Microb Ecol (1996) 32:263–281

MICROBIAL ECOLOGY

Problems and Promises of Assaying the Genetic Potential for Nitrogen Fixation in the Marine Environment

J.P. Zehr,[1] D.G. Capone[2]

[1] Department of Biology MRC 303, Rensselaer Polytechnic Institute, Troy, New York 12180-3590, USA
[2] Chesapeake Biological Laboratory, University of Maryland, Solomons, Maryland 20688, USA

Received: 22 November 1995; Accepted: 6 December 1995

Abstract. Nitrogen fixation in the sea has attracted the attention of ecologists for decades. Much is known about the habitats in which it occurs and some of the factors that limit N_2 fixation activity in different environments, but we still know little about the organisms that fix nitrogen, and what limits the growth and distribution of these organisms in marine environments. Molecular biology technological developments have provided tools for detecting and characterizing N_2-fixing organisms in the environment. These techniques hold great promise for unraveling the mysteries and paradoxes of N_2 fixation in the sea. In this review, we address the theoretical basis for the use of a molecular approach to N_2 fixation, highlight the strengths and weaknesses of the approach, and provide case studies that demonstrate the potential contribution of molecular biology approaches to studies of N_2 fixation in the sea.

Introduction

Biological N_2 fixation, the reduction of atmospheric N_2 gas to biologically available ammonium, is a crucial component of the global nitrogen (N) cycle [28, 68]. In many terrestrial, freshwater, and marine environments, N is believed to be in short supply, and N_2 fixation can provide the mechanism for alleviating N limitation of ecosystem production [81]. N_2 fixation, however, is an energetically demanding process, requires molybdenum and iron, and is inhibited by oxygen. For these reasons, N_2 fixation, although a potential mechanism for obtaining N for microorganisms, can itself be limited by other biochemical or environmental parameters [18, 42].

Rates of N_2 fixation can be measured using ^{15}N as a tracer, or by using the C_2H_2 reduction technique [18]. The measurement of N_2-fixation rates can provide information on the flux rate at the time of measurement, but provides limited

Correspondence to: J.P. Zehr.

information on the regulating factors or organisms responsible for this activity. Such activity measurements cannot provide insight into the reasons for lack of N_2 fixation when it cannot be detected [88]. To understand the ecosystem-level controls on N_2 fixation, it is necessary to distinguish between mechanisms that limit the distribution of organisms that have the genetic capability to fix N_2, and mechanisms that regulate the expression of the N_2-fixation apparatus. Molecular biological approaches provide a suite of techniques that can provide basic information on the presence or absence of N_2-fixing organisms, their types, and the factors that regulate the expression of N_2 fixation activity [27, 46].

The marine environment is composed of a variety of habitat types, including the benthos, coral reefs, marshes, estuaries, ocean margin water columns, and the open ocean. The extent of N_2 fixation in these habitats varies [16] and dinitrogenase activity has been detected in all of them, including the deepest ocean sediments (refs. in [18]). The ecological questions surrounding the N_2-fixation issue differ among habitats.

N_2 fixation is catalyzed by the enzyme dinitrogenase, a multisubunit protein that is highly regulated by transcriptional and post-translational controls [68]. The genetics of N_2 fixation have been well studied in *Klebsiella pneumoniae,* an enteric bacterium that can be genetically manipulated (reviewed in Merrick [53]). Research on the genetics of N_2 fixation in cyanobacteria [39] and purple bacteria [72] has also rapidly progressed in the last ten years, although the N_2 fixation apparatus is somewhat less well known than that in *Klebsiella.* The results of studies of N_2 fixation in *Klebsiella, Rhodospirillum rubrum,* and *Anabaena* sp. PCC 7120 have provided models for the genetics and regulation of N_2 fixation that can be used as a starting point for investigations of regulation of N_2 fixation in organisms in the environment.

Subsequent to characterization of *nif* structural protein genes (*nifHDK*) in *Klebsiella,* it was found that dinitrogenase genes are highly conserved [74] and are present in numerous prokaryotic taxa representing all the major groups of the prokaryotes (see review by Young [87]). The marine environment contains diverse prokaryotic populations, many of which have yet to be represented in culture collections [35]. Many types of marine microorganisms are likely to be involved in N_2 fixation [62]. Since dinitrogenase genes can be found in virtually any group of prokaryotes, but are not present in all representatives of any group, taxonomic information provides little information on whether or not N_2 fixation capability is present in natural populations of uncultivated species.

In the open ocean environment, inorganic N and phosphorus (P) concentrations are low, and there is still question over the relative importance of the availability of P and N in limiting productivity [40, 41]. Analysis of particulate C:N:P and total N and P in the mixed layer of the oceans indicates that N could limit productivity, at least at some times and in some places. Global distributions of dissolved inorganic N (nitrate + nitrite) and P concentrations show that the oligotrophic ocean gyres have low N:P ratios indicative of potential N limitation [30]. The geochemical argument for P limitation is based on the assumption that N_2 fixation should alleviate any N deficit [69]. Whether nitrogen or phosphorus actually limits productivity, either perspective argues that N_2 fixation should occur in the low-nutrient environment of the open sea, yet few studies have identified N_2-fixing organisms [37, 44, 51, 85] or measured N_2-fixation rates in situ. A recent nitrogen

budget analysis for the Atlantic Ocean strongly indicates that there is an unidentified source of N, speculated to be N_2 fixation [54]. The planktonic filamentous nonheterocystous cyanobacterium *Trichodesmium* is one of the few organisms known to fix N_2 in the open ocean, and may be one source of this "missing" N [24]. Molecular techniques provide an approach to identifying other organisms that may contribute to oceanic N_2 fixation, by making it feasible to detect the genes that are required for N_2 fixation to occur. This approach has the benefit that it is possible to detect the genetic potential for the conversion of N_2 to ammonium, without experimental manipulation or cultivation.

In some environments, such as microbial mats, N_2 fixation can be easily measured and we know that N_2 fixation is an important source of fixed nitrogen in these environments. These communities contain a rich assemblage of diverse microorganisms. It can be extremely difficult to identify the organisms responsible for N_2 fixation in such environments, and identification is dependent upon culturing (which then raises questions as to the importance of those organisms in situ) or the results of experimental manipulations of physiologically relevant parameters (e.g., light, O_2 or organic supply) or the use of inhibitors [4, 63]. Interpretation of these experiments can be difficult, primarily because of the potential for consortial interactions and interdependence of organisms in mats and sediments. For example, when dark incubation conditions or DCMU result in reduced N_2-fixation rates, the reduction may result from direct inhibition of photosynthetic organisms responsible for N_2 fixation or of heterotrophic bacteria dependent upon photosynthate from photosynthetic organisms. Molecular techniques provide an approach to answering these questions directly through phylogenetic analysis of N_2-fixation genes present in the microbial community. Thus, a molecular approach to N_2 fixation in the environment can provide answers to questions regarding the presence of N_2-fixing organisms (detection), and species and functional group identification (characterization of the types of N_2-fixing organisms).

In this review we will detail the strengths and weaknesses of the approach of characterizing genes for the N_2 fixation apparatus in natural populations, and illustrate in several examples how this information may provide information on the limitation of N_2 fixation in the environment. We will describe the theoretical considerations of the PCR approach, and, using examples from the open ocean and benthic environments where research along these lines has been initiated, will demonstrate the power and utility of this approach to provide insight into ecological questions of N_2-fixation regulation in the marine environment.

Dinitrogenase Genes and the Use of Dinitrogenase Sequences for Species Identification

The conserved nature of the dinitrogenase genes means that the DNA and amino acid sequence of the dinitrogenase (*nif*) genes share a high degree of similarity among organisms. In particular, the DNA and deduced amino acid sequences of genes for the structural proteins dinitrogenase (the Mo protein) and dinitrogenase reductase (the Fe protein) have been conserved through evolution. The dinitrogenase reductase gene is the most highly conserved, and there are more dinitrogenase reductase gene (*nifH*) sequences than dinitrogenase gene (*nifDK*) sequences available

in the sequence databases. This makes the *nifH* gene a good choice for diazotrophic species detection and identification.

The conserved nature of these proteins indicates that some features of the protein sequences are particularly important for biochemical activity, and replacements at specific sites have been restricted throughout evolution. The sequences contain less-conserved regions, which can provide taxonomic information as well, in the same way that the conserved ribosomal RNA genes have been used to define evolutionary relationships of organisms [60, 83]. The major difficulty in using *nif* sequences for detection and identification of organisms is the ability to rapidly obtain sequences from environmental samples containing numerous types of organisms, often in low concentrations. The polymerase chain reaction, which can amplify DNA sequences between two known short DNA sequences [57, 75], such as those in highly conserved regions, provides a tool for obtaining these sequences efficiently and rapidly from environmental samples [5], as well as from cultures of known organisms.

Several regions in the sequence of *nifH,* which encodes the Fe protein, are very highly conserved, occurring in virtually every *nifH* sequence determined thus far, including some from the Archaea. Two short DNA sequences, which flank a DNA sequence of about 325 nucleotides, have been used to design degenerate oligonucleotide primers that provide for every possible codon that could encode the conserved amino acid sequences [90]. The conserved features, particularly at the primer locations, ensure that most if not all *nifH* genes can be amplified, unless there exists a yet to be characterized dinitrogenase that is substantially different from the three dinitrogenases that have been characterized. The amplified *nifH* segment encodes several potentially important regions in the structure of the Fe protein including conserved cysteins involved in coordination of the Fe-S center, and an ADP ribosylation site [67] important in regulation of activity of the protein [34]. Figure 1 shows the amino acid sequence encoded by the amplified DNA fragment for diverse organisms, including uncultivated organisms in the environment. The upstream primer site may be involved in ATP hydrolysis. The upstream part of the sequence is particularly divergent among organisms and may be related to the distinctive properties of dinitrogenase from different species [34] (Fig. 1).

The use of the PCR for analyzing mixed assemblages of nitrogen fixing organisms is based on the amplification of a mixed set of genes derived from multiple organisms, cloning of the mixed amplification products, and sequencing clones containing individual sequences derived from one operon within one genome. The amplification product will contain products of different templates. In theory, the different products should be obtained in the same ratio as the different templates. The mixture of amplification products cannot be sequenced directly, but must be cloned into a recombinant vector for analysis of individual sequences. Recombinant clones contain individual sequences, and the composition of the recombinant library should be representative of the composition of the template mixture. However, amplification of 16S rRNA genes has previously indicated that amplification can select for certain types of sequences [71]. The validity of the assumption that the PCR technique amplifies different *nifH* sequences with the same efficiency has been evaluated by amplifying mixtures of known templates that vary in GC content with the degenerate primers. Different *nifH* templates of varying GC content might be expected to amplify with different efficiencies, but our results indicate that the

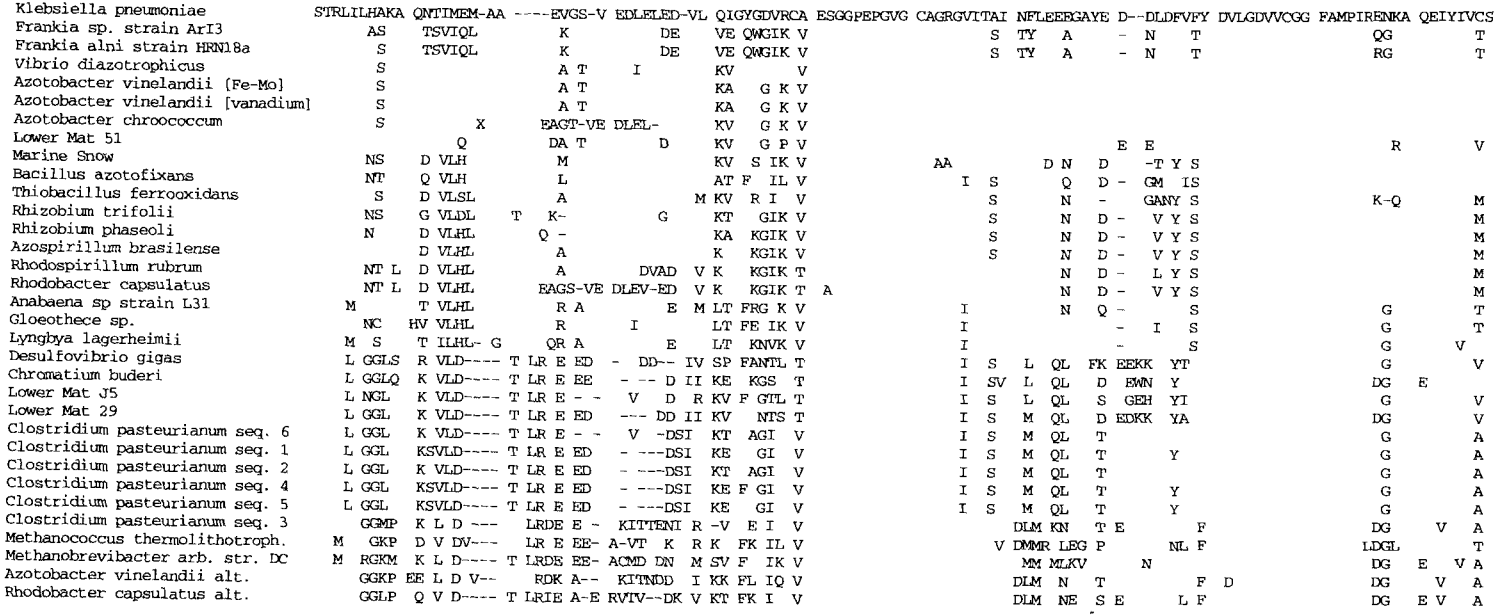

Fig. 1. Deduced amino acid sequences for region of *nifH* amplified with *nifH* degenerate PCR primers. Sequences representing major groups of microorganisms are shown, demonstrating the variability in sequence of this region of *nifH*. Sequences are compared to the *nifH* sequence from *Klebsiella pneumoniae,* with exact matches indicated by blank spaces.

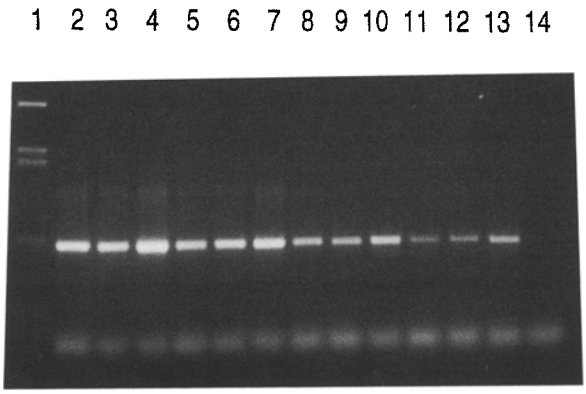

Fig. 2. Amplification competition experiments using *nifH* templates with varying G + C content in primer sequences. Amplification of two templates containing different G + C contents of the primer region produce expected doubling of product (see lanes 4, 7, 10, 13). *Lane 1,* size marker; *lane 2,* 10^7 copies template 1; *lane 3,* 10^7 copies template 2; *lane 4,* 10^7 copies template 1 + 10^7 copies template 2; *lane 5,* 10^7 copies template 3; *lane 6,* 10^7 copies template 4; *lane 7,* 10^7 copies template 3 + 10^7 copies template 4; *lane 8,* 10^5 copies template 1; *lane 9,* 10^5 copies template 2; *lane 10,* 10^5 copies template 1 + 10^5 copies template 2; *lane 11,* 10^5 copies template 3; *lane 12,* 10^5 copies template 4; *lane 13,* 10^5 copies template 3 + 10^5 copies template 4; *lane 14,* negative control. Template 1, 53% G + C upstream primer site; Template 2, 71% G + C upstream primer site; Template 3, 53% G + C downstream primer site; Template 4, 71% G + C downstream primer site.

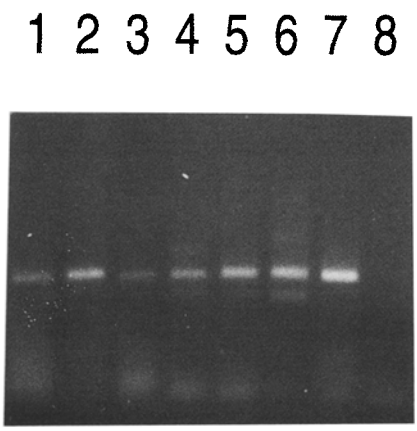

Fig. 3. Amplification of *nifH* fragment from diverse microorganisms. *Lane 1, Nostoc muscorum,* heterocystous cyanobacterium; *lane 2, Chlorobium limicola,* green bacterium; *Lane 3, Gloeothece* sp., unicellular cyanobacterium; *lane 4, Plectonema boryanum,* filamentous nonheterocystous cyanobacterium; *lane 5, Chromatium buderi,* purple sulfur bacterium; *lane 6, Vibrio diazotrophicus,* heterotrophic γ proteobacterium; *lane 7, Methanobrevibacter arboriphilicus* strain DC; *lane 8,* negative control.

amplification is not sensitive to GC content of the target (Fig. 2). The degenerate primer sequences include the sequences of all of the bacterial *nifH* sequences in the GenBank database, except for certain species of methanogens. Nonetheless, we have been successful in amplifying *nifH* (or *nifH*-like) genes from cultures of methanogens (Fig. 3).

A wide variety of *nifH* genes can be amplified with the *nifH* primer set, including purple sulfur bacteria, heterocystous and nonheterocystous cyanobacteria, vibrios, and other heterotrophic bacteria (Fig. 3). At this time, there is little reason to suspect that the PCR approach is biased for different types of templates. The *nifH*

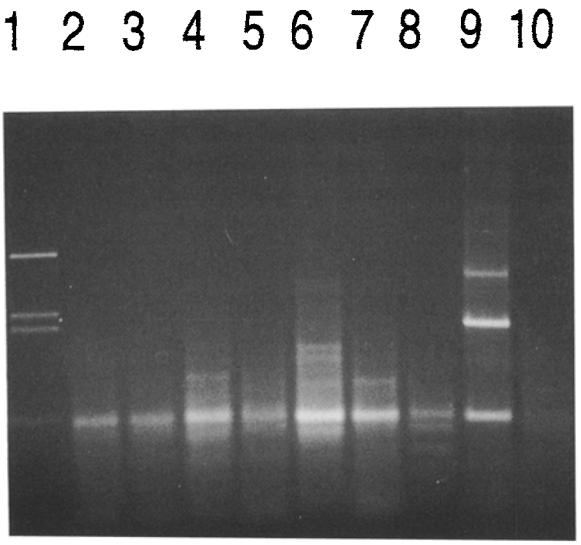

Fig. 4. Amplification of the *nifH* fragment from diverse marine samples. *Lane 1,* Size markers; *lane 2,* mangrove sediments from Bahamas; *lane 3,* Little Lake, a saline lake on San Salvador Island, Bahamas; *lane 4,* marine cyanobacterial mat sediment from North Carolina; *lane 5,* stromatolites from Lee Stocking Island, Bahamas; *lane 6, Lyngbya*-dominated cyanobacterial mat from North Carolina; *lane 7, Lyngbya* sp. culture isolated from North Carolina mat; *lane 8,* marine copepod (*Acartia* sp., provided by L. Proctor); *lane 9,* positive control; *lane 10,* negative control.

primers have also successfully amplified a fragment of *nifH* from a variety of sample types, including benthic mats and stromatolites (Fig. 4). A few other studies have used a similar approach to amplify *nifH* in the terrestrial environment [79, 80].

Sequence and Phylogenetic Analysis

The sequence information obtained by cloning and sequencing the amplification products can be used to identify the organisms from which the sequences were derived. DNA and deduced amino acid sequences show that similar organisms have features that can identify groups of organisms (Fig. 1). For very similar organisms, high values of identity (>85% on the DNA level, >90% on the amino acid level) between sequences can be used to identify sequences [6]. Phylogenetic tools designed to determine the evolutionary relationships among organisms provide a more definitive way to recognize similarities among sequences. The phylogeny of *nifH* sequences (from the portion of *nifH* amplified with the primers of Zehr and McReynolds [90]) is largely consistent with the phylogeny of microorganisms derived from sequences of the 16S rRNA gene (Fig. 5). In particular, cyanobacterial *nifH* sequences cluster together, with heterocystous cyanobacterial sequences clustering tightly (Fig. 5). Although the use of the amplified *nifH* sequence for identification is not necessarily dependent upon the similarities between the 16S rRNA and *nifH* phylogenetic trees, the correspondence of the trees provides some assurance that similarities between sequences are not usually due to the lateral transfer of *nifH* genes. However, there are a couple of interesting discrepancies between the *nifH* phylogeny and the 16S rRNA phylogeny that could be due to the lateral transfer of *nifH*. *nifH* sequences from some anaerobic organisms including *Clostridium* (low GC firmicute), *Desulfovibrio gigas* (sulfate-reducing delta proteobacterium) and *Chromatium buderi* (photosynthetic purple sulfur γ proteobacterium) do not cluster with their respective groups, but together form a cluster of deeply

branching sequences (Fig. 5). One of the characteristics of *nifH* sequences within this cluster is a difference in sequence and gaps in a region near the upstream end of the amplified fragment (Fig. 1), but masking this region for phylogenetic analysis does not affect the clustering of these organisms. We do not yet know why these sequences from anaerobes cluster together, but the simplest interpretation is that lateral transfer occurred at some point in time. These organisms are found in the same habitats (e.g., muds and soils), and the close proximity of cells in these habitats may have enhanced the probability of transfer of genetic material between cells.

There are several characteristics of the *nifH* gene that are particularly important in interpretation of *nifH* sequence data obtained from the environment. In some organisms, the *nifH* gene is present in multiple copies. In *Clostridium,* not all of the *nifH* copies are expressed. If such sequences were obtained from the environment, they potentially could be confused as being from different organisms, thus inflating the apparent diversity of N_2-fixing organisms. However, the multiple copies for *Clostridium* are closely related to each other. Multiple copies probably most often arise from duplication events, and therefore will be related to each other. Multiple sequences within the anaerobic cluster containing *Clostridium* were obtained from amplification of DNA extracted from cyanobacterial mats, which indicated a high diversity of heterotrophic N_2 fixing microorganisms [93]. The sequences are less similar to each other than the multiple copies in *Clostridium* are to each other, indicating that these sequences probably were derived from different genera, and were not multiple copies within the same organisms. However, the possibility of multiple copies must be considered when closely related sequences are obtained from one sample from natural assemblages.

In addition to multiple copies of nonfunctional *nifH* genes, there are alternative *nif* operons. The alternative dinitrogenases are referred to as the V and Fe dinitrogenases, in light of the requirement for V or Fe cofactors in place of Mo in the conventional dinitrogenase. These alternative operons differ extensively in the proteins encoded by the dinitrogenase sequence (*nifDGK* instead *nifDK*), but the alternative dinitrogenase reductase genes (*nifH*) from the alternative operons (V and Fe dinitrogenases) are similar to that of the conventional *nifH* [11]. The conserved amino acid sequences in the conventional dinitrogenase reductase sequence also occur in the alternative dinitrogenase reductase sequences. Phylogenetic analysis of the alternative *nifH* sequences shows that the V nitrogenase (first alternative) *nifH* is most closely related to the conventional *nifH* from the same organism, but that the Fe dinitrogenase (second alternative) *nifH* sequences group together, consistent with an independent evolution (Fig. 5). Microorganisms (e.g., *Azotobacter*) can contain all three dinitrogenase operons [12]; when sequences from these organisms are obtained, the conventional and nitrogenase *nifH* sequences are most closely related to the Fe dinitrogenase reductase sequences of other organisms. The V dinitrogenase *nifH* may be difficult to detect, but will probably be closely related to the conventional *nifH* from the same organism (as in *Azotobacter*), and therefore is an issue only when similar sequences are obtained from the same environment. Eventually, these operons should be made distinguishable by the development of a downstream primer in the more divergent *nifD* (or *vnfD*; *anfD* in the alternative operons), which would amplify both *nifH* and a portion of *nifD*.

One final consideration in using the amplification and sequencing approach for

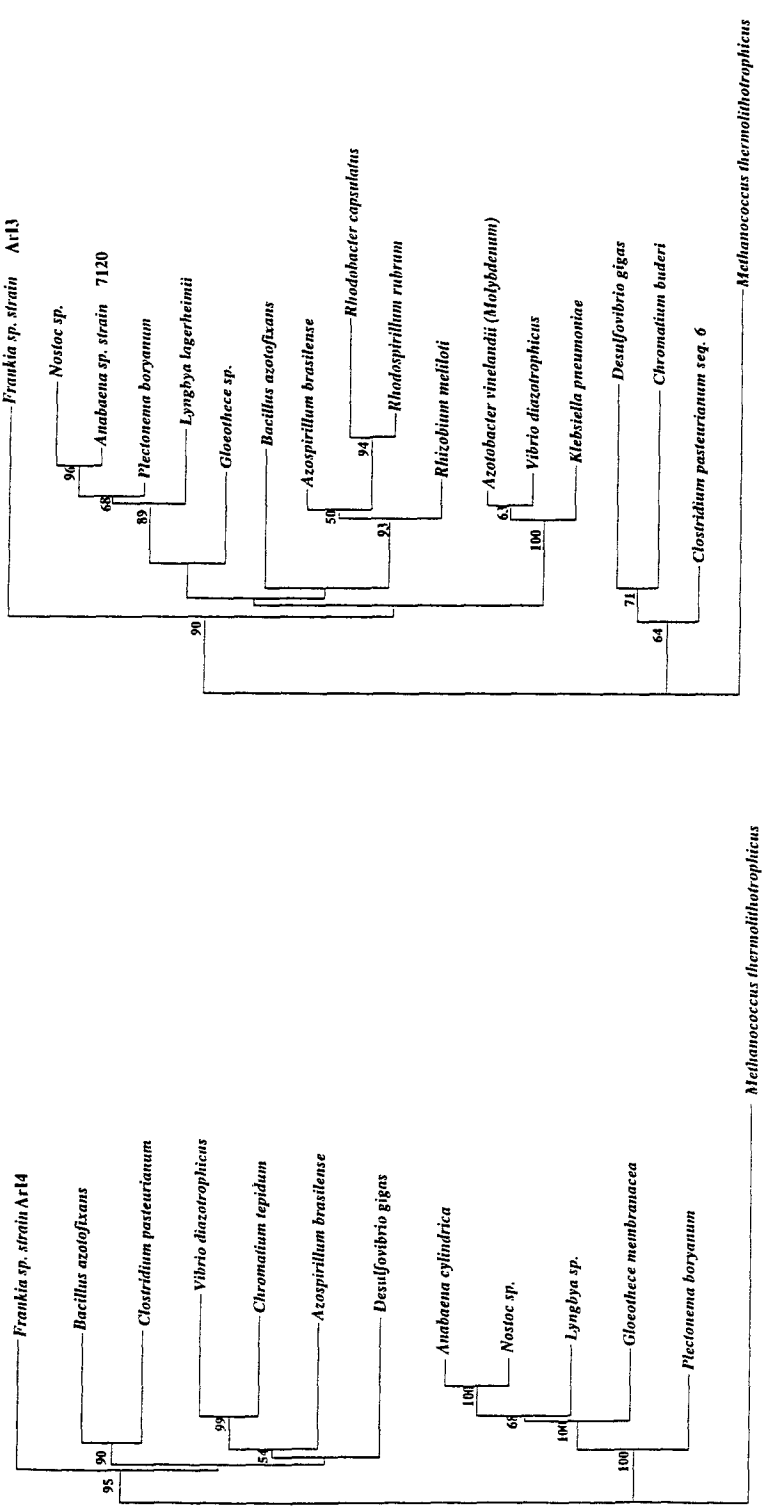

Fig. 5. Phylogenetic analysis of *nifH* and 16S rRNA sequences from diverse genera. *nifH* sequences (*left*) were obtained from GenBank or have been reported in Zehr et al. [93]. 16S rRNA sequences (*right*) and alignments from similar species or strains were obtained from the Ribosomal Database Project [50]. Phylogenetic analysis was performed using distance methods and neighbor-joining algorithms of PHYLIP 3.5 [31]. Trees were bootstrapped 100 times and bootstrap values greater than 50% are indicated at their respective nodes. Clusters in both trees are similar, except that anaerobes including *Desulfovibrio* and *Clostridium* cluster together in the *nifH* tree. Gram-positive bacteria, (firmicutes) cluster together in the 16S rRNA tree.

detecting and characterizing *nifH* genes is the existence of other closely related non-*nif* genes. It has been reported that the *frxC* gene, which encodes a chlorophyllide reductase, has significant homology to *nifH* [33]. However, homology of the *frxC* sequence to *nifH* encoded by the sequence fragment amplified by PCR is less than 40%. There is little chance of confusing the *nifH* sequence with a *frxC* sequence, even if the correct size fragment is amplified. The amino acid sequence encoded by the downstream primer used for PCR does not occur in *frxC*, and therefore it is not clear that *frxC* could even be amplified using this primer set.

Case Study 1: N_2 Fixation in the Open Ocean

Much of the surface ocean in tropical and subtropical regions is nutrient depleted. Furthermore, these regions are generally considered to be N limited, that is, N is in the least supply relative to other nutrients required for planktonic growth [20, 29]. Thus, N_2 fixation would confer a competitive advantage for those organisms capable of it [81]. There is one prominent N_2 fixer identified in these open ocean waters, the cyanobacterium *Trichodesmium* [23]. However, other diverse cyanobacteria, bacteria, and Archaea exist in these environments and may also contribute to N_2 fixation. Molecular approaches have been applied to *Trichodesmium*, and have greatly increased our understanding of the importance of this organism, as well as providing important insight into the features of its N_2-fixing system [89, 91]. Analysis of *nifH* DNA sequences provided direct evidence that *Trichodesmium* itself fixed N_2 [90], and gave information on the relationships of species and strains [7]. Analysis of the Fe protein of dinitrogenase in *Trichodesmium* has lent clues to the mechanisms involved in regulating the diel cycle of N_2 fixation [22, 92].

Our knowledge of the other N_2 fixers in these environments is meager. There are abundant prokaryotic organisms in the water column, including unicellular cyanobacteria and the related, recently discovered *Prochlorococcus* [25], as well as approximately 10^6 cells ml^{-1} of unidentified heterotrophic bacteria. Unicellular N_2-fixing cyanobacteria have been brought into culture (e.g., *Synechococcus;* [55]), but these strains have not yet been shown to be important in the water column. A number of vibrio cultures were isolated by Guerinot and Colwell [37], although these were obtained from relatively nearshore locations. Therefore, the presence of N_2-fixing strains in the open ocean remains to be demonstrated.

The development of PCR primers that amplify a wide variety of organisms has facilitated the study of other N_2-fixing organisms in the open ocean environment. Traditional culturing studies have indicated that N_2-fixing organisms, particularly heterotrophic bacteria, can be present in marine waters [37, 44]. However, it is difficult to interpret the results of culturing studies when it is clear that cultivation may select for specific strains and may not assess the abundance or diversity of populations in situ [3, 35, 82]. The amplification of *nifH* genes directly from natural populations provides information on the "genetic potential" for N_2 fixation in natural populations. In theory, the PCR can be used, quantitatively, and even semiquantitative order of magnitude estimates of *nif* gene abundance would shed light on the potential for N_2 fixation in the open ocean [89]. Phylogenetic analysis of the amplified *nifH* sequences (or the deduced amino acid sequences) can be

used to identify unknown organisms (Fig. 5), providing that fairly closely related sequences are available in the database.

Amplification of *nifH* from cells collected in the tropical Atlantic Ocean produced a product that could represent a number of cells per liter equivalent to that of *Trichodesmium* [89]. Although this represents a relatively small percentage of the total prokaryotic population, N_2 fixation by *Trichodesmium* has been estimated to potentially contribute a large fraction of the nitrogen input into the surface layer [24].

Analysis of *nifH* sequences from the water column show that there are heterotrophic species that contain the *nifH* gene. *nifH* sequences obtained from marine snow aggregates (samples collected from the Santa Barbara Channel and generously provided by E. DeLong) are most closely related to those of α proteobacteria [89]. Sequences obtained from picoplankton samples and a bacterial culture isolated from *Trichodesmium* aggregates are related to γ proteobacteria and could be vibrio-related organisms [89] (Zehr unpublished data). *nifH* sequences derived from cyanobacteria other than *Trichodesmium* have yet to be found in the open ocean but studies are still in progress.

The presence of bacterial *nifH* genes in the plankton raises the issue of whether these genes are expressed, and what limits the growth and distribution of these species. It seems likely that heterotrophic N_2 fixation in the water column would be limited by the availability of organic carbon for the energy-intensive N_2-fixation process. The question will be resolved only by experimental manipulations designed to determine the factors limiting the growth of these organisms, and by using an assay for the actual expression of specific *nifH* genes. Procedures are available for assaying mRNA with the polymerase chain reaction (e.g., Pichard and Paul [66]), and preliminary results indicate that this technique can be adapted for detecting and characterizing the mRNA transcribed from *nifH* DNA (Fig. 6).

Case Study 2: N_2 Fixation in Marine Sediments and Mat Communities

N_2 fixation, as determined by acetylene reduction, has been reported in and upon a relatively broad range of marine sediments. Cyanobacterial mats on salt marsh and coral sediments are recognized as important sites of N_2 fixation [16, 18]. Sediments colonized by rooted macrophytes, such as seagrasses, as well as unvegetated systems have also been reported as sites of nitrogenase activity (NA) [16–18, 42].

Sediments. Despite relatively high concentrations of ammonium often observed in vegetated sediments (e.g., Short [78]), the high demand for N by the plant, the observation and isolation of root-associated diazotrophs (e.g., Capone and Budin [19], Capone [17]), and the agreement obtained between $^{15}N_2$ fixation and C_2H_2 reduction in several studies [19, 58, 65] has generally been interpreted as confirmation of the presence of N_2 fixers in these systems. The precise spatial and metabolic relationships between N_2-fixing bacteria in the rhizosphere and in the plant roots have been debated and remain to be elucidated.

In unvegetated sediments, there are some conflicting results and controversy over the presence of active N_2-fixing microorganisms [18, 42]. Marine sediments,

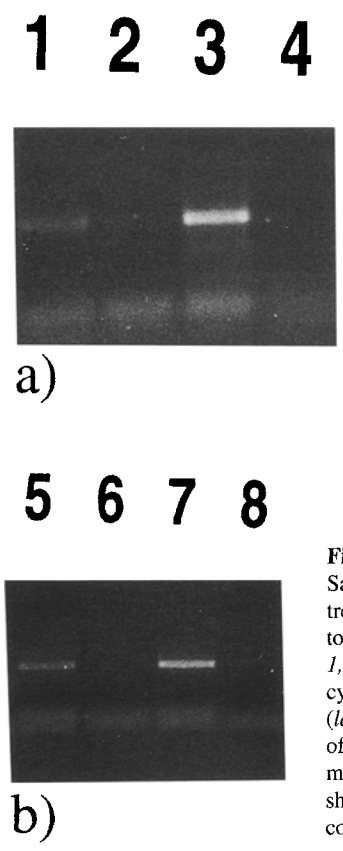

Fig. 6. Amplification of *nifH* mRNA from *Trichodesmium* sp. Samples were amplified with *nifH* degenerate primers after treating with reverse transcriptase (*lanes 1, 3, 5,* and *7*), or prior to treating with reverse transcriptase (*lanes 2, 4, 6,* and *8*). *Lanes 1, 2, 3,* and *4: Trichodesmium* sp., culture at beginning of light cycle. Culture incubated under low (*lanes 1* and *2*) or high (*lanes 3* and *4*) light. *Lanes 5, 6, 7,* and *8:* Natural populations of *Trichodesmium* from the Caribbean Sea. Levels of *nifH* mRNA amplified by RT-PCR at 0830 and 1230 hours. Samples show consistent amplification of *nifH* mRNA, with no DNA contamination.

particularly fine-grained organic-rich sediments, are often anoxic within a few millimeters of the sediment-water interface [70] and, therefore, are appropriate habitats for strictly anaerobic diazotrophs. As a result, these environments often have millimolar levels of NH_4^+, a repressor of nitrogenase synthesis that should severely limit or prevent NA. Moreover, NH_4^+ is a preferred N source because energy and reductant need not be expended for its assimilation. Hypothetically, long-term existence of microbial populations in high NH_4^+ environments should cause selection against N_2-fixers.

C_2H_2 reduction has been reported in sediments ranging from deep sea sediments to shallow, organic-rich, nearshore sediments [38]. The C_2H_2 reduction procedure is a highly sensitive assay. Measurable activity in these sediments may be minor compared to other N transformations. Indeed, area-extrapolated estimates of N_2-fixation for mid-latitude estuarine sediments based on C_2H_2 reduction are about 0.4 g N m^{-2} y^{-1} [16] compared to NH_4^+ fluxes across the sediment-water interface typically in excess of 10 g N m^{-2} y^{-1} [47]. In contrast, several recent studies [45, 59] (D. Capone, unpublished data) indicate that sediment NA in tropical carbonate sediments (which are typically organic poor) may account for a relatively large fraction of sediment NH_4^+ production and efflux.

Only one study we are aware of has attempted to confirm and calibrate C_2H_2 reduction rates in nonvegetated sediments [77]. However, the investigators were

unable to observe appreciable ^{15}N enrichment in sediments after exposure to $^{15}N_2$, despite measurable rates of C_2H_2 reduction. The derived ratios they reported ranged from 10 to 100, compared to the theoretical 3 (or 4) for the amount of C_2H_2 reduction to the amount of N_2 fixed. They suggested that observed C_2H_2 reduction in this environment may not be assaying NA, in a quantitative sense. Possible reasons for this are discussed by Howarth et al. [42] and Capone [18], but the existence of NA in organic-rich sediments is still questioned. On the other hand, O'Donohue et al. [58] compared $^{15}N_2$ uptake and C_2H_2 reduction in tropical seagrass sediments and obtained good correspondence to theoretical values.

Several independent pieces of evidence support the contention that C_2H_2 reduction in sediments, independent of its quantitative significance, is a direct result of NA. The supposition that NA in sediments is predominantly associated with heterotrophic anaerobes is corroborated by the observation in a wide range of studies that C_2H_2 reduction can be stimulated by the reduction in O_2 tension or the addition of organic substrates [18]. A number of studies have examined the effect of NH_4^+ additions on sediment C_2H_2 reduction, with several reporting a suppression of activity with these additions [18]. In their studies using a perfusion method, Capone and Carpenter [21] found that perfusion of C_2H_2-saturated deoxygenated seawater without NH_4^+ through sediment cores resulted in a large stimulation of C_2H_4 production, compared to parallel samples perfused with NH_4^+ at levels typical of pore waters (0.5 mM). Capone [18] and Yoch and Whiting [86] reported that the addition of methionine sulfoximine (MSX), a derepressor of NA [36] that interferes with the control of dinitrogenase synthesis by NH_4^+ through a complex chain of effectors, stimulated acetylene reduction in sediments. The stimulatory effects of NH_4^+ removal and of MSX additions indicate that rates of NA in sediments are far below the potential for these systems. Interestingly, it has been suggested (M. Scranton, personal communication) that the "residual" NA observed in NH_4^+-rich marine sediments could be a result of natural analogs of MSX that act as derepressors of the *nif* operon.

We have recently examined a variety of marine sediments (Burns, Capone, and Zehr, unpublished data) and mat [93] communities for the presence of *nifH.* In organic-rich Chesapeake Bay sediments, *nifH* is consistently amplified in sediments that exhibit NA. We are currently examining the downcore and horizontal patterns of distribution of amplified *nifH* sequences to determine the relationship between NA and the spatial distribution of N_2-fixing species.

Seagrasses and Cyanobacterial Mats. Seagrass beds provide a potential site for N_2 fixation, as the photosynthetic carbon input by the plant can provide sufficient energy for associated microbes. Preliminary studies did indeed demonstrate that heterotrophic nitrogen-fixing bacteria are associated with seagrasses [46]. Bacterial isolates from *Ruppia* beds were shown to contain a *nifH* gene related to the γ proteobacteria, perhaps a vibrio [93]. Amplification from DNA extracted from *Halodule* roots produced *nifH* sequences [46] that we now know are in the anaerobic cluster containing *Clostridium, Desulfovibrio,* and *Chromatium* [93].

Cyanobacterial mats in nearshore environments are another place where N_2 fixation is often observed. N_2 fixation is often attributed to the cyanobacteria, but recent studies have demonstrated that, at least at certain times of the year, the mats can be dominated by a diverse assemblage of heterotrophic bacteria that contain

the *nifH* gene. The expression of these *nifH* genes has yet to be evaluated, but when the mat was dominated by a *Lyngbya* sp., rather than *Microcoleus,* the *nifH* sequences that were obtained were dominated by one that was most closely related to the *Lyngbya lagerheimii* sequence [93]. Again, the *nifH* sequences that were obtained fell largely into two clusters, the γ proteobacteria and the anaerobes [93]. Interestingly, a similar study of rice soils produced similar types of sequences [80]. Clearly, there may be a large number of N_2-fixing species in both terrestrial and aquatic environments, of which we know little. Now that we have these *nifH* sequences, we have markers to determine the distribution of these organisms, as well as identify related cultivated representatives of these unidentified organisms.

Case Study 3: Potential Use of nif *Gene Probes as Paleo-Indicators of Nitrogen Limitation*

While paleo-ecological and -climatological studies have been pursued for many years, the issue of global warming has intensified efforts to develop the ability to predict future trends. Hence, there is an intense and broad effort worldwide to examine the geochemical and biological record of the past, contained, for instance, in aquatic sediments [8] and ice cores [10]. With respect to the world's oceans, while the details may be debated, it is generally accepted that major changes in sea level and large-scale circulation are driven by climatological cycles (e.g., glacial-interglacial cycles) [13]. As a corollary, dramatic changes in the planktonic populations of the sea over these time scales are also accepted facts, and the paleontological record of species shifts is used as direct evidence to support the inferred climatological trends (e.g., [14]).

Implicit in large-scale temperature excursions, planktonic population shifts, and modification of ocean basin geomorphometry and circulation are dramatic changes in oceanic productivity (e.g., [9, 14]) and nutrient cycling [52]. Nutrient delivery to the oceans from land is presumed to change dramatically during periods of shelf exposure (glacial), compared to interglacial periods, and scenarios for the salient differences in nutrient cycling between such periods have been put forth. For instance, McElroy [52] suggests that during cooler, glacial periods, N delivery to the seas is enhanced, with greater productivity during the beginning of these periods. The perceived, present-day imbalance of the oceanic N cycle has led some to propose that a balance is achieved only on time scales of 10^6 to 10^7 years [26]. Specifically, the major complementary processes of denitrification and N_2 fixation respond on different time scales, and the current deficit may be the result of the more rapid response of denitrification to recent increases in organic flux, or to warmer temperatures. Hence, the severity of N limitation should vary over these time scales.

Geochemical and paleontological indicators presently used have provided important insight into the details of sea temperature change (e.g., [49]) and taxonomic composition [56, 76] with respect to oceanic productivity. However, the relative importance of specific nutrient cycling processes can only be inferred. Recently, Altabet and Francois [1] have observed in two oceanic transects that the $\delta\ ^{15}N$ of PON in deep surficial sediments varies inversely along gradients of NO_3^- availability in overlying waters. That is, higher $\delta\ ^{15}N$ values underlie zones of NO_3^-

depletion. Francois and Altabet [32] have also examined downcore trends of $\delta\ ^{15}N$ of PON and have proposed that they may be indicative of the historical variation in NO_3^- availability. More recently, Altabet et al. [2] have inferred trends in water-column denitrification over 10,000-year time scales in the Arabian Sea based on variations in the $\delta\ ^{15}N$ in cores. Similarly, inferences into the modes of C metabolism (C_3 vs. C_4) have been made with reference to the sedimentary record of $\delta\ ^{13}C$ in organic matter [43].

The tools of molecular biology have recently entered into the repertoire of paleontology. A current area of research interest is the use of DNA amplification and phylogenetic analysis to determine the identity of ancient organisms and their relatedness to their present-day descendants [15, 48, 61, 84].

The facts that DNA preserves well in sediments and that with modern DNA amplification techniques DNA sequence information can be obtained even from badly degraded DNA make it feasible to use genes in marine sediments as markers of past environmental conditions. Thus we suggest that isolation and characterization of DNA from sediment cores can potentially be used as a new and direct paleo-indicator of relevant processes and functional groups. Depending on downcore trends, if identified, this approach could provide a new dimension to paleo-reconstructions.

As a gene that, on the one hand, has been highly conserved, but within which sufficient divergence has occurred to allow for differentiation of the diverse types of microbes, *nif* is an appropriate candidate for such analyses. The current availability of probes for several of the *nif* genes make this system all the more attractive.

The ability to amplify DNA from surficial marine and freshwater sediments and to quickly screen amplified sequences for cyanobacterial sequences [6] support the proposition that similar *nif* gene sequences may be amplified from sediment profiles. These can be differentiated from the sequences of contemporary, active, heterotrophic bacteria that may be found deep in cores (e.g., [64]) and so are suitable for use as a paleoindicator.

Identification of discrete layers of cyanobacterial *nif* genes sequences downcore would provide evidence of past environmental conditions in the overlying water that selected for N_2-fixing species. With reference to other indicator organisms preserved in discrete sediment layers (whether assessed by conventional microscopy or also by molecular methods), the relative dominance of cyanobacterial N_2 fixers may be surmised. When correlated with appropriate geochemical markers of sea surface temperature (alkenones, e.g., [49, 73]), population structure (carbonates, silicates, indicator species, e.g., [56, 76]) and erosion/deposition, inferences about the predominance of N limitation may also be ventured. By dating with geochronological markers, a historical record of nutrient (nitrogen) limitation in that system may potentially be reconstructed. Ideally, procedures that would allow for quantitative assessment of gene presence within sediment horizons would be of greatest utility.

Acknowledgments. We would like to thank E. Carpenter and H. Paerl for discussions and encouragement and J. Burns for helpful comments. We are particularly grateful to M. Mellon and S. Braun for their excellent technical assistance and M. A. Sessions and J. Slater for manuscript preparation and review. Also we thank L. Sherman and L. Kerkhof for reviewing the manuscript. This work was

supported by NSF DEB-9410325, OCE-9202106 and OCE-9101399 to JZ, and OCE-91-03843, 93-14252 and DEB-94-10326 to DGC.

References

1. Altabet MA, Francois R (1994) Sedimentary nitrogen isotopic ratio as a recorder of surface ocean nitrate utilization. Global Biogeochem Cycles 8:103–106
2. Altabet MA, Francois R, Murray DW, Prell WL (1995) Climate-related variations in denitrification in denitrification in the Arabian Sea from sediment N-15/N-14 ratios. Nature 373:506–509
3. Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Revs 59:143–169
4. Bebout BM, Fitzpatrick MW, Paerl HW (1993) Identification of the sources of energy for N_2 fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. Appl Environ Microbiol 59:1495–1503
5. Bej AK, Mahbubani MH (1992) Applications of the polymerase chain reaction in environmental microbiology. PCR Meth Appl 1:151–159
6. Ben-Porath J, Zehr JP (1994) Detection and characterization of cyanobacterial *nifH* genes. Appl Environ Microbiol 60:880–887
7. Ben-Porath J, Carpenter EJ, Zehr JP (1993) Genotypic relationships in *Trichodesmium* (cyanophyceae) based on *nifH* sequence comparisons. J Phycol 29:806–810
8. Berger WH, Herguera JC (1992) Primary productivity and biogeochemical cycles in the sea. In: Falkowski PG, Woodhead AD (eds) Reading the sedimentary record of the ocean's productivity. Plenum Press, New York, pp 455–486
9. Berger WH, Smaetacek VS, Wefer G (1989) Productivity of the oceans: present and past. In: Berger WH, Smaetacek VS, Wefer G (eds) Ocean productivity and paleoproductivity—an overview. J. Wiley, Chichester, pp 1–34
10. Berner W, Oeschger H, Stauffer B (1980) Information on the CO_2 cycle from ice core studies. Radiocarbon 22:227–269
11. Bishop PE, Premakumar R (1992) Alternative N_2 fixation systems. In: Stacey G, Burris RH, Evans HR (eds) Biological N_2 fixation. Chapman and Hall, New York, pp 736–762
12. Bishop PA, MacDouglas SI, Wolfinger ED, Shermer CL (1990) N_2 fixation: achievements and objectives. In: Gresshoff PM, Roth LE, Stacey G, Newton WE (eds) Genetics of alternative N_2 fixation systems in *Azotobacter vinelandii*. Chapman and Hall, New York, pp 789–795
13. Broecker WS, Peng TH (1986) Carbon cycle: 1985. Glacial to interglacial changes in the operation of the global carbon cycle. Radicarbon 28:309–327
14. Burckle LH, Cirilli J (1987) Origin of diatom ooze belt in the Southern Ocean: implications for late quaternary paleooceanograph. Micropaleont 33:86–86
15. Cano RJ, Poinar H, Pieniazek N, Acra A, Poinar Jr, GO (1993) Amplification and sequencing of DNA from a 120–135 million-year-old weevil. Nature 363:536–539
16. Capone DG (1983) Benthic N_2 fixation. In: Carpenter EJ, Capone DG (eds) Nitrogen in the marine environment. Academic Press, New York, pp 105–137
17. Capone DG (1983) N_2 fixation in seagrass communities. Mar Tech Soc J 17:32–37
18. Capone DG (1988) Benthic N_2 fixation. In: Blackburn TH, Sorensen J (eds) Nitrogen cycling in coastal marine environments. John Wiley and Sons, pp 85–123
19. Capone DG, Budin J (1982) N_2 fixation associated with rinsed roots and rhizomes of the eelgrass, *Zostera marina*. Plant Physiol 70:1601–1604
20. Capone DG, Carpenter EJ (1982) N_2 fixation in the marine environment. Science 217:1140–1142
21. Capone DG, Carpenter EJ (1982) A perfusion method for assaying microbial activities in estuarine sediments: Applicability to studies of N_2 fixation by C_2H_2 reduction. Appl Environ Microbiol 43:1400–1405
22. Capone DG, O'Neil JM, Zehr J, Carpenter EJ (1990) Basis for diel variation in nitrogenase activity in the marine planktonic cyanobacterium *Trichodesmium thiebautii*. Appl Environ Microbiol 56:3532–3536

23. Carpenter EJ (1982) Physiology and ecology of marine planktonic *Oscillatoria* (*Trichodesmium*). Mar Biol Lett 4:69–85
24. Carpenter EJ, Romans K (1991) Major role of the cyanobacterium *Trichodesmium* in nutrient cycling in the North Atlantic Ocean. Science 254:1356–1358.
25. Chisholm FP, Frankel SL, Goericke R, Olson RJ, Palenik B, Waterbury, JB, West-Johnsrud L, Zettler ER (1992) *Prochlorococcus marinus* nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing divinylchlorophyll *a* and *b*. Arch Microbiol 157:297–300
26. Codispotti L (1989) Phosphorus vs. nitrogen limitation of new and export production. In: Berger WH, Smetacek VS, Wefer G (eds) Productivity of the ocean: present and past. S Bernhard, Dahlem Konferenzen, pp 377–394
27. Currin CA, Paerl HW, Suba GK (1990) Immunofluorescence detection and characterization of N_2-fixing microorganisms from aquatic environments. Limnol Oceanogr 35:59–71
28. Delwiche CC (1981) The nitrogen cycle and nitrous oxide. In: Delwiche CC (ed) Denitrification, nitrification, and atmospheric nitrous oxide. Wiley & Sons, New York, pp 1–15
29. Dugdale RC (1967) Nutrient limitation in the sea: Dynamics, identification and significance. Limnol Oceanogr 12:685–695
30. Fanning KA (1992) Nutrient provinces in the sea: concentration ratios, reaction rate ratios, and ideal covariation. J Geophysical Res 97:5693–5712
31. Felsenstein J (1993) PHYLIP-Phylogeny Inference Package (Version 3.5c). Distributed by the author. Department of Genetics, University of Washington, Seattle
32. Francois R, Altabet MA (1992) Glacial to interglacial changes in surface nitrate utilization in the Indian sector of the Southern Ocean as recorded by sediment $d^{15}N$. Paleoceanography 7:589–606
33. Fujita Y, Takahashi Y, Chuganji M, Matsubara H (1992) The *nifH*-like (*frxC*) gene is involved in the biosynthesis of chlorophyll in the filamentous cyanobacterium *Plectonema boryanum*. Plant Cell Physiol 33:81–92
34. Georgiadis MM, Komiya H, Chakrabarti P, Woo D, Kornuc JJ, Rees DC (1992) Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. Science 257:1653–1659
35. Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63
36. Gordon JK, Brill WJ (1974) Depression of nitrogenase synthesis in the presence of excess NH_4^+. Biochem Biophys Res Comm 59:967–971
37. Guerinot ML, Colwell RR (1985) Enumeration, isolation, and characterization of N_2-fixing bacteria from seawater. Appl Environ Microbiol 50:350–355
38. Hartwig EO, Stanley SO (1978) N_2 fixation in Atlantic deep-sea and coastal sediments. Deep-Sea Res 25:411–417
39. Haselkorn R, Buikema WJ (1992) N_2 fixation in cyanobacteria. In: Stacey G, Burris RH, Evans HJ (eds) Biological N_2 fixation. Chapman and Hall, New York, pp 166–190
40. Hecky RE, Kilham R (1988) Nutrient limitation of phytoplankton in freshwater and marine environments: a review of recent evidence on the effects of enrichment. Limnol Oceanogr 33:796–822
41. Hecky RE, Campbell P, Hendzell LL (1993) The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. Limnol Oceanogr 38:709–924
42. Howarth RW, Marino R, Cole JJ (1988) N_2 fixation in freshwater estuarine and marine ecosystems. 2. Biogeochemical controls. Limnol Oceanogr 33:688–701
43. Jasper JP, Gagosian RB (1989) Glacial-interglacial climatically forced $d^{13}C$ variations in sedimentary organic matter. Nature 342:60–62
44. Kawai A, Sugahara I (1971) Microbiological studies on N_2 fixation in aquatic environments. III. On the nitrogen-fixing bacteria in offshore regions. Bull Jpn Soc Sci Fish 37:981–985
45. King GM, Carlton RG, Sawyer TE (1990) Anaerobic metabolism and oxygen distribution in the carbonate sediments of a submarine canyon. Mar Ecol Prog Ser 58:275–285
46. Kirshtein JD, Paerl HW, Zehr J (1991) Amplification, cloning, and sequencing of a *nifH* segment from aquatic microorganisms and natural communities. Appl Environ Microbiol 57:2645–2650
47. Klump V, Martens C (1983) Benthic nitrogen regeneration. In: Carpenter EJ, Capone DG (eds) Nitrogen in the marine environment. Academic Press, New York, pp 411–457
48. Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362:709–715

49. Lyle MW, Prahl FG, Sparrow MA (1992) Upwelling and productivity changes inferred from a temperature record in the central equatorial Pacific. Nature 355:812–815
50. Maidak BL, Larsen N, McCaughey MJ, Overbeek R, Olsen GJ, Fogel K, Blandy J, Woese CR (1994) The ribosomal database project. Nucleic Acids Res 22:3485–3487
51. Martinez L, Silver MW, King JM, Alldredge AL (1983) N_2 fixation by floating diatom mats: a source of new nitrogen to oligotrophic ocean waters. Science 221:152–154
52. McElroy MB (1983) Marine biological controls on atmospheric CO_2 and climate. Nature 302:328–329
53. Merrick MJ (1992) Regulation of N_2 fixation genes in free-living and symbiotic bacteria. In: Stacey G, Burris RH, Evans HJ (eds) Biological N_2 fixation. Chapman and Hall, New York, pp 835–876
54. Michaels A, and 8 others (1996) Inputs, losses, and transformations of nitrogen and phosphorus in the deep North Atlantic Ocean. Biogeochemistry in press.
55. Mitsui A, Kumazawa S, Takahashi A, Ikemoto H, Cao S, Arai T (1986) Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. Nature 323:720–722
56. Mortlock RA, Charles CD, Froelich PN, Zibello MA, Saltzman J, Hays JD, Burckle LH (1991) Evidence for lower productivity in the Antarctic Ocean during the last glaciation. Nature 351:220–223
57. Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Meth Enzymol 155:335–350
58. O'Donohue, MJ, Moriarty DJW, Mac Rae IC (1991) N_2 fixation in sediments and the rhizosphere of the seagrass *Zostera capricorn.* Microb Ecol 22:53–64
59. O'Neil JM, Capone DG (1989) Nitrogenase activity in tropical carbonate marine sediments. Mar Ecol Prog Ser 56:145–156
60. Olsen GJ, Woese CR, Overbeek R (1994) The winds of (evolutionary) change: breathing new life into microbiology. J Bacteriol 176:1–6
61. Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci USA 86:1939–1943
62. Paerl HW (1990) Physiological ecology and regulation of N_2 fixation in natural waters. In: Marshall KC (ed) Advances in microbial ecology. Plenum Publishing, pp 305–344
63. Paerl HW, Bebout BM, Prufert LE (1989) Naturally occurring patterns of oxygenic photosynthesis and N_2 fixation in a marine microbial mat: physiological and ecological ramifications. In: Cohen Y, Rosenberg E (eds) Microbial mats: physiological ecology of benthic microbial communities. Am Soc Microbiol, pp 326–341
64. Parks RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ, Harvey SM (1994) Deep bacterial biosphere in Pacific Ocean sediment. Nature 371:410–413
65. Patriquin D, Knowles R (1972) N_2 fixation in the rhizosphere of marine angiosperms. Mar Biol 16:49–58
66. Pichard SL, Paul JH (1993) Gene expression per gene dose, a specific measure of gene expression in aquatic microorganisms. Appl Environ Microbiol 59:451–457
67. Pope MR, Murrell SA, Ludden PW (1985) Convalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphoshoribosylation of a specific arginine residue. Proc Natl Acad Sci USA 82:3173–3177
68. Postgate JR (1982) The fundaments of N_2 fixation. Cambridge University Press, Cambridge, Massachusetts, pp 252
69. Redfield AC (1958) The biological control of chemical factors in the environment. Am Sci 46:205–222
70. Revsbech NP, Sorensen J, Blackburn TH, Lomholt J (1981) Distribution of oxygen in marine sediments measured with microelectrodes. Limnol Oceanogr 25:403–411
71. Reysenbach AL, Giver LJ, Wickham GS, Pace NR (1992) Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol 58:3417–3418
72. Roberts GP, Ludden PW (1992) N_2 fixation by photosynthetic bacteria. In: Stacey G, Burris RH, Evans HJ (eds) Biological N_2 fixation. Chapman and Hall, New York, pp 135–165
73. Rostek F, Ruhland G, Bassinot FC, Muller PJ, Labeyrle LD, Lancelot Y, Bard E (1993) Reconstructing sea surface temperature and salinity using $\delta^{18}O$ and alkenone records. Nature 364:319–323

74. Ruvkun GB, Ausubel FM (1980) Interspecies homology of nitrogenase genes. Proc Natl Acad Sci USA 77:191–195
75. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491
76. Sancetta C (1992) Primary production in the glacial North Atlantic and North Pacific oceans. Nature 360:249–251
77. Seitzinger SP, Garber JH (1987) N_2 fixation and $^{15}N_2$ calibration of the acetylene reduction assay in coastal marine sediments. Mar Ecol Prog Ser 37:65–73
78. Short FT (1983) The seagrass *Zostera marina* L.: plant morphology and bed structure in relation to sediment ammonium in Izembeck lagoon Alaska. Aquat Bot 16:149–161
79. Simonet P, Grosjean MC, Misra AK, Nazaret S, Cournoyer B, Normand P (1991) *Frankia* genus-specific characterization by polymerase chain reaction. Appl Environ Microbiol 57:3278–3286
80. Ueda T, Suga Y, Yahiro N, Matsuguchi T (1995) Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J Bacteriol 177:1414–1417
81. Vitousek PM, Howarth RW (1991) Nitrogen limitation on land and in the sea: how can it occur? Biogeochemistry 13:87–115
82. Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numberous uncultured inhabitants in a natural community. Nature 345:63–65
83. Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271
84. Woodward SR, Weyand NJ, Bunnell M (1994) DNA sequence from Cretaceous period bone fragments. Nature 266:1229–1231
85. Wynn-Williams DD, Rhodes ME (1974) N_2 fixation in seawater. J Appl Bacteriol 37:203–216
86. Yoch DC, Whiting GJ (1986) Evidence for NH_4^+ switch-off regulation of nitrogenase activity by bacteria in salt marsh sediments and roots of the grass *Spartina alterniflora*. Appl Environ Microbiol 51:143–149
87. Young JPW (1992) Phylogenetic classification of nitrogen-fixing organisms. In: Stacey G, Burris RH, Evans HJ (eds) Biological N_2 fixation. Chapman and Hall, New York, pp 43–86
88. Zehr JP (1992) Molecular biology of nitrogen fixation in natural populations of marine cyanobacteria. In: Carpenter EJ, Capone DG, Rueter JG (eds) Marine pelagic cyanobacteria *Trichodesmium* and other diszotrophs. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 249–264
89. Zehr JP (1994) N_2 fixation in the sea: why only *trichodesmium?* In: Joint I, Carr N, Falkowski P (eds) NATO ASI series. Springer-Verlag.
90. Zehr JP, McReynolds LA (1989) Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl Environ Microbiol 55:2522–2526
91. Zehr JP, Ohki K, Fujita Y (1991) Arrangement of nitrogenase structural genes in an aerobic filamentous nonheterocystous cyanobacterium. J Bacteriol 173:7055–7058
92. Zehr JP, Wyman M, Miller V, Duguay L, Capone DG (1993) Modification of the Fe protein of nitrogenase in natural populations of *Trichodesmium thiebautii*. Appl Environ Microbiol 59:669–676
93. Zehr JP, Mellon M, Braun S, Litaker W, Steppe T, Paerl HW (1995) Diversity of heterotrophic N_2 fixation genes in a marine cyanobacterial mat. Appl Environ Microbiol 61:2527–2532