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REVIEW

Nitrogenase genes in non-cyanobacterial plankton: prevalence, diversity and regulation in marine waters

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ABSTRACT: Marine waters are generally considered to be nitrogen (N) limited and are therefore favourable environments for diazotrophs, i.e. organisms converting atmospheric N_2 into ammonium or nitrogen oxides available for growth. In some regions, this import of N supports up to half of the primary productivity. Diazotrophic *Cyanobacteria* appear to be the major contributors to marine N_2 fixation in surface waters, whereas the contribution of heterotrophic or chemoautotrophic diazotrophs to this process is usually regarded inconsequential. Culture-independent studies reveal that non-cyanobacterial diazotrophs are diverse, widely distributed, and actively expressing the nitrogenase gene in marine and estuarine environments. The detection of *nifH* genes and *nifH* transcripts, even in N-replete marine waters, suggests that N_2 fixation is an ecologically important process throughout the oceans. Because this process is highly sensitive to and inhibited by molecular oxygen (O_2), diazotrophy requires efficient scavenging of intracellular O_2 or growth in environments with low ambient O_2 concentration. Particles with interior low-O_2 micro-zones and oceanic oxygen minimum zones are just 2 potential habitats suitable for N_2 fixation by non-cyanobacterial diazotrophs. Our ignorance about the regulation of N_2 fixation by non-*Cyanobacteria* in their natural marine environments currently prevents an evaluation of their importance in marine N cycling and budgets. A review of the molecular data on distribution and expression of *nifH* genes in non-*Cyanobacteria* suggests that further study of the role of these *Bacteria* in N cycling at local, regional and global scales is needed.

KEY WORDS: N_2 fixation · Nitrogen fixation · Non-*Cyanobacteria* · *nifH* · Nitrogenase

INTRODUCTION

Biological nitrogen (N) fixation, the conversion of nitrogen gas (N_2) into ammonium, is widespread in the tropical and subtropical oligotrophic oceans (e.g. Capone et al. 2005), where it may fuel up to ~50% of new primary production (Karl et al. 1997). In contrast, this process is believed to be rather insignificant in temperate or cold pelagic oceanic regions (LaRoche & Breitbarth 2005). Biological N_2 fixation (or diazotrophy) is confined to the prokaryotic domains *Bacteria* and *Archaea*. N_2-fixing *Archaea* appear, thus far, to be limited to the methanogens (e.g. Chien & Zinder 1996, Mehta et al. 2005), but diazotrophy is widely distributed among diverse phenotypes and genotypes of *Bacteria* (Paerl & Zehr 2000, Zehr et al. 2003b). Given the importance of N_2 fixation in marine N cycling, identification of the organisms involved and the factors that determine their activity is essential for our understanding of ecosystem productivity and for N biogeochemistry (Horner-Devine & Martiny 2008).

Cyanobacteria are the most well-studied marine diazotrophs, and filamentous forms such as the free-living *Trichodesmium* spp. (Capone et al. 1997) and

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the diatom symbiont *Richelia* spp. (Carpenter et al. 1999) were long thought to be the only important diazotrophs in the ocean. However, molecular analyses of the *nifH* gene, coding for the iron (Fe) protein subunit of the nitrogenase enzyme (Zehr & McReynolds 1989), has provided important new insights into the diversity, distribution and activity of diazotrophs. For example, Zehr et al. (2001) found evidence that N_2 fixation by unicellular *Cyanobacteria* may equal or even exceed that of *Trichodesmium* in some tropical regions. The same molecular methods have also revealed putative N_2-fixing non-*Cyanobacteria* (possessing or even expressing the *nifH* gene) in diverse aquatic environments (Zehr et al. 1998, Braun et al. 1999, Zani et al. 2000, Mehta et al. 2003, Steward et al. 2004b, Boström et al. 2007a) and an almost ubiquitous distribution of these diazotrophs in marine waters (Farnelid & Riemann 2008). Still, knowledge about their importance and ecological role in the marine water column is scant, partly limited by ignorance about how N_2 fixation is regulated in these organisms and where, and on what space and time scales, they are actively fixing N_2.

Here we review the most recent information on the composition and distribution of non-cyanobacterial diazotrophs in marine waters. We then discuss the distribution and expression of nitrogenases in the context of environmental constraints on N_2 fixation and speculate on potential loci for N_2 fixation by non-*Cyanobacteria* in pelagic marine environments.

NITROGENASE GENES FROM NON-*CYANOBACTERIA* ARE WIDESPREAD IN MARINE ENVIRONMENTS

The *nifH* gene contains regions of sequence that are sufficiently conserved across phylogenetic groups such that they can serve as 'universal' PCR priming sites using moderately degenerate primers (Zehr & McReynolds 1989, Kirshtein et al. 1991). Therefore, molecular studies of the diversity and composition of diazotrophic organisms have been largely based on analyses of this gene, generating one of the largest non-ribosomal gene datasets on uncultivated microorganisms (Zehr et al. 2003b). *NifH* genes are present in a broad spectrum of prokaryotes, and the phylogenetic relationships among these organisms based on *nifH* sequences are largely congruent with those determined from 16S rRNA gene sequences (Zehr et al. 2003b). This suggests that horizontal gene transfer of the *nifH* gene has been limited (Zehr et al. 2003b), although it has been observed for several bacterial species (Raymond et al. 2004, Kechris et al. 2006, Bolhuis et al. 2010). Consequently, *nifH* sequences are used to tentatively infer phylogenetic affiliations of diazotrophs in microbial communities. A single microorganism can, however, have multiple phylogenetically distinct paralogous versions of *nifH* (Zehr et al. 2003b). This somewhat limits the inferences that can be made about diazotroph species richness in a sample based solely on data from a clone library.

NifH gene clone libraries show that non-cyanobacterial diazotrophs are distributed throughout the 4 canonical *nifH* clusters (Chien & Zinder 1996) and are widespread in lakes, estuaries and marine waters (reviewed by Zehr et al. 2003b). Proteobacterial *nifH* phylotypes within Cluster I (molybdenum nitrogenases from *Cyanobacteria* and *Proteobacteria* [*Alpha-*, *Beta-* and *Gamma-* subgroups] and vanadium nitrogenases [*vnfH*] from *Gammaproteobacteria*), are particularly prevalent in marine waters (Fig. 1). For instance, similar *Gammaproteobacteria* have been detected in the Pacific and Atlantic Oceans, and the Arabian and South China Seas (Bird et al. 2005, Langlois et al. 2005, 2008, Church et al. 2005a, 2008, Moisander et al. 2008). Alpha- and betaproteobacterial *nifH* genes are also widespread and commonly detected (e.g. Zehr et al. 1998, Falcón et al. 2004, Man-Aharonovich et al. 2007, Hewson et al. 2007a, Moisander et al. 2008, Rees et al. 2009).

Few sequences within Cluster II (archaeal nitrogenases and bacterial iron nitrogenases, *anfH*) have been obtained from the environment (Zehr et al. 2003b), although members of both Cluster II and, more commonly, Cluster III (diverse distantly related anaerobic *Bacteria* such as *Clostridium* and sulphate reducers, *Deltaproteobacteria*) have been detected in coastal or estuarine waters (Affourtit et al. 2001, Jenkins et al. 2004, Steward et al. 2004a, Moisander et al. 2007, Man-Aharonovich et al. 2007, Farnelid et al. 2009), or near hydrothermal vents (Mehta et al. 2003). Cluster III phylotypes are rather uncommon in the open ocean, but do appear occasionally in clone libraries from this habitat (Langlois et al. 2005, 2008). Since the cultivated representatives within Cluster III are mostly, if not all, strict anaerobes (Zehr et al. 2003b), it has been speculated that the Cluster III phylotypes reside in anoxic microzones in the oxygenated water column, for instance on particles or in association with zooplankton (Braun et al. 1999, Church et al. 2005a, Man-Aharonovich et al. 2007). This Cluster is, however, characterised by large distances between sequences (Zehr et al. 2003b) and it may therefore be plausible, and ecologically meaningful, if many of these planktonic phylotypes in fact represent facultative anaerobic *Bacteria*. This possibility is supported by the fact that they are found in both the free-living and particle-attached fractions of pelagic waters (Church et al. 2005a, Farnelid et al. 2009). Finally, Cluster IV, including diver-

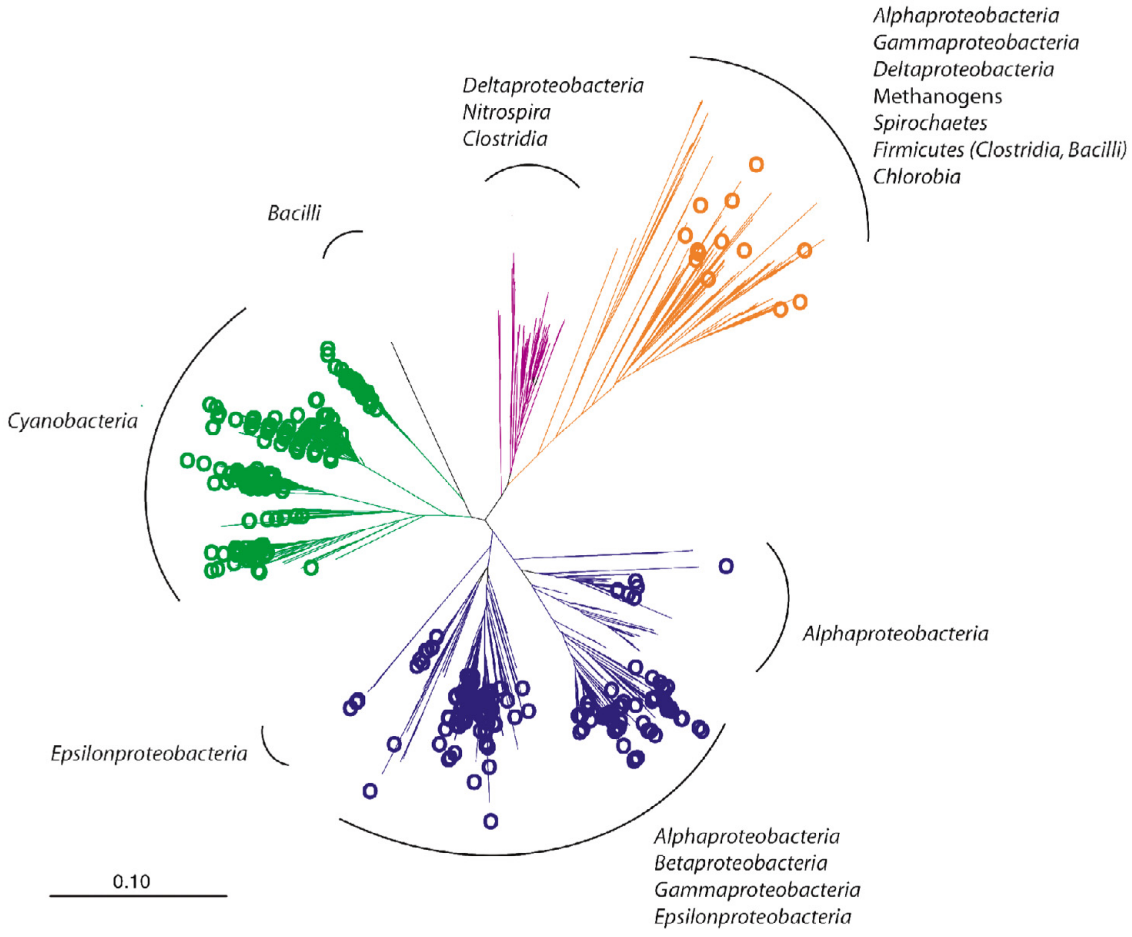

Fig. 1. Phylogenetic tree illustrating the diversity among 2570 *nifH* genes amplified from microorganisms in plankton samples from marine and estuarine environments. Branches derived from sequences (n = 809) from open ocean samples are marked with a "O". Green and blue branches belong to a loosely affiliated group of sequences designated as Cluster I (Chien & Zinder 1996), orange branches are affiliated with Clusters II and III. Purple branches are not assigned to the traditionally defined clusters. The tree (neighbour-joining, with no distance corrections) was generated using Arb (Ludwig et al. 2004) and is based on amino acid residues 46 to 151 (*Azotobacter vinelandii* numbering) translated from PCR-amplified fragments of *nifH* genes or transcripts. Sequences were downloaded as an Arb database that was last updated on 30 March 2009 (http://pmc.ucsc.edu/~wwwzehr/research/database/) and aligned by a hidden Markov model for *nifH* available at the Pfam web site (Finn et al. 2010). Sequences from marine and estuarine samples (oceans, seas, bays, gulfs, harbours, lagoons) were retrieved from the database, and those from sediments, hydrothermal vents or associated with sessile plants and animals were excluded. Open ocean samples include those from the Atlantic and Pacific Oceans and the Arabian Sea, excluding nearshore environments (gulfs, bays, harbours, fjords) and inland seas (Baltic, Mediterranean). Clusters are labelled to indicate the phylogenetic affiliations of cultivated microorganisms whose *nifH* sequences most closely match those from the uncultivated microorganisms shown in the tree. A total of 66% of all sequences shown are non-*Cyanobacteria*, and 36% of those from the open ocean are non-*Cyanobacteria*

gent non-functional archaeal *nifH* homologues, have been reported from the deep sea (Mehta et al. 2003).

Based on clone libraries, *nifH* sequences from non-*Cyanobacteria* appear to be not only diverse, but also abundant relative to those of *Cyanobacteria*. This is particularly evident from a recent compilation of published marine *nifH* gene clone libraries (Farnelid & Riemann 2008) showing that 73 to 91% (average 83%) of the total number of *nifH* sequences obtained were related to non-*Cyanobacteria*. The data compiled here (Fig. 1) similarly show that 80% of the sequences from coastal marine and estuarine plankton samples derive from non-*Cyanobacteria*. In the open ocean the proportion is lower, with ca. 36% of the sequences deriving from non-*Cyanobacteria*, but this likely underestimates their numerical contribution, since many oceanographic studies sample most intensively in the euphotic zone, and clone libraries from below ~200 m consist almost exclusively of non-cyanobacterial *nifH* genes (e.g. Hewson et al. 2007a).

While *nifH* clone libraries provide convincing evidence for a near-ubiquitous distribution of non-

cyanobacterial diazotrophs in marine waters, the information can only be considered qualitative, since endpoint PCR is not suitable for quantitative analyses (von Wintzingerode et al. 1997). In most cases, the inserts for these libraries were prepared by nested PCR amplification using degenerate primers and many cycles (≥60), which means that the resulting clone libraries are susceptible to amplification biases (Polz & Cavanaugh 1998). Primer bias has been observed in *nifH* amplifications, and the severity varies among primer sets (Demba Diallo et al. 2008). High cycle numbers, which are often required because diazotrophs typically make up a small proportion of microbial communities, also increase the chance of amplifying trace contaminants in the PCR reagents (Zehr et al. 2003a, Goto et al. 2005). It is likely that some proteobacterial *nifH* sequences reported to be from the environment actually originated from contaminants in PCR reagents, but this is difficult to prove conclusively. Those sequences most likely to be derived from reagent contaminants can be identified by amplification, cloning and sequencing of no-template controls and removed from sample libraries (Boström et al. 2007b).

Metagenomics is an alternative means of analysing genetic diversity that can circumvent the problems inherent in PCR. Unfortunately, this approach has not proven practical for surveys of *nifH*, because of the relatively low abundance of diazotrophs in assemblages of marine bacterioplankton (Johnston et al. 2005). More promising is the use of Real-Time PCR for quantification of specific *nifH* phylotypes. This approach circumvents some of the biases of endpoint PCR and may provide quantitative data on the spatio-temporal distribution of individual phylotypes. A significant number of recent studies have applied this approach to quantify diazotrophs in marine waters (Table 1). These data support the conclusion drawn from clone libraries that, like N_2-fixing *Cyanobacteria*, non-cyanobacterial diazotrophs are widespread and abundant in marine waters.

In some cases *Cyanobacteria* are the dominant diazotrophs in marine planktonic communities (Church et al. 2005b, Zehr et al. 2007, Langlois et al. 2008, Moisander et al. 2008), but heterotrophic phylotypes are sometimes just as abundant, even in the surface ocean, as the most common cyanobacterial diazotrophs, *Trichodesmium* and the unicellular cyanobacterial Groups A and B (Church et al. 2005a, Fong et al. 2008). The specific diazotrophs represented in marine microbial communities vary as a function of depth. For instance, a clone library from the subtropical Atlantic Ocean suggested a shift from dominance by filamentous *Cyanobacteria* in the surface waters to dominance by unicellular *Cyanobacteria* and/or heterotrophic *Bacteria* in deeper waters (Langlois et al. 2005). Similarly, in the subtropical North Pacific Ocean, a Cluster III phylotype was almost as abundant as the Group A *Cyanobacteria* in the upper water column (~10^4 to 10^5 *nifH* copies l^{-1}), and was dominant at 100 to 200 m depth (Church et al. 2005a). Such observations suggest that N_2 fixation may be underestimated if only illuminated surface waters are considered (Hewson et al. 2007a).

The increasing proportion of non-cyanobacterial diazotrophs with depth suggests that the distribution of these *Bacteria* is not constrained by light or, in contrast to oceanic *Cyanobacteria* (Stal 2009), by temperature. Although many of the investigations of nitrogenase gene diversity have been conducted in tropical or subtropical regions, cyanobacterial and heterotrophic diazotrophs have also been reported from temperate waters (Affourtit et al. 2001, Wasmund et al. 2001, Holl

Table 1. *In situ* abundance of nitrogenase (*nifH*) genes from non-*Cyanobacteria* quantified using real-time PCR

NifH genes (copies l^{-1} seawater)	Phylotype	Sea area	Depth (m)	Source
34000	Gammaproteobacterial isolate	Baltic Sea	Surface	Boström et al. (2007a)
60000[a]	*Alphaproteobacterium*	South China Sea	Epipelagic	Moisander et al. (2008)
700[a]			Mesopelagic	
5000[a]	*Gammaproteobacterium*		Mesopelagic	
1000 – 100000	Two phylotypes	Chesapeake Bay	1 – 20	Short et al. (2004)
1000 – 10000	*Gammaproteobacterium*	Northern Atlantic Ocean	5 – 120	Langlois et al. (2008)
16000[a]	Cluster III			
1000 – 100000[b]	Cluster III	Northern Pacific Ocean	25 – 200	Church et al. (2005a)
0 – 92000 ± 27000	*Gammaproteobacterium*	Northern Pacific Ocean	25	Zehr et al. (2007)
10000 – 100000	*Gammaproteobacterium*	Northern Pacific Ocean	0 – 100	Fong et al. (2008)
1 – 43	Three phylotypes	Sargasso Sea	1000 – 5948	Hewson et al. (2007a)
80 – 700	*Gammaproteobacterium*, cDNA	Northern Pacific Ocean	0 – 175	Church et al. (2005b)

[a]Maximum abundance
[b]Most abundant phylotypes below 100 m

et al. 2007, Man-Aharonovich et al. 2007, Needoba et al. 2007, Farnelid et al. 2009, Rees et al. 2009). For instance, in the Baltic Sea a cultivated *Gammaproteobacterium* was found at 3.0×10^4 *nifH* gene copies l^{-1} (Boström et al. 2007a), while in Chesapeake Bay 2 proteobacterial *nifH* phylotypes were present at $\geq 1.4 \times 10^5$ copies l^{-1} (Short et al. 2004). In summary, molecular analyses of samples from pelagic marine waters strongly suggest that non-cyanobacterial diazotrophs are diverse, widely distributed, and relatively abundant, but do they actually express the nitrogenase gene? If so, which factors regulate presence and *nifH* expression for these phylotypes *in situ*?

EXPRESSION OF NON-CYANOBACTERIAL *nifH* GENES

Presence of non-cyanobacterial *nifH* genes does not necessarily imply that these microorganisms are contributing to N_2 fixation. From what is known from cultivated organisms, however, the presence of *nifH* mRNA is a reasonable indicator of active N_2 fixation (e.g. Chien & Zinder 1996, Eady 1996, Sicking et al. 2005). For this reason, recent studies have begun to investigate the diversity among *nifH* transcripts in marine systems. Clone libraries based on PCR-amplified *nifH* cDNA from the Atlantic and Pacific Oceans as well as the Mediterranean and Arabian Seas have documented the expression of *nifH* by diverse non-cyanobacterial (particularly proteobacterial) diazotrophs (Zehr et al. 2001, Falcón et al. 2004, Bird et al. 2005, Church et al. 2005b, Man-Aharonovich et al. 2007, Fong et al. 2008, Rees et al. 2009). For instance, non-cyanobacterial genes accounted for 88% of the 105 *nifH* cDNA clones sequenced from the Mediterranean Sea (Man-Aharonovich et al. 2007). In the North Pacific Subtropical Gyre, 17% of 135 *nifH* cDNA clones were related to non-*Cyanobacteria* (Church et al. 2005b). Only a few studies have examined the spatial distribution of these expressed phylotypes. In the North Pacific, transcript concentrations for the targeted gammaproteobacterial phylotype, on average 400 *nifH* cDNA copies l^{-1} (Church et al. 2005b), were found to be lower than those of the targeted cyanobacterial transcript concentrations in samples from <100 m depth (Church et al. 2005b, Zehr et al. 2007). However, the specific expression (i.e. transcripts normalised to *nifH* gene concentration) for a gammaproteobacterial phylotype was comparable to or even exceeded values for targeted groups of *Cyanobacteria* (Zehr et al. 2007). Similarly, Real-Time PCR-derived inventories of *Proteobacteria*-like *nifH* copies in this region were as great as the inventories of the unicellular *Cyanobacteria* and some of the heterocystous *Cyanobacteria*, suggesting that these phylotypes account for a large fraction of the *nifH* containing plankton (Fong et al. 2008). Also, data from the Gulf of Aqaba in the Red Sea suggested an important role for proteobacterial diazotrophs in local N_2 fixation (Foster et al. 2009). Hence, based on the relative abundance and expression of *nifH* genes, non-*Cyanobacteria* could make a significant contribution to overall N_2 fixation.

A curious feature of the expression data is that only few of the phylotypes present in DNA clone libraries are present in cDNA libraries prepared from the same sample (Zani et al. 2000, Man-Aharonovich et al. 2007, Zehr & Paerl 2008, Rees et al. 2009). Similar results have been observed in *nifH*-based oligonucleotide microarray studies of microbial mats and rice roots (Moisander et al. 2006, Zhang et al. 2007). These data suggest that relatively few of the diazotrophs possessing *nifH* in a particular environment actively transcribe the gene. The high rate of mutation in microbial populations suggests, however, that only functional genes providing a selective advantage should be fixed in a population (Berg & Kurland 2002). Consequently, conserved genes, like *nifH*, should at least occasionally be expressed and encode functions of cellular importance (e.g. Tyson et al. 2004). The contrast between the DNA and cDNA data suggests that we still have much to learn about the regulation of *nifH* gene expression and the evolutionary pressures relevant to maintenance of this gene.

Even for those *Bacteria* that have been shown to transcribe the nitrogenase gene *in situ*, an open question is how these *Bacteria* cope with the conditions in the open ocean that would appear to be unfavourable for N_2 fixation. For most diazotrophs, *nifH* expression is coordinated in response to the availability of reduced N and the presence of O_2 (reviewed by Dixon & Kahn 2004); however, the pelagic realm is oligotrophic, generally well oxygenated, and the bulk of the ocean's interior is replete with inorganic N sources. The mechanisms by which cyanobacterial diazotrophs cope with life in the open ocean has been well studied (e.g. see reviews by Bergman et al. 1997, Stal & Zehr 2008, Stal 2009), but whether the lessons learned for cyanobacterial diazotrophs apply to non-*Cyanobacteria* is unknown. Below, we discuss a few selected factors that may be relevant for understanding the regulation of N_2 fixation by non-*Cyanobacteria*.

REGULATION OF NON-CYANOBACTERIAL DIAZOTROPHS *IN SITU*: CARBON AND NITROGEN

Most information on the regulation of N_2 fixation in heterotrophic *Bacteria* comes from soil studies (Gallon

1992, Marchal & Vanderleyden 2000, Karl et al. 2002), but some heterotrophic N_2-fixing *Bacteria* have been isolated from seawater (Maruyama et al. 1970, Werner et al. 1974, Wynn-Williams & Rhodes 1974, Guerinot & Colwell 1985, Tibbles & Rawlings 1994, Boström et al. 2007a). Transcription and translation of the nitrogenase genes, and the activity of the resulting enzyme, generally appear to be tightly regulated (Paerl & Zehr 2000), and post-translational mechanisms regulating nitrogenase activity have also been described (e.g. Kanemoto & Ludden 1984, Pope et al. 1985, Zehr et al. 1993). However, to what extent these findings are representative of processes occurring in the diazotrophs that inhabit marine pelagic environments is unknown.

The availability of organic carbon may limit N_2 fixation in non-cyanobacterial diazotrophs due to the high energy requirements of the reaction (Zehr & Capone 1996). Interestingly, Church et al. (2005b) observed that *nifH* expression by a gammaproteobacterial phylotype exhibited a slight diurnal periodicity, which may reflect a linkage to organic matter substrates provided by photoautotrophs (Zehr & Paerl 2008). However, in clone libraries from the subtropical North Pacific, *nifH* transcripts clustering with *Alpha*- and *Gammaproteobacteria* were found in both day and night samples (Falcón et al. 2004). Presently, the available data from marine waters are too sparse to draw any firm conclusion about the potential carbon limitation of N_2 fixation by non-*Cyanobacteria*.

Some marine *Bacteria*, in addition to growing heterotrophically, can use light-driven proton pumps to generate ATP (Béja et al. 2000). The extra energy available to photoheterotrophs growing in the light may facilitate growth in oligotrophic environments (Gómez-Consarnau et al. 2007). Some facultatively photoautotrophic *Bacteria*, e.g. *Rhodospirillum*, *Rhodopseudomonas* and *Rhodobacter* species, are able to grow diazotrophically (Meyer et al. 1978, Oelze & Klein 1996), and *nifH* sequences clustering with these species have been obtained from marine waters (Zehr et al. 2003b, Moisander et al. 2008, H. Farnelid & L. Riemann unpubl.). Moreover, photoautotrophic diazotrophs have been isolated from marine copepods (Proctor 1997). Hence, it is tempting to speculate that phototrophy helps support the high energy demands associated with N_2 fixation by some non-cyanobacterial diazotrophs in the photic zone.

Unlike *Cyanobacteria*, putative diazotrophic non-*Cyanobacteria* are not confined to the photic zone. Rather, their relative abundance increases with depth (e.g. Church et al. 2005a). In fact, *nifH* genes (e.g. Short et al. 2004, Moisander et al. 2006, Zehr et al. 2007, Hewson et al. 2007b, Langlois et al. 2008) and *nifH* transcripts (e.g. Bird et al. 2005, Short & Zehr 2007, Zehr et al. 2007, Man-Aharonovich et al. 2007) from these organisms are commonly detected in environments where microbial growth would not be assumed to be limited by reduced N. For instance, *nifH* phylotypes were found in the vicinity of hydrothermal vents, an environment rich in reduced N (Mehta et al. 2003). Further, *nifH* transcripts of *Gammaproteobacteria* have been detected in samples from below the nutricline at nitrate/nitrite concentrations of >5 µM and in surface waters at productive coastal sites (Bird et al. 2005). For communities, noteworthy examples include measurable N_2 fixation in the tropical Atlantic at nitrate concentrations of ~10 µM (Voss et al. 2004) and the observation that addition of ammonium and nitrate did not reduce N_2 fixation in salt marsh microbial communities (Hanson 1977). Insensitivity to the presence of dissolved inorganic N has also been observed experimentally for some marine communities and individual isolates. For instance, Boström et al. (2007a) measured acetylene reduction by a gammaproteobacterial Baltic Sea isolate in medium amended with ammonium (0.5 mM), and Tibbles & Rawlings (1994) found that nitrogenase activity was only repressed by nitrate in 1 of 3 heterotrophic marine isolates. When initially grown with N_2 as the sole N source, the nitrogenase activity of *Trichodesmium* was not suppressed after addition of 2 mM nitrate or 0.02 mM ammonium (Ohki et al. 1991), although other studies have reported inhibition of N_2 fixation in *Trichodesmium* by nitrate (Holl & Montoya 2005), ammonium (Capone et al. 1990) and other N sources (Mulholland & Capone 2000). Overall, these observations contrast with the prevailing perception that N_2 fixation is highly regulated by the availability of reduced inorganic N (Klugkist & Haaker 1984 and references therein). Hence, it appears that factors other than just N availability influence the distribution and expression of *nifH*.

It has been proposed that a lack of tight ammonium regulation is a general feature of diazotrophic organisms from tropical and subtropical waters (Bird et al. 2005). Similarly, it appears that high nitrate concentrations do not select against all diazotrophs (Langlois et al. 2008). Interestingly, recent genomic data suggest that one explanation for continued N_2 fixation in the presence of reduced N sources is that some *Bacteria* are simply not equipped to use other N sources. The genomes of 2 isolates in the marine *Roseobacter* clade lacked both nitrate and nitrite transporters, and experimental studies demonstrated that 1 of the strains (*Silicibacter pomeroyi*) could not assimilate nitrate (Moran et al. 2007). Similarly, Moore et al. (2002) found that their *Prochlorococcus* isolates were not able to grow with nitrate as the only N source, and Allen et al. (2001) observed that structural genes for nitrate assimilation (*nasA*) could only be amplified from certain

marine heterotrophic *Bacteria*. Hence, the lack of assimilatory enzymes to utilise either nitrate or nitrite may potentially explain nitrogenase expression in N-replete environments, as was suggested for a gammaproteobacterial diazotrophic clade found in the Arabian Sea (Bird et al. 2005). It has been suggested that organisms designed for diazotrophy have few ecological reasons to invest in a switch to nitrate uptake, since this reduction is energetically more expensive than the reduction of N_2 (Karl et al. 2002). Thus, the regulation of nitrogenase expression by reduced N is not straightforward and most likely involves species-specific responses to specific forms of reduced inorganic N.

OXYGEN-DEPRIVED LOCI FOR N_2 FIXATION BY NON-*CYANOBACTERIA* IN THE MARINE WATER COLUMN

The most abundant component of our atmosphere, N_2, is the substrate for the nitrogenase enzyme, while the next most abundant component, O_2, not only inactivates and destroys nitrogenase, but also represses nitrogenase synthesis. Thus, protecting the nitrogenase from O_2 is crucial in order for N_2 fixation to occur. Using cultivation approaches based on semi-solid N-deficient media with an O_2 gradient, Guerinot & Colwell (1985) isolated a wide variety of aerobic, facultative anaerobic and microaerophilic diazotrophs from seawater, and Boström et al. (2007a) found that heterotrophic diazotrophs from the Baltic Sea required low O_2 (0 to 6% air saturation) or anaerobic conditions for N_2 fixation. Similar results using other approaches have been reported (Wynn-Williams & Rhodes 1974, Urdaci et al. 1988; see also review by Zehr et al. 2006). Hence, as has been observed for most soil *Bacteria* (e.g. Oelze & Klein 1996, Marchal & Vanderleyden 2000), marine non-cyanobacterial diazotrophs generally require anaerobic or low O_2 conditions to perform N_2 fixation.

Where are such conditions present in the well-oxygenated pelagic realm of the oceans? The interior of organic-rich aggregates or detritus could potentially be suitable microhabitats for N_2 fixation. Particle-associated *Bacteria* account for <10 to 15% of total bacterial biomass and/or production (Turley & Mackie 1994, Griffith et al. 1994), but during phytoplankton post-bloom conditions the fraction of *Bacteria* attached to organic-rich aggregates can be very high (Smith et al. 1995, Riemann et al. 2000). These loci may be perceived as metabolic hot spots (Azam & Long 2001) where extensive microbial growth (Alldredge & Gotschalk 1990, Smith et al. 1995, Riemann et al. 2000) and thereby respiration can lead to reduced O_2 concentrations in the particle interiors (Paerl & Prufert 1987, Ploug et al. 1997). The isolation of strictly anaerobic *Bacteria* (Bianchi et al. 1992) together with measurements of products of anaerobic processes (Shanks & Reeder 1993) indicate that even anoxic conditions can occur within marine aggregates, although this may be ephemeral (Ploug et al. 1997). Microbial remineralisation of freshly produced aggregates in surface waters is evident as enriched ammonia levels relative to the surrounding water (Alldredge & Gotschalk 1990), but over time, and with depth, the microbial activity within aggregates will not only consume O_2, but will also result in selective loss of N and phosphorus due to high rates of enzymatic hydrolysis that is uncoupled from uptake (Smith et al. 1992). Thus, the interiors of aging aggregates, with their tendency to be rich in carbon (Maruyama et al. 1970), but depleted in O_2 and N, could be suitable sites for N_2 fixation.

While this idea is not new (Paerl & Prufert 1987, Paerl 1990), experimental or field-based interrogations of this phenomenon remain scarce. However, it appears that both live and dead particulate matter could potentially serve as loci for N_2-fixing non-*Cyanobacteria* in the marine water column (Fig. 2). In an early study, Guerinot & Colwell (1985) found that their pure culture of a heterotrophic bacterium was only capable of N_2 fixation *in situ* in the presence of particulate matter >8.0 µm. This suggests that these *Bacteria* have the ability to rapidly colonise particulate matter (Kiørboe et al. 2003) and generate low-O_2 conditions suitable for N_2 fixation. Indeed, Paerl (1985) observed that N_2 fixation by *Cyanobacteria* and possibly heterotrophic *Bacteria* paralleled the formation of low O_2 microzones in aggregates. This is consistent with the more frequent detection of sequences within *nifH* Cluster III (related to anaerobic or facultative anaerobic *Bacteria*) in coastal or estuarine waters (Affourtit et al. 2001, Jenkins et al. 2004, Steward et al. 2004a, Moisander et al. 2007, Man-Aharonovich et al. 2007, Farnelid et al. 2009), which are rich in particulate matter relative to offshore environments. Non-cyanobacterial diazotrophs may also be associated with zooplankton (Proctor 1997, Braun et al. 1999) or phytoplankton. Light-driven respiration (Mehler activity) reduces O_2 tension within cells and colonies of *Trichodesmium*, presumably allowing for N_2 fixation in actively photosynthesising cells (Kana 1993). Such a mechanism may contribute to the formation of low O_2 zones associated with live phytoplankton cells. We recently found diverse *nifH* genes from non-*Cyanobacteria* associated with single cells of heterotrophic dinoflagellates in the Indian Ocean (Fig. 2A–C). Light and transmission electron microscopy confirmed the presence of large heterotrophic *Bacteria* attached to the surfaces of the dinoflagellates (Farnelid et al. 2010). Similarly, putative diazotrophic non-*Cyanobacteria* have been observed on

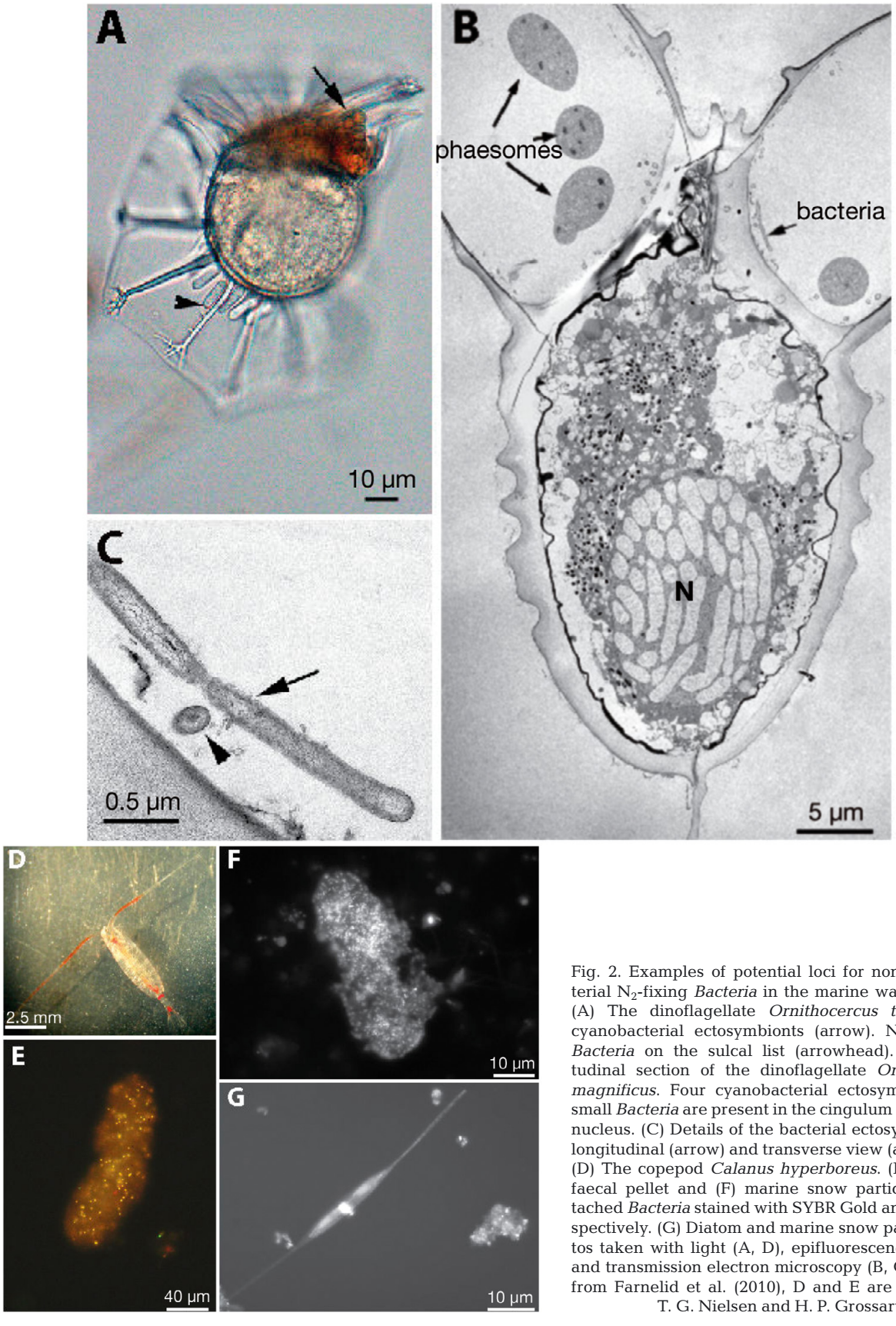

Fig. 2. Examples of potential loci for non-cyanobacterial N_2-fixing *Bacteria* in the marine water column. (A) The dinoflagellate *Ornithocercus thumii* with cyanobacterial ectosymbionts (arrow). Notice large *Bacteria* on the sulcal list (arrowhead). (B) Longitudinal section of the dinoflagellate *Ornithocercus magnificus*. Four cyanobacterial ectosymbionts and small *Bacteria* are present in the cingulum (arrows). N: nucleus. (C) Details of the bacterial ectosymbionts, in longitudinal (arrow) and transverse view (arrowhead). (D) The copepod *Calanus hyperboreus*. (E) Copepod faecal pellet and (F) marine snow particle with attached *Bacteria* stained with SYBR Gold and DAPI, respectively. (G) Diatom and marine snow particle. Photos taken with light (A, D), epifluorescence (E, F, G) and transmission electron microscopy (B, C). A–C are from Farnelid et al. (2010), D and E are courtesy of T. G. Nielsen and H. P. Grossart

and within suspended N_2-fixing diatom mats in the eastern Pacific (Martínez et al. 1983).

Interestingly, Dore et al. (2002) estimated higher N_2 fixation rates from sedimenting particles in traps relative to rates determined from tracer incubations. The trap-derived rates agreed well with previous estimates (Deutsch et al. 2001), lending support to their accuracy. Dore et al. (2002) attributed the discrepancy to the possibility that tracer incubation underestimates true rates due to a patchy distribution of cyanobacterial diazotrophs or due to disruption of consortia (Martínez et al. 1983, Dore et al. 2002). Another source of error could be a patchy distribution of marine snow particles harbouring N_2-fixing microbial assemblages. A patchy distribution of active sites of N_2 fixation could help explain the discrepancy between the DNA and cDNA libraries mentioned earlier.

Considering the information summarised here, we find it conceivable that marine snow particles are ephemeral loci for N_2 fixation by non-*Cyanobacteria*. One conceptual problem is that extensive surface-associated growth is required to generate low O_2 microzones on marine snow particles, but mineralisation at the same time generates ammonium, which may inhibit N_2 fixation. Eventually, organic N in a particle would be exhausted, however, and N_2 fixation might be possible for a short period until readily utilisable carbon is also depleted and the respiration rate is no longer sufficient to maintain low O_2 conditions in the particle interior (Ploug et al. 1997). It is also possible, as noted earlier, that diazotrophs on particles are simply insensitive to the presence of fixed nitrogen.

In addition to suspended particles, interfaces between oxic and anoxic layers in the water column could be loci for N_2 fixation by non-cyanobacterial diazotrophs. Anoxic basins are well known in fjords (Ramsing et al. 1996), estuaries (Hannig et al. 2006), inland seas (Lam et al. 2007) and from oceanic oxygen minimum zones (OMZs; Fig. 3), where O_2 concentrations are low enough to induce anaerobic metabolism. The OMZs account for 8% of the global ocean area and play an essential role in the global N cycle (Paulmier & Ruiz-Pino 2009), primarily due to loss of fixed N through denitrification (e.g. Ward et al. 2009) and anaerobic ammonia oxidation (e.g. Lam et al. 2009). In sediment studies, parallel occurrence of denitrification and N_2 fixation has been shown (Fulweiler et al. 2007), yet these processes are thought to be spatially segregated in the marine water column. Recently, the co-occurrence of denitrification and N_2 fixation was demonstrated in a

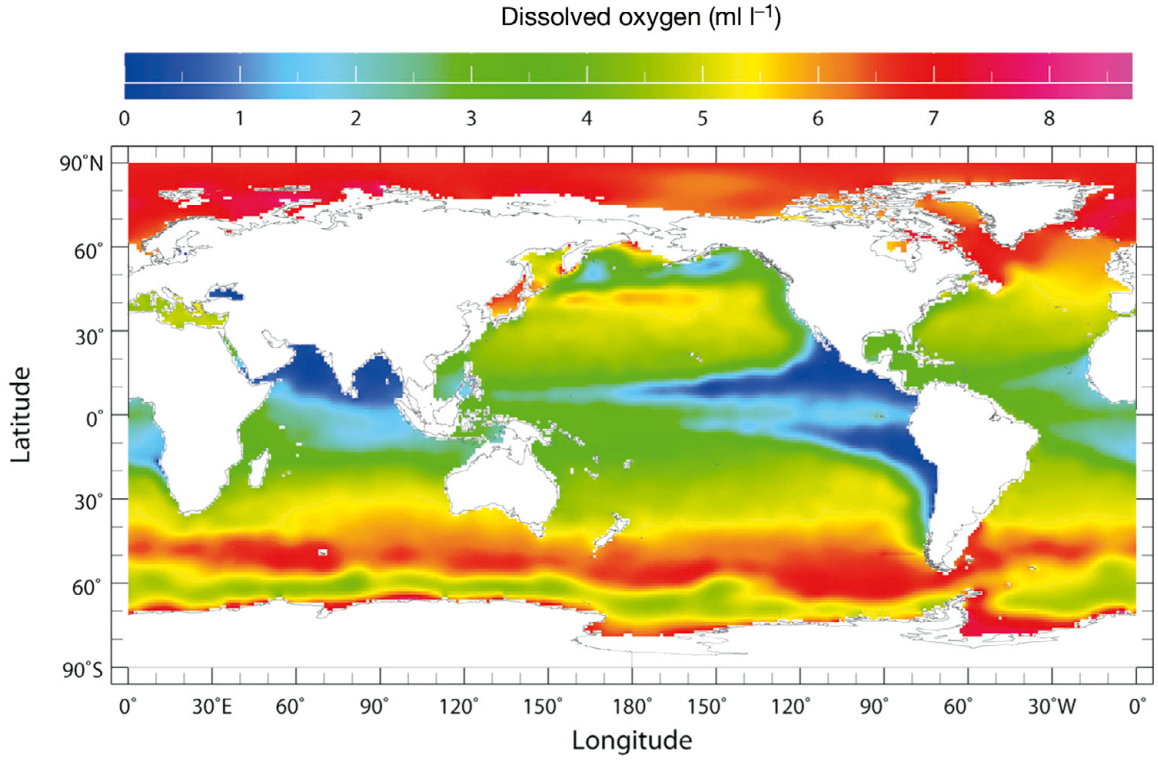

Fig. 3. Global map of major oceanic oxygen minimum zones. Annual mean dissolved oxygen levels at 200 m below the surface are illustrated as a colour contour plot. Areas in dark blue indicate regions with particularly pronounced subsurface oxygen minima. Data from the IRI/LDEO Climate Data Library, Columbia University (http://iridl.ldeo.columbia.edu/). Original raw data from World Ocean Atlas 2005 (Garcia et al. 2006)

lake in association with the chemocline (Halm et al. 2009). The authors found low inorganic N concentration in the oxic zone, while in the lower anoxic zone ammonium concentrations were high. Between these zones, inorganic N was below detection, N_2 fixation was substantial, and high levels of *nifH* gene expression were detected. Hence, in this system N loss and N gain occurred at the same space and time, which is very different from the conventional view of N cycling in the modern ocean (Halm et al. 2009).

The extent to which N_2 fixation could be associated with the low O_2 conditions occurring in the upper part of oceanic OMZs is currently unknown. However, the study by Halm et al. (2009) combined with the ubiquity of non-cyanobacterial diazotrophs in the ocean (Zehr et al. 2003b), even in the deep sea (Mehta et al. 2003), indicate that examination of OMZ-associated N_2 fixation could be a promising avenue.

CONCLUDING REMARKS

It is a common view that N_2 fixation by non-cyanobacterial diazotrophs is not quantitatively important in the ocean. Evidence to the contrary does not yet exist, because rate measurements of N_2 fixation by non-*Cyanobacteria*, and the presumed linkage between *nifH* gene expression and nitrogen fixation, have not been confirmed *in situ*. However, an increasing number of studies reporting on the wide distribution and expression of nitrogenase genes from non-*Cyanobacteria* in marine pelagic waters indicates that these microorganisms are ecologically significant. Although the environmental conditions in the open ocean appear to be unfavourable for N_2 fixation by non-*Cyanobacteria* when considered at the macroscale, the pelagic habitat is heterogeneous at small scales. N_2 fixation in discrete micro-scale loci may have important consequences for carbon and nutrient cycling at local, and perhaps even global, scales.

The discovery of an unusual O_2-insensitive N_2 fixation pathway in a terrestrial bacterium (Ribbe et al. 1997) is a reminder that there may also be other pathways for N_2 fixation in the ocean not captured by our current molecular tools. That observation, and the continued discovery of novel microbial ecotypes with major influences on marine biogeochemical cycling and productivity (e.g. Béja et al. 2000, Zehr et al. 2001, 2008), reflect the extraordinary metabolic innovation that characterises the prokaryotic world. Consequently, it is reasonable to expect that strategies to circumvent the presumed chemical and physical constraints on N_2 fixation in the oligotrophic open oceans have evolved among some non-*Cyanobacteria*. We anticipate that the future of this field will soon be one in which we are no longer asking whether non-*Cyanobacteria* fix N_2 in the open ocean, but are determining how they do so and at what magnitude.

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