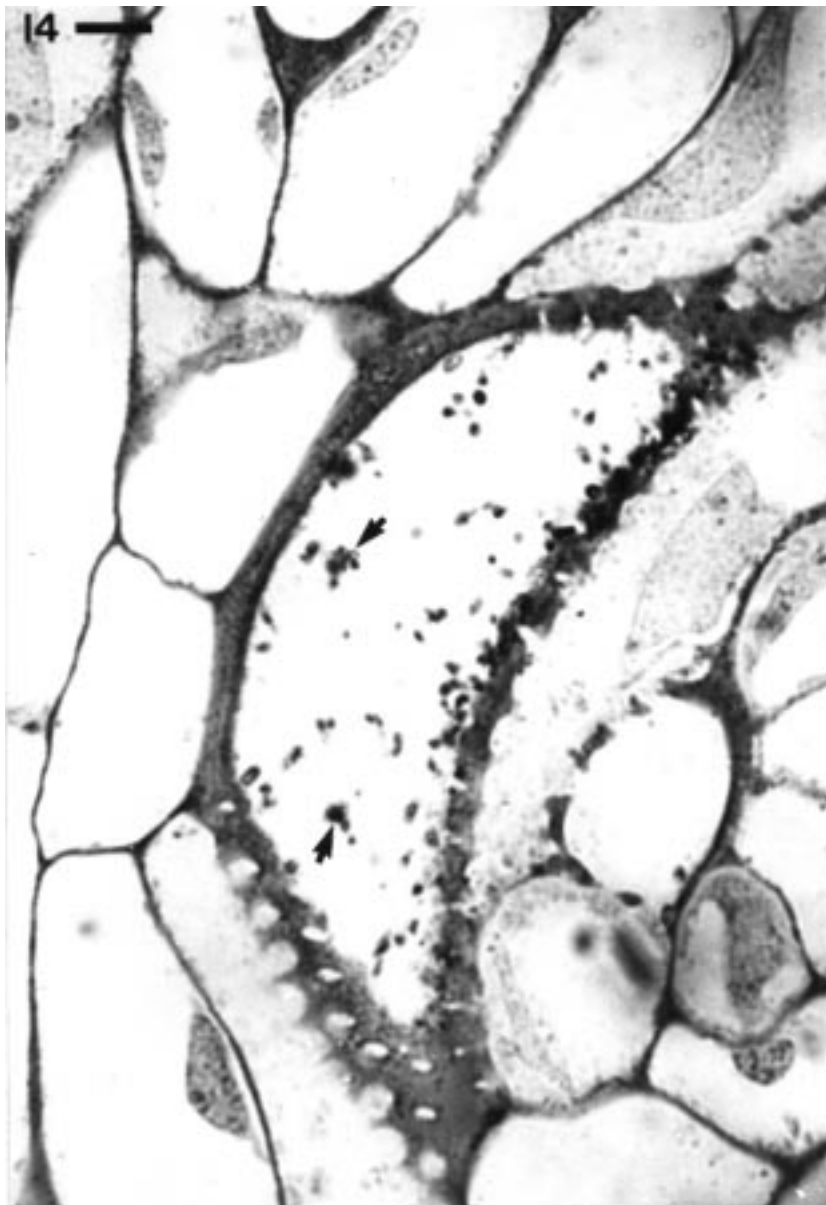
FIGURE 14. Light micrograph of a TS of the base of the stem of sugar cane 15 dai with *Acetobacter diazotrophicus* (see James et al., 1994 for details). This section was immunogold labeled (followed by silver enhancement) with an antibody raised against *A. diazotrophicus*. Note that the bacteria (arrows) are located within a xylem vessel. (Bar = 5 μm.)

the fact that the centrifugation method used by Dong et al. (1994) to obtain apoplastic fluid inevitably yields a high proportion of xylem sap (Raven, 1983). Indeed, Dong et al. (1994) admitted that their "apoplastic fluid" could actually have consisted of up to 20% by volume of xylem sap, and hence the *A. diazotrophicus* cultures that they claimed to
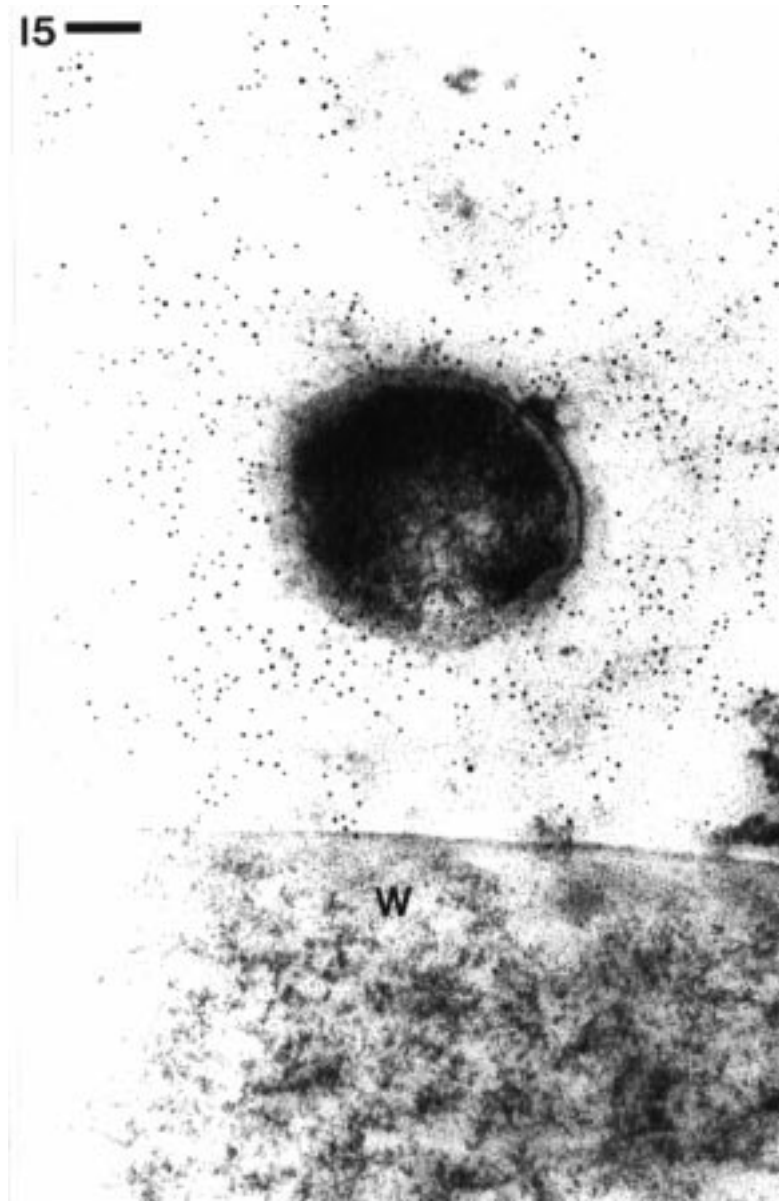
FIGURE 15. TEM of *A. diazotrophicus* within a xylem vessel at the base of the stem of a sugar cane plantlet. This section was immunogold labeled with an antibody raised against *A. diazotrophicus*; note that the antibody mainly recognizes extracellular material (EPS). W = xylem vessel wall. (Bar = 100 nm.)

have isolated exclusively from the intercellular apoplast actually probably originated from a mixture of both intercellular and vascular-dwelling bacteria.

How then does *A. diazotrophicus* enter sugar cane seedlings/plantlets and move within the adult plants? A possible mechanism was suggested by the study of James et al.

(1994), who grew sugar cane plantlets axenically in a modified plant growth medium containing 2 g/l sucrose and inoculated the medium with *A. diazotrophicus*. After 4 d, the bacteria had consumed much of the sucrose in the medium and began to colonize the root surfaces. By 15 d after inoculation, the bacteria had entered the root apoplast via lateral root junctions and the loose cells of the root cap. These results have since been confirmed by Reis Jnr et al. (1995), and in both studies the bacteria in the plantlets were confirmed to be *A. diazotrophicus* by using immunogold labeling with antibodies specific to the bacterium, immunolabeling being necessary as sugar cane contains many different species of pathogenic and endophytic bacteria (see earlier sections). No root hair infection by *A. diazotrophicus* was observed by James et al. (1994) or Reis Jnr et al. (1995), but it was reported by Bellone et al. (1997). James et al. (1994) also showed that some bacteria entered root tip cells via "infection thread-like" structures, but these bacteria then appeared to be subsequently lysed by the host. Infection thread-like structures in sugar cane root tips have also been reported by Bellone et al. (1997), although they stated that the *A. diazotrophicus* were not lysed but were surrounded by a host cell membrane, as with bacteroids in the legume-*Rhizobium* symbiosis (Brewin, 1991). Bellone et al. (1997) also suggested that these intracellular bacteria were the source of N_2 fixation within sugar cane. The occurrence of at least part of the *A. diazotrophicus* population within sugar cane cells has also been suggested by Fuentes-Ramirez et al. (1997), who showed (using scanning electron microscopy; SEM) *A. diazotrophicus* colonizing cells adjacent to stem vascular bundles. However, the intriguing claims of Bellone et al. (1997) and Fuentes-Ramirez et al. (1997) await confirmation. For example, the experiments of Bellone et al. (1997) were performed under non-sterile greenhouse conditions, and there was no indication that immunological

or molecular techniques were used to establish the identity of the bacteria in the micrographs. In the case of the SEMs of Fuentes-Ramirez et al. (1997), cell contents are usually so severely disrupted by the preparation of material for conventional SEM, that SEMs by themselves are not reliable indicators of *intra*-cellular bacteria (Sprent and James, 1995), and need to be confirmed by transmission electron microscopy.

As discussed above, James et al. (1994) showed that *A. diazotrophicus* had entered the xylem of the sugar cane plantlets by 15 d after inoculation. Although the exact mechanism for entry into the xylem was not elucidated, it has been suggested by Patriquin et al. (1983) and Sumner (1990) that the stele/primordial vascular tissue is insufficiently differentiated or thickened at the root tip to allow for bacterial entry. Moreover, Patriquin and Döbereiner (1978) and Kloepper et al. (1992) have suggested that developing vascular tissue at lateral root junctions may also be a site of entry (also, see the section on *Herbaspirillum* and Vasse et al., 1995). Therefore, with respect to bacterial entry into developing xylem, it may be significant that James et al. (1994) showed that *A. diazotrophicus* accumulated particularly at root tips and lateral root junctions. It is likely that once it is in the xylem, *A. diazotrophicus* then moves up the stem in the transpiration stream as the sugar cane plant grows. This has been shown with *Herbaspirillum* (Figure 13), *C. xyli* subsp. *xyli* (Kao and Damann, 1980; Harrison and Davis, 1988), and *X. albilineans* (Comstock, 1992), as well as being suggested by the studies of Patriquin et al. (1980). Graciolli and Ruschel (1981) and Reis (1991) have also shown that, in common with *C. xyli* subsp. *xyli* (Kao and Damann, 1980), *A. diazotrophicus* is particularly concentrated in the stem nodes, probably because the xylem is convoluted at these points and thus impedes the flow of solutes (plus bacteria) through the xylem into the next internode (Bull et al., 1972;

Teakle et al., 1977; Clements, 1980; Harrison and Davis, 1988). Therefore, as the bacteria seem to accumulate at nodes it is probable that they then escape from the xylem to colonize the intercellular space apoplast (Dong et al., 1994). Indeed, this has been demonstrated with *Herbaspirillum* (Figures 1, 3, 4; and Olivares et al., 1997), and is also suggested by the study of Patriquin et al. (1980).

Although it is considered that in the field the infection mechanisms described by James et al. (1994) and Reis Jnr et al. (1995) are unnecessary due to *A. diazotrophicus* being spread from generation to generation via the vegetative propagation of setts, the fact that the bacterium is not always present in setts (Li and MacRae, 1992) suggests that other mechanisms of plant infection must also occur. Indeed, the presence of *A. diazotrophicus* in the sugar cane rhizosphere (Li and MacRae, 1991, 1992) and in trash (Reis et al., 1994) is an obvious source of root inoculum and hence the processes described by James et al. (1994) and Reis Jnr et al. (1995) may also occur in the field. As well as being spread from generation to generation via setts (Döbereiner et al., 1993; Reis et al., 1994), Paula et al. (1991, 1992) have shown that sugar cane, sweet potato, and sweet sorghum (*Sorghum vulgare*) roots can be infected by *A. diazotrophicus* when the bacteria are co-inoculated with the mycorrhizal fungus *Glomus clarum* and have suggested that spores of the fungus contain the bacterium. These results have since been repeated by Bellone et al. (1995) using *Glomus* and *Gigaspora gigantea* isolated from sugar cane in Argentina.

An intriguing discovery was made recently by Ashbolt and Inkerman (1990), who isolated *A. diazotrophicus*, along with a number of other *Acetobacter* spp., from the pink sugar cane mealy bug. This insect feeds on the "sterile" meristematic tissue between the sugar cane leaf sheath and the stem (commonly known as the leaf sheath "pocket"), and *Acetobacter* spp. were particularly abundant in mealy

bugs feeding on aerial sucrose storage tissue in the summer. The bacterium has also been isolated recently from mealy bugs in Mexico, Brazil, and Australia, and taxonomic studies have shown that the mealy bug bacteria are a subset of the sugar cane bacterial population (Caballero-Mellado et al., 1995). *Acetobacter* spp. were also isolated from leafhoppers (*Perkinsiella saccharicida* Kirk) that feed in the same place as the mealy bugs. By contrast, other sugar cane-associated insects, for example, linear bugs, aphids, and ants, which feed on leaves further up from the leaf sheath pocket, did not contain *Acetobacter*s (Ashbolt and Inkerman, 1990). The honeydew of the pink mealy bug is very high in sugars such as fructose and glucose but low in sucrose, with a low pH of 2.9 to 3.2 due to the conversion (by *Acetobacter*s) of sugars and ethanol to organic acids (Ashbolt and Inkerman, 1990). The high sugar composition of the honeydew suggests that the pink sugar cane mealy bug is a phloem feeder (Raven, 1983), in common with other Pseudococcidae (Calatayud et al., 1994). In contrast, the leaf hoppers observed by Ashbolt and Inkerman (1990) are probably xylem feeders, as suggested by the low sugar content of their honeydew, and its high pH (due to the neutralization of organic acids; Raven, 1983), although it is not unusual for insect plant vascular parasites to feed on both xylem and phloem and hence divisions between "xylem feeders" and "phloem feeders" can be blurred (Ullman and McLean, 1988; Purcell, 1989; Press and Whittaker, 1993; Purcell, personal communication). The fact that *Acetobacter*s are found within sap-feeding insects is *circumstantial* evidence that they may be involved in transferring them from plant to plant (Ashbolt and Inkerman, 1990; Caballero-Mellado and Martinez-Romero, 1994; Döbereiner et al., 1995; Boddey et al., 1995b). In particular, the very high sugar content of the mealy bug honey dew, as well as its low pH, makes the gut of this insect a potentially good place for

the bacteria to live in, allowing them to survive for prolonged periods outside of host plants and thus increasing the potential for the mealy bugs as bacterial vectors (Purcell, 1989; Barbehenn and Purcell, 1993). However, we must stress that the accumulation of bacteria within insects is not by any means *conclusive* evidence that they are actually transmitted by them (Purcell, personal communication, and see earlier discussion of *C. xyli* subsp. *xyli*), and hence more work is clearly required to confirm the putative transmission of *A. diazotrophicus* within and between sugar cane populations by mealy bugs.

A final interesting note about the mealy bug–*Acetobacter* association: when Ashbolt and Inkerman (1990) tested portions of mealy bug-infested stems for ARA they were all negative, suggesting that neither the bacteria within the insects nor within the insect-infested stems expressed nitrogenase.

C. *Azospirillum* spp.

The genus *Azospirillum* was first described by Tarrand et al. (1978) and, at that time, it consisted of two species: *A. brasilense* and *A. lipoferum*. Since then, three more species have been described: *A. amazonense*, *A. halopraeferans*, and *A. irakense* (see reviews by Michiels et al., 1989; Bashan and Levanony, 1990; Vande Broek and Vanderleyden, 1995). *Azospirillum* spp. are usually isolated from the roots of grasses and are motile, growing best in a semi-solid medium with formation of a pellicle (Tarrand et al., 1978; and see sections on *Herbaspirillum* and *A. diazotrophicus*), as their nitrogenase expression/activity is sensitive to pO_2s above 2% (Vande Broek et al., 1996). The preferred C sources for all *Azospirillum* spp. are organic acids, for example, malate and succinate (Tarrand et al., 1978), although the pattern of carbohydrate use differs markedly between species and only *A. amazonense* is capable of utilizing disaccharides (Michiels et al., 1989). There have been numerous reviews on the potential benefits that *Azospirillum* may confer on the plants with which they are associated (see above), and there is much evidence that hormonal effects, as well as BNF, may be the major factors involved in plant growth promotion (Okon and Kapulnik, 1986; Boddey and Döbereiner, 1988, 1995; Bashan and Levanony, 1989; Sumner, 1990; Vande Broek and Vanderleyden, 1995).

Although they are generally regarded as being rhizosphere bacteria, colonizing mainly the elongation and root hair zones of roots (Okon and Kapulnik, 1986; Döbereiner et al., 1995b; Vande Broek and Vanderleyden, 1995; Bashan and Holguin, 1995), some *Azospirillum* strains can also be endophytic, being found within the roots of some Gramineae, including sugar cane (Patriquin et al., 1983; Okon and Kapulnik, 1986; Michiels et al., 1989; Sumner, 1990; Bashan and Levanony, 1990; Kloepper and Beauchamp, 1992; Kennedy and Tchan, 1992; Bellone and Bellone, 1994; Döbereiner et al., 1995b; Boddey and Döbereiner, 1995; Vande Broek and Vanderleyden, 1995). For example, using light microscopy and tetrazolium staining, Patriquin and Döbereiner (1978) observed *Azospirillum lipoferum* within the roots of maize, sorghum, wheat, *P. maximum*, and *D. decumbens*. Moreover, some *A. brasilense* strains (e.g., Sp 109) have also been seen within the leaves and seeds of rice (Baldani et al., 1993). Other strains, such as *A. brasilense* strain Sp 245 and *A. lipoferum* strain Sp S82, have been isolated only from surface-sterilized maize, sorghum, wheat, and rice roots, suggesting that they are possibly obligate endophytes (Baldani et al., 1983, 1986b; Pereira et al., 1988). This contrasts with the two closely related *A. brasilense* strains Sp 7 and Cd, which were originally isolated from soil associated with *D. decumbens* and *Cynodon dactylon*, respectively (Tarrand et al., 1978), and tend only to colonize the root surface, with few bacteria being isolated from within inoculated plants (Baldani et al. 1986b; Bashan and Levanony,

1990; Kennedy and Tchan, 1992; Döbereiner et al., 1995a). The difference between endophytic strains such as Sp 245 on the one hand and surface colonizers such as Sp 7 on the other was confirmed recently by the studies of Schloter et al. (1994) and Assmus et al. (1995). Using strain-specific molecular probes and monoclonal antibodies (Hartmann et al., 1995), they showed that strain Sp 245 colonized the interior of root hairs, as well as the xylem vessels of wheat roots, whereas strain Sp 7 only colonized the root surface. Interestingly, and in contrast to Schloter et al. (1994) and Assmus et al. (1995), Levanony et al. (1989) observed strain Cd colonizing the interior of wheat roots, that is, within cortical intercellular spaces. However, unlike Sp 245, strain Cd was never seen within the root endodermis or vascular system (Levanony et al., 1989). These observations of strain specificity as regards endophytic *Azospirillum* support the suggestion of Bashan and Levanony (1990) that, although *A. brasilense* is not a plant-specific bacterium per se (Bashan and Holguin, 1995), internal root colonization by *A. brasilense* (and possibly *A. lipoferum*) does occur in certain specific plant-strain interactions (Baldani et al., 1997).

In previous sections we have discussed how endophytic bacteria can penetrate the root endodermis and enter the xylem, and these arguments also apply to endophytic *Azospirillum*. For example, several authors (e.g., Patriquin et al., 1983; Michiels et al., 1989; Bashan and Levanony, 1990) have suggested that *Azospirillum* can enter at lateral root junctions, and also through root hairs (Assmus et al., 1995). There has also been some pectolytic activity demonstrated by *Azospirillum* (Umali-Garcia et al., 1978; Okon and Kapulnik, 1986; Plazinski and Rolfe, 1985; Bellone and Bellone, 1994), and hence the bacteria may actually enter via active enzymic degradation of the host cell wall middle lamellae (Levanony et al., 1989), as observed in some other endophytic, symbiotic, and pathogenic plant-bacterial interactions (Gross and Cody, 1985; Huang,

1986; Sprent and de Faria, 1989; Hurek et al., 1994; Boher et al., 1995; Vasse et al., 1995). Baldani et al. (1993) have also reported *A. brasilense* strain Sp 109 alongside *H. seropedicae* within rice seeds, and Sundaram et al. (1988) have also reported *Azospirillum* spp. in seeds of various grasses. Therefore, the bacteria could be passed from generation to generation via seeds, as suggested for *Herbaspirillum* (see earlier).

D. Other Bacteria

At present, *A. diazotrophicus* and *Herbaspirillum* spp., and possibly *Azospirillum* spp., are the only bacteria found living within grasses, such as sugar cane, which may be in sufficient numbers to account for the observed N_2 fixation rates (Boddey et al., 1995a). However, there are other diazotrophs associated with grasses (particularly rice, maize, and wheat) that may also be important. These include *Klebsiella* spp. (Fisher et al., 1992; Kennedy and Tchan, 1992; McInroy and Kloepper, 1995; Palus et al., 1996), *Alcaligenes faecalis* (You and Zhou, 1989), *Pantoea agglomerans* (Ruppel et al., 1992), *Rhizobium leguminosarum* biovar *trifolii* (Yanni et al., 1997) and diazotrophic *Pseudomonas*, *Enterobacter* and *Bacillus* spp. (Graciolli and Ruschel, 1981; Lindberg et al., 1985; Watanabe et al., 1987), and more endophytic diazotrophs are still being discovered, for example, *Azoarcus* (Hurek et al. 1994) and *Burkholderia vietnamiensis* (Gillis et al., 1995).

The newly named diazotroph, *Azoarcus* (Reinhold-Hurek et al., 1993; Hurek et al., 1994), was isolated from within the roots of Kallar grass (*Leptochloa fusca*) and will also infect rice, living in the intercellular spaces, xylem vessels, and dead root cells. *Azoarcus* can also be systemically spread within the plants that it infects (Hurek et al., 1994). The bacterium has a relatively high tolerance to oxygen, fixing N_2 in pO_2s of up to 6.5% (Vande Broek et al., 1996) but, although it

will fix N_2 in free-living cultures, Hurek et al. (1994) have shown that *Azoarcus* benefits rice via a mechanism other than N_2 fixation (see next section), and have thus suggested that it cannot be termed an "endophytic diazotroph" according to the definition of Döbereiner et al. (1993). However, more recent results from Hurek et al. (1997a) have shown that not only will intercellularly and intracellularly located (probably within dead host cells) *Azoarcus* express nitrogenase within its original host (Kallar grass) but that it may actually directly benefit the plant via its N_2 fixation (Hurek et al., 1997b).

An N_2-fixing *Burkholderia* sp. (*B. vietnamiensis*), originally isolated from rice roots, has been described recently by Gillis et al. (1995). Moreover, Hartmann et al. (1995) have suggested that "isolate E", a diazotroph isolated from within sugar cane, sweet potato, and rice (Boddey et al., 1995a), is also of the genus *Burkholderia*, but is not *B. vietnamiensis*. Recent work by Baldani (1996) (and see also Baldani et al., 1997) has shown that rice inoculated with the latter *Burkholderia* sp. can be colonized extensively by the bacteria and will fix substantial amounts of N_2.

Finally, it is worth mentioning the highly specific association between *Azorhizophilus paspali* (formerly *Azotobacter paspali*) and the tropical grass *Paspalum notatum* (Döbereiner et al., 1972; Thompson and Skerman, 1979; Kennedy and Tchan, 1992). The latter has been shown (via ARA and ^{15}N isotope dilution) to fix N_2 (Döbereiner et al., 1972; de Polli et al., 1977; Boddey and Victoria, 1986), and a strong candidate responsible for this is *A. paspali*, which has been shown to be present in the rhizosphere only of cultivars of *Paspalum notatum*, which have shown BNF (Patriquin et al., 1983). However, it is likely that *A. paspali* is not endophytic and probably only colonizes the epidermis of roots and the associated soil (Döbereiner et al., 1972) and does not actually penetrate the roots (Kennedy and Tchan, 1992). The key to its ability to fix N_2 in the rhizosphere may be its very high tolerance to O_2 (Döbereiner et al., 1972; Vande Broek et al.; 1996). For example, in a detailed study, the latter authors compared several plant-associated diazotrophs for their ability to reduce acetylene and to express *NifH* under different pO_2s and showed that *A. paspali* had significant ARA at O_2 levels above 8.5% and gave optimal nitrogenase activities and expression of *NifH* at pO_2s up to 6.5% O_2. These pO_2s were far higher than any of the other bacteria examined, including *Acetobacter* diazotrophicus, *Herbaspirillum*, and *Azospirillum* spp. (Vande Broek et al., 1996).

V. ARE ENDOPHYTIC DIAZOTROPHS RESPONSIBLE FOR BIOLOGICAL N_2 FIXATION IN GRASSES?

Sugar cane is essential for the Brazilian alcohol program, which is the largest liquid biofuel production program in the world (Boddey, 1995). One of the reasons for its cost-effectiveness is a positive energy balance partly brought about via the low N-fertilizer requirements of the cultivars used (Ruschel and Vose, 1982; Urquiaga et al., 1988, 1992; Boddey et al., 1995a,b; Döbereiner et al., 1995a). Indeed, it has long been suspected that some Brazilian sugar cane varieties will fix N_2, for example, as determined by $^{15}N_2$ gas incorporation (Ruschel et al., 1975) and ARA (Patriquin et al., 1980). This was confirmed recently at EMBRAPA/CNPAB (Seropedica, Rio de Janeiro) by ^{15}N-aided N-balance experiments in large pots (Lima et al., 1987), and in a concrete tank (Urquiaga et al., 1992). These latter studies showed that the varieties CB 45–3, SP 70–1143 and the variety of *S. spontaneum*, Krakatau, were capable of fixing up to 80% of their N requirements under ideal conditions of water and nutrient (except N) supply; extrapolation to the field suggested inputs of over 150

kg/ha/year (Boddey et al., 1995a). The studies of Lima et al. (1987) and Urquiaga et al. (1992) have been confirmed recently in the field using the ^{15}N natural abundance technique (Yoneyama et al., 1997). Interestingly, the varieties that showed the most N fixation were those that had been bred under conditions of low N fertilizer, with the varieties bred for high N-inputs showing less N_2 fixation (Urquiaga et al., 1992). Indeed, most Brazilian varieties show poor response to N fertilizer (Boddey et al., 1995a), unlike those from Cuba, Mexico, Venezuela, and Hawaii, where more than three times as much N-fertilizer is applied than in Brazil (Döbereiner et al., 1995). However, Brazilian varieties will respond to increases in molybdenum (Urquiaga et al., 1995). This is strong supporting evidence for N_2 fixation, as it is usually only N_2-fixing crops, with their requirements for the Mo-containing nitrogenase enzyme that respond so markedly to applied Mo (Johansen et al., 1977). Urquiaga and Boddey (personal communication) rule out the possibility that this Mo-response is due to increased nitrate reductase synthesis, as there was no concomittant increase in mineral N uptake.

It has long been suspected that wetland rice receives at least some of its N requirements from N_2 fixation (see review by Boddey et al., 1995a), and this has been confirmed in some varieties by $^{15}N_2$ incorporation (Eskew et al., 1981), N-balance studies (App et al., 1986), delta ^{15}N (Watanabe et al., 1987), and ARA (Watanabe et al., 1978). Sorghum is often grown in the Third World in soils deficient in N, and this has led to suggestions that genotypes have been inadvertently selected for N_2-fixing ability (Giller et al., 1986). Indeed, Wani et al. (1983) and Giller et al. (1984) have shown, using ARA and $^{15}N_2$ incorporation, respectively, that some varieties of this plant will fix N_2. There is also evidence, via $^{15}N_2$ and ARA, that maize will fix N_2 (Boddey, 1987; Alexander and Zuberer, 1989). Tropical pasture grasses, such as *Brachiaria*, *Digi-*

taria, *Panicum*, and *Paspalum* spp., have also all demonstrated some ability to fix N_2, depending on the species (de Polli et al., 1977; Boddey and Victoria, 1986; Miranda and Boddey, 1987). For example, *Brachiaria* pastures cover a vast area of South America and, despite the lack of fertilizer, do not degrade if maintenance fertilization with phosphorus and potassium is practiced. Indeed, Boddey and Victoria (1986) have shown, using ^{15}N isotope dilution, that two species (*B. humidicola*, *B. decumbens*) were capable of obtaining up to 40% of their N from N_2 fixation, although other *Brachiaria* spp. appeared not to have significant BNF.

Although Urquiaga et al. (1992) suggested that *A. diazotrophicus* was responsible for the measured BNF in sugar cane, there is actually no evidence as to the causal organism(s) of BNF in any gramineous species. Moreover, no correlation between bacterial numbers and BNF has been demonstrated, and expression of nitrogenase by any bacterium in field-grown sugar cane has yet to be shown unambiguously. Indeed, so far there is little evidence that diazotrophs such as *A. diazotrophicus*, *Herbaspirillum* spp., and *Azospirillum* spp. will fix N_2 *in planta*. The situation may be complicated by the possibility that endophytic diazotrophs may affect plants in a manner other than via N_2 fixation. For example, Hurek et al. (1994) have shown that *Azoarcus* in rice and Kallar grass has similar behavior to *Herbaspirillum* and *A. diazotrophicus* within rice and sugar cane, that is, it colonizes root intercellular spaces and subsequently enters the xylem vessels from where it is translocated. Moreover, *Azoarcus* has been shown by immunogold labeling to express dinitrogenase reductase within the roots. However, using Nif$^-$ mutants, Hurek et al. (1994) showed that increases in growth and N-accumulation in inoculated rice seedlings were due to a process other than BNF. Indeed, it is possible that endophytic *Azospirillum* spp., *Pantoea agglomerans*, and *A. dia-*

zotrophicus may give positive growth responses in inoculated plants due to a number of processes, including plant hormone production, improvement in mineral uptake, and bacterial nitrate reductase (Okon and Kapulnik, 1986; Boddey and Döbereiner, 1988; Bashan and Levanony, 1989; Sumner, 1990; Ruppel et al., 1992; Fuentes-Ramirez et al. 1993). Therefore, a simple growth response by an inoculated plant and/or ability of a particular bacterium to fix N_2 *ex planta* does not necessarily mean that the bacteria associated with the plant are fixing N_2, or passing the products of BNF to the plant. For example, although authors of some recent studies have suggested that the simple presence of *A. diazotrophicus* in a sugar cane cultivar is evidence of an actual N_2-fixing "symbiosis" (for example, cvs. Ja 60-5 and Media Luna; Dong et al., 1994), clearly more evidence is needed before such claims can be made. In addition, although many cultivars will not respond well to nitrate-containing fertilizer (Ruschel and Vose, 1982; Abellan et al., 1994; Döbereiner et al., 1995a), the fact that sugar cane will recycle much of its N (Ruschel and Vose, 1982) shows that this also cannot be used as a reliable indicator of BNF. The same arguments can also be applied to other crops (e.g., sorghum), as Giller et al. (1986) have shown that gains in N content attributed to BNF can also be attributed to efficient scavenging of N from the soil by the plant. Therefore, is there *any* evidence that endophytic diazotrophs actually fix N_2 *in planta* and, if so, where is the BNF taking place, and are the products of N_2 fixation transferred to the plant?

James et al. (1994) showed ARA in *A. diazotrophicus*-infected plantlets of the cultivar NA 56–79, and Reis et al. (1994) have shown ARA in *A. diazotrophicus*-inoculated setts of cvs. SP 70–1143 and SP 79–2312. All these cultivars have been shown previously by ^{15}N isotope dilution to fix N_2 in the field (Urquiaga et al., 1992). Although these small studies are indirect supporting evidence for

A. diazotrophicus fixing N_2 in these cultivars, they should not be extrapolated too far, and clearly more work is needed on examining BNF by diazotrophs in field-grown plants. However, such studies do give some indication as to where N_2-fixing *A. diazotrophicus* and other diazotrophs may be located. For example, the nodes are a possible location for the expression of nitrogenase by the bacteria, as the tissue is dense and hence will have a low pO_2 (Clements, 1980; James et al., 1994), although the internodes of mature plants may also be dense (Clements, 1980). Within either the nodes or the internodes, the bacteria could be living in the intercellular apoplast surrounding the storage parenchyma, as this is filled with a solution containing a high sucrose concentration (pH 5.5), which is a very suitable medium for the growth of *A. diazotrophicus* (Dong et al., 1994). However, against this, in a recent study of bacteria within the apoplast of field-grown sugar cane, the bacteria in the micrographs of Dong et al. (1994) were few in number, were not identified as being *A. diazotrophicus* (via antibodies or molecular markers), and no evidence was presented that they were expressing nitrogenase. James et al. (1994), Döbereiner et al. (1995b) and Sprent and James (1995) have suggested that the xylem vessels may be a possible location for nitrogenase expression as the pO_2 is low (Clements, 1980). However, as with *A. diazotrophicus* colonizing the intercellular apoplast (James et al., 1994; Dong et al., 1994; Fuentes-Ramirez et al., 1997), xylem-dwelling *A. diazotrophicus* has not yet been shown to express nitrogenase (or *Nif* genes; Fuentes-Ramirez et al., 1997). On a more positive note, although it has not been observed in *A. diazotrophicus*, nitrogenase expression by *H. rubrisubalbicans* has been demonstrated (using immunogold labeling) in dense colonies within sugar cane leaf intercellular spaces (Olivares et al., 1997), and in the protoxylem of sorghum leaves (James et al., 1997). Moreover, Hurek et al. (1994,

1997a,b) have demonstrated nitrogenase expression by *Azoarcus* within rice and Kallar grass (see earlier). Olivares et al. (1997) and James et al. (1997) suggested that the low pO_2 conditions necessary for expression of nitrogenase proteins (Gallon, 1992) was possibly satisfied by a high respiratory O_2 uptake by the large number of bacteria within the colonies and/or by xylem vessels having an inherently low pO_2 (Clement, 1980; Sprent and James, 1995). In support of this, earlier studies by Patriquin et al. (1980) have shown (unidentified) tetrazolium-reducing bacteria in the xylem and intercellular spaces of sugar cane stems showing ARA, and Patriquin and Döbereiner (1978) showed tetrazolium-reducing bacteria (probably *A. lipoferum*) within the roots of maize, sorghum, wheat, *P. maximum*, and *D. decumbens*. Therefore, it appears that both the intercellular apoplast and the xylem vessels are possible locations for nitrogenase expression by bacteria within grasses.

Even though nitrogenase activity by endophytes within grasses may be a possibility, this does not automatically mean that the products of N_2 fixation are released by the bacteria in a form that is available for use by the host plant, except for when the bacteria die. However, there is some circumstantial evidence that specific endophytic diazotrophs can release NH_4^+/fixed N. For example, on the basis of ^{15}N isotope dilution experiments with axenically grown rice inoculated with the bacteria, Baldani et al. (1995) showed that *H. seropedicae* strain Z94 contributed over 50% of the total N accumulated by the plants, and Reis Jnr (unpublished data) has shown a positive effect of inoculation by *A. diazotrophicus* and *H. seropedicae* on root growth of sugar cane cv. NA 56–79. In addition, in model yeast/bacterial systems, *A. diazotrophicus* has been shown to release up to 48% of the N that it fixes (Cojho et al., 1993). This is an essential prerequisite if endophytic diazotrophs are to be of any benefit to the plants that they inhabit (Boddey et al., 1995b) and, again, the

xylem vessels, as the main sites of N-transport within the plant, would be an advantageous location (at least for the plant) for this to occur (Sprent and James, 1995).

Some work has also been done with *Azospirillum* in this respect, for example, increased N-accumulation in ^{15}N-labeled soil by some maize varieties has been shown recently after inoculation by various Argentinian *A. brasilense* and *A. lipoferum* strains (see Boddey and Döbereiner, 1995). The endophytic *A. brasilense* strain Sp 245 has also been shown to increase the N-accumulation and yield of wheat and sorghum compared with the root surface-colonizing strains Sp 7 and Cd (Baldani et al., 1986b,c). However, although *nifA* expression and enhanced ARA (Katupitiya et al., 1995), as well as release of NH_4^+ (Christiansen-Weniger, 1992; Christiansen-Weniger and Vanderleyden, 1994), has been shown by mutant *A. brasilense* strains in association with wheat roots, there is still no direct evidence that wild-type *Azospirillum* in the field are fixing N_2 and releasing it to host plants.

VI. FUTURE WORK

Clearly, much work has yet to be done to confirm that endophytic diazotrophs are responsible for BNF by sugar cane and other grasses. In particular, field-grown plants of sugar cane cultivars showing different BNF abilities (Lima et al., 1987; Urquiaga et al., 1988, 1992) should be examined more critically to see if (1) there is a correlation between bacterial numbers and BNF, and (2) that there are sufficient numbers of bacteria within the plants to be of significance.

Large numbers of N_2-fixing bacteria are actually of critical importance, as, for example, one large soybean (*Glycine max*) nodule contains 10^9 bacteria (Baldani et al., 1986b). Theoretically, a "symbiotic" sugar cane plant should have a similar concentration of N_2-fixing bacteria, at least for part of the growing season, if they are to fix up to 80% of

their N requirements for that season (Urquiaga et al., 1992). This number of bacteria should be clearly visible by microscopy, as well as by normal enumeration methods such as counts using selective semi-solid media (Döbereiner et al., 1993; Boddey et al., 1995a,b), and ELISA with species and strain-specific antibodies (Li and MacRae, 1992; Schloter et al., 1992; Boddey et al., 1995b). Counts using these methods should be done over at least one growing season to check whether there is ontogenic variation in bacterial numbers throughout all the plant tissues (da Silva et al., 1995). It may be that there is only an early "flush" of BNF followed by recycling of the fixed N thereafter (Ruschel and Vose, 1982). With respect to bacterial numbers, it is worth mentioning at this point that a recent study by Fuentes-Ramirez et al. (1997), combined with the studies of James et al. (1994) and Dong et al. (1994), observed very low numbers of an *A. diazotrophicus* strain labeled with a *GUS*-fusion within inoculated sugar cane plants, but they suggested that if the bacteria were distributed evenly throughout large plants the *total* number of bacteria may be sufficient to supply at least some of the N-needs of the host plant. It should also be noted that the % N of sugar cane is very low compared with an N_2-fixing legume such as soybean. Therefore, demands for N are not as high, and hence the actual numbers of diazotrophic bacteria required to support it may not need to be quite so high as in legume nodules. For example, green sugar cane leaves typically only have between 1.0 and 1.2% N, and this can be reduced to as little as 0.2 to 0.4% N in stems and senescent leaves (Urquiaga et al., 1988), whereas soybean can have from 2 to 6% N per plant (Ryle et al., 1978).

Once simple enumeration has established that all or some endophytic diazotrophs could be of importance, it will be necessary to examine plants more closely to see if, when, and where "symbiotic" structures (and/or large bacterial concentrations) are present, and if the bacteria within these structures/concentrations express nitrogenase and transfer the products of BNF to the plants. Bacteria could be recognized using specific antibodies (Levanony et al., 1989; James et al., 1994, 1997; Hurek et al., 1994; Schloter et al., 1994; Olivares et al., 1997), and nitrogenase expression could be examined *in situ* using ARA and immunolabeling with nitrogenase-specific antibodies (Hurek et al., 1994; Olivares et al., 1997; James et al., 1997). Moreover, in addition to field-grown material, it would be useful also to examine axenically grown plants of sugar cane cultivars showing BNF. These could be separately inoculated with *A. diazotrophicus*, *Herbaspirillum* spp., and *Azospirillum* spp and be examined for their ability to incorporate $^{15}N_2$. This would provide proof that individual endophytic diazotrophs could fix N_2 *in planta* and transfer the products to the plant (Giller et al., 1984; Boddey, 1987). Nif⁻ mutants of these diazotrophs, if and when they are available, would also assist in determining if BNF by the bacteria, or some other mechanism, was responsible for growth and N-accumulation (Hurek et al., 1994, 1997b).

A concerted program of research combining the field and laboratory techniques described above should answer many of the questions concerning BNF in grasses. This would enable these systems to be managed more efficiently and economically, and may also assist in their introduction to other regions.

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