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Nitrogen-Fixing Phylotypes of Chesapeake Bay and Neuse River Estuary Sediments

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ABSTRACT

Sediments often exhibit low rates of nitrogen fixation, despite the presence of elevated concentrations of inorganic nitrogen. The organisms that potentially fix nitrogen in sediments have not previously been identified. Amplification of *nifH* genes with degenerate primers was used to assess the diversity of diazotrophs in two distinct sediment systems, anoxic muds of Chesapeake Bay and shallow surficial sediments of the Neuse River. Phylogenetic analysis revealed that sequences obtained from mid-Chesapeake Bay, which receive high organic loading and are highly reducing, clustered closely with each other and with known anaerobic microorganisms, suggesting a low abundance of aerobic or facultative diazotrophs in these sediments. Sulfate reduction dominates in the surface, but methanogenesis becomes more important with depth. A thin (<1 cm) oxidized layer is present only in the spring. No archaeal *nifH* sequences were obtained from Chesapeake Bay. Sequences of *nifH* amplified from surficial sediments of the Neuse River were distant from Chesapeake Bay sequences and included *nif* phylotypes related to sequences previously reported from marine mats and the *Spartina* rhizosphere. Differences in environmental site characteristics appear to select for different types of sediment diazotrophs, which is reflected in the phylogenetic composition of amplified *nifH* sequences.

Introduction

Biological nitrogen fixation, the conversion of atmospheric dinitrogen (N_2) to ammonium (NH_4^+), is an important source of new nitrogen in the marine environment [13]. N_2 fixation is mediated by the nitrogenase enzyme, a multisubunit protein encoded by the *nif* gene operon [30]. Organisms that possess the nitrogenase genes are limited to prokaryotes and represent a wide range of physiological groups within the Archaea and Bacteria [30, 45].

Low levels of nitrogenase activity, as determined by the acetylene (C_2H_2) reduction method [11], have been detected in a variety of marine environments, including near-shore and estuarine sediments [5, 9, 20, 24, 27]. These latter observations are somewhat enigmatic as high levels of NH_4^+ (millimolar), which can inhibit synthesis of the nitrogenase enzyme [30], are often found in sediments [15, 17, 18, 21, 39].

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One attempt using a direct $^{15}N_2$ tracer procedure in an estuarine sediment to confirm that this activity was indeed due to nitrogenase was largely unsuccessful and questioned whether the results of the C_2H_2 procedure in these types of environments were accurate [33]. Capone [10] noted that there are inherent problems with the application of the $^{15}N_2$ method in organic-rich sediments. Furthermore, other studies have provided data to corroborate that the activity associated with C_2H_4 production from C_2H_2 is indeed associated with nitrogenase. For example, there was an increase in activity after NH_4^+-rich sediment porewater removal [12], stimulation of activity with addition of methionine sulfoximine (MSX), which derepresses N_2 fixation in the presence of NH_4^+ [10, 39], and the stimulation of C_2H_4 production from C_2H_2 with removal of O_2 or addition of organic substrates [10, 18, 39]. The latter observation suggested that heterotrophic diazotrophs were involved in this process. Recently, Piceno and Lovell [29] described a stable diazotrophic assemblage associated with rhizosphere of *Spartina* that seems independent of nutrient concentrations. In the same system, Bagwell and Lovell [3] found culturable diazotrophs to be of a diverse physiology and suggest that the community is divided into specialized niches allowing nitrogen fixation to occur over a broad range of environmental conditions.

The measurement of nitrogenase activity does not provide information on the phylogenetic identity or diversity of microorganisms in these environments that may contribute to N_2 fixation. Until the advent of modern molecular techniques, identification of diazotrophs in microbial communities was dependent on isolation and culturing of organisms [19, 31]. While culturing of organisms is a useful tool for obtaining subjects for detailed physiological studies, it is difficult to ascertain the importance of a specific isolate *in situ* and, therefore, their ecological relevance. Isolates may represent only a fraction of the natural diversity of marine microbes and, because of biases in isolation procedures, may not be representative of active populations [2, 16, 38, 40] The use of PCR, and other molecular techniques, provides additional information to further the understanding of microbial diversity and community structure.

The analysis of *nif* structural genes has revealed these genes to be highly conserved [30, 32]. Despite this, *nifH* sequences provide useful taxonomic information [4] and can be used to determine phylogenetic relationships [44]. Degenerate PCR primers are effective in amplifying a segment of the *nifH* gene, which encodes for the Fe protein of the nitrogenase enzyme [22, 43]. In addition, DGGE has been utilized with PCR to reveal the diversity of diazotrophs in the rhizosphere of *Spartina alterniflora* [23].

In order to explore further the basis for nitrogenase activity in organic- and NH_4^+-rich estuarine sediments, we attempted to obtain and identify nitrogenase genes from the surficial sediments of two temperate estuaries. DNA was extracted from anoxic muds obtained from the mesohaline portion of the Chesapeake Bay and sandy sediments from the oligohaline portion of the Neuse River and was amplified for sequences of a portion of the *nif* gene. Phylogenetic analysis of the resulting sequences obtained revealed a diverse assemblage of diazotrophs belonging to groups of organisms with physiological characteristics consistent with the known biogeochemistry of the study sites.

Methods

Site Description

Sediment samples were obtained from the Maryland portion of the Chesapeake Bay, specifically from a station in the mesohaline portion of the bay (38°33.4.1'N, 76°26.6'W). This site, which is dominated by sulfate reduction, is organic-rich and highly reducing and experiences seasonal water-column anoxia [25]. NH_4^+ porewater concentrations approach 2 mM with depth and DOC approaches 1.5 mM [7]. This site has an average bottom water salinity range of 10–20 psu and has an average sediment porosity of 0.91. Samples from the Neuse River (NC) were obtained from the oligohaline portion of the river that experiences periodic water-column hypoxia. The sediment, which was sandy, was obtained from a water depth of 3 m and was relatively low in total organic matter [14]. Average bottom water salinity is 0 psu (range from 0 to 20 psu) and has an approximate sediment porosity of 0.6 (MODMON data set, University of North Carolina [1]. Biogeochemical and physical data are not available from the time of sampling.

Sample Collection

Chesapeake Bay sediments were collected by modified Bouma box corer with Plexiglass liners (9 cm i.d. × 34 cm). Intact cores were maintained at ambient bottom water temperatures until processing (2–3 hr). One core for each sampling event was extruded in 2-cm increments and subsamples placed in appropriate containers by use of Teflon-coated spatula and/or 3-cc cutoff plastic syringe. Samples for nitrogenase activity and PCR analysis were taken from the same core. Separate cores were taken for biogeochemical analysis [25]. Neuse River samples were collected by a single sediment grab and frozen (−80°C) until processed.

Table 1. Nitrogenase activity associated with Chesapeake Bay sediments

	Nitrogenase activity 0–2 cm nmol C_2H_4 (g dry sediment)$^{-1}$ h^{-1}						Nitrogenase Activity 11–13 cm nmol C_2H_4 (g dry sediment)$^{-1}$ h^{-1}					
Date	*x*	*SE*	*x*	*SE*	% of control	One-way ANOVA $\alpha = 0.05$	*x*	*SE*	*x*	*SE*	% of control	One-way ANOVA $\alpha = 0.05$
			+20 mM molybdate						+20 mM molybdate			
Apr 93	0.76	0.08	***0.036***	***0.015***	***4.70%***	$p = 0.008$	0.42	0.1	***0.022***	***0.012***	***5.20%***	$p = 0.01$
July 93	1.16	0.03	***0.036***	***0.015***	**3.10%**	$p < 0.001$	0.76	0.1	***0.022***	***0.012***	***2.90%***	$p < 0.001$

Nitrogenase Activity

Acetylene reduction assays were carried out as described by Capone [11]. Sediment samples from the Chesapeake Bay site were placed in 14 mL serum bottles, capped with a rubber stopper, and sealed. Vials were then purged with N_2 gas to remove O_2. One mL of C_2H_2 was injected into the headspace with a gas-tight syringe. Samples were incubated at ambient bottom-water temperatures. A 100 μL subsample was taken by gas-tight syringe and injected into a gas chromatograph with a flame ionization detector to measure the production of ethylene (C_2H_4) over time.

No assays were conducted with Neuse River sediments.

Molybdate Additions

Sodium molybdate was added to certain samples to a final concentration of 20 mM [8, 28] in order to abolish nitrogenase activity associated with sulfate reducers. Solutions were purged with N_2 to remove O_2 and salinities were adjusted to match ambient bottom water.

DNA Extraction

Whole sediment samples were stored in TE buffer (10 mM Tris, pH 8, 100 mM EDTA, pH 8, 0.3 M NaCl) at −80°C. DNA was extracted from 1 g of wet sediment using the protocol of Tsai and Olson [36]. The concentration of DNA in extracts ranged from 1 to 6 μg/μL.

PCR

Degenerate *nifH* primers described by Zehr and MacReynolds [43] were used for amplification of an approximately 359 bp region. Each PCR reaction contained 1 μL of DNA extract, 1.0 U AmpliTaq polymerase (PerkinElmer), 0.5 μM of each primer, 6.25 mM $MgCl_2$, and 200 μM each of dATP, dGTP, dTTP, dCTP and diluted to a final volume of 50 μL. Samples were overlaid with mineral oil and placed in a M.J. Research, Inc. PTC-100 thermocycler (Watertown, MA). DNA was amplified using 30 cycles of 1 min at 93°C, 1 min at 64°C, and 1 min at 72°C. PCR amplification products were cloned into a plasmid vector (pT7blue, Novagen, Madison, WI). For each sample, 15 to 20 clones containing *nifH* fragments were sequenced by Sequenase 2.0 DNA sequencing kit (US Biochemical, IL) or ABI automated sequencer. Plasmids were sequenced in both directions.

Phylogenetic Analysis of Recovered nifH Sequences

DNA sequences (320–330 bp excluding primer sequences) were translated and the deduced amino acid sequences were aligned using ClustalW [35] and GeneDoc software [26]. Phylogenetic trees were constructed using TREECON for Windows [37] utilizing distance matrix methods. Sequences were compared against NCBI GenBank *nifH* sequences using BLAST and representative organisms from marine environments were chosen for final tree construction.

Results

Acetylene Reduction

Acetylene reduction assays of sediments from Chesapeake Bay indicate low-level nitrogenase activity (Table 1). The addition of 20 mM sodium molybdate (final concentration), an inhibitor of sulfate reduction [28], lowered ethylene production for both surficial (0–2 cm) and downcore sediments (11–13 cm). This suggests that sulfate-reducing bacteria are a significant portion of the microbes responsible for the observed nitrogenase activity.

PCR Analysis

PCR amplification of DNA extracted from whole sediment samples yielded several unique sequences (Table 2, Figs. 1 and 2). None of the sequences were identical to *nifH* genes from known microorganisms. All 10 sequences obtained from the Chesapeake Bay Estuary clustered with those of anaerobic organisms and each other. Sequences MB57S2 and MB57S3 cluster with the sequence of *Desulfobacter curvatus* (a δ-proteobacterium). Several sequences (MB57S5, MB57S6, MB57S9, MB57S10) cluster with

Table 2. GenBank accession numbers for clone sequences and collection depth

Clone name	Accession number	Collection depth (cm)
MB57S1	AF329991	11–13
MB57S2	AF329992	0–2
MB57S3	AF329993	0–2
MB57S4	AF329994	11–13
MB57S5	AF329995	0–2
MB57S6	AF329996	0–2
MB57S7	AF329997	11–13
MB57S8	AF329998	11–13
MB57S9	AF329999	0–2
MB57S10	AF330000	0–2
NRS1605	AF374342	Surface
NRS1607	AF518561	Surface
NRS1609	AF374343	Surface
NRS1278	AF374338	Surface
NRS1279	AF374339	Surface
MRS1280	AF374340	Surface
MRS1299	AF374341	Surface

unidentified bacterial sequence from marine microbial mats. Sequences MB57S8 grouped with a sequence from *Acetobacterium woodii* (gram-positive fermentative bacterium) and sequence MB57S7 grouped with a sequence from *Chlorobium tepidum* (green sulfur bacterium). Sequence MB57S4 is in a clade with sequences from unidentified marine bacteria associated with zooplankton and a sequence obtained from the water column of the Neuse River. Sequence MB57S1 is highly divergent and does not cluster with known *nifH* sequences (Fig. 1).

Neuse River sequences are highly divergent and clustered with deeply branching sequences from anaerobic environments (Figs. 1 and 2). Sequence NRS1280 groups with an unidentified mat bacterium. Sequence NRS1607 groups with a sequence obtained from bacteria associated with the rhizosphere of *Spartina* sp. Four sequences are in a clade with β- and γ-proteobacteria. Two of these sequences cluster closely with sequences obtained from *Azoarcus* spp. and sequences obtained from the water column of the Neuse River (NRS1278, NRS1605). Sequence NRS1279 is deeply branching in a clade with *Marichromatium purpuratum*. Sequence NRS1609 is highly divergent. Sequence NRS1299 clusters closely with sequences obtained from a freshwater lake.

Discussion

We successfully obtained diazotroph sequences from organic, NH_4^+-rich Chesapeake Bay sediments that cluster closely with each other and known anaerobic diazotrophs. Sequences obtained from the same depth horizon also clustered more closely to each other (MB57S5 and MB57S6; MB57S2 and MB57S3; MB57S9 and MB57S10). Several sequences specifically group with N_2-fixing, sulfate-reducing bacteria (SRB), consistent with the anaerobic biogeochemistry of this site [25] and our observation of substantial inhibition of nitrogenase activity with molybdate (Table 1). This suggests that SRBs are important diazotrophs at this site.

Observed acetylene reduction rates for the mid-bay region of Chesapeake Bay are consistent with reported values for temperate estuaries [10]. Utilizing the same assumptions as previous studies, namely a 3:1 C_2H_4 to N_2 ratio and a liner rate of nitrogenous activity for each depth segment, integrated to 20 cm, a nitrogen fixation rate of 0.4 ± 0.2 g N m^{-2} $year^{-1}$ is predicted. Examining a number of earlier studies, Capone [9] calculated an average estuarine sediment nitrogen fixation rate of 0.40 ± 0.07 g N m^{-2} $year^{-1}$.

Sequences amplified from the Neuse River cluster more closely to each other than to those amplified from the Chesapeake Bay. Several sequences clustered with anaerobes. This observation is also consistent with a sediment environment which experiences periodic hypoxic events in the overlying water column and has rapid oxygen depletion down-core. Affourtit et al. [1] found a wide distribution of nitrogen-fixing organisms in the surface waters of the Neuse River. Several of the sequences (NRS1278, NRS1605, NRS1609) cluster with sequences from organisms found in the water column of the same oligohaline region of the Neuse River [1].

No archaeal sequences were amplified from either site. The degenerate PCR primers used do not appear to be biased against archaeal sequences [42]. This would suggest a low relative abundance of this diazotrophic group. Methanogens are known to be an important component of the down-core microbial community at the mesohaline site in Chesapeake Bay as sulfate becomes depleted [25]. In April and July 1993, porewater sulfate levels were 11.8 and 7.0 mmol/L in the surface and 0.24 and 0.74 mmol/L at 12 to 14 cm, respectively. For the same site, methane levels increased from 0.02 mmol/L wet sediment at the surface to 3.46 mmol/L wet sediment at 12 to 14 cm in April [Marvin, 1996, Ph.D. thesis, University of Maryland]. Sulfate levels of 1 to 3 mM are generally cited as limiting to sulfate reduction [6, 34]. It is possible that diazotrophic methanogens were present in low abundance but were not amplified or that the methanogens at this site are not

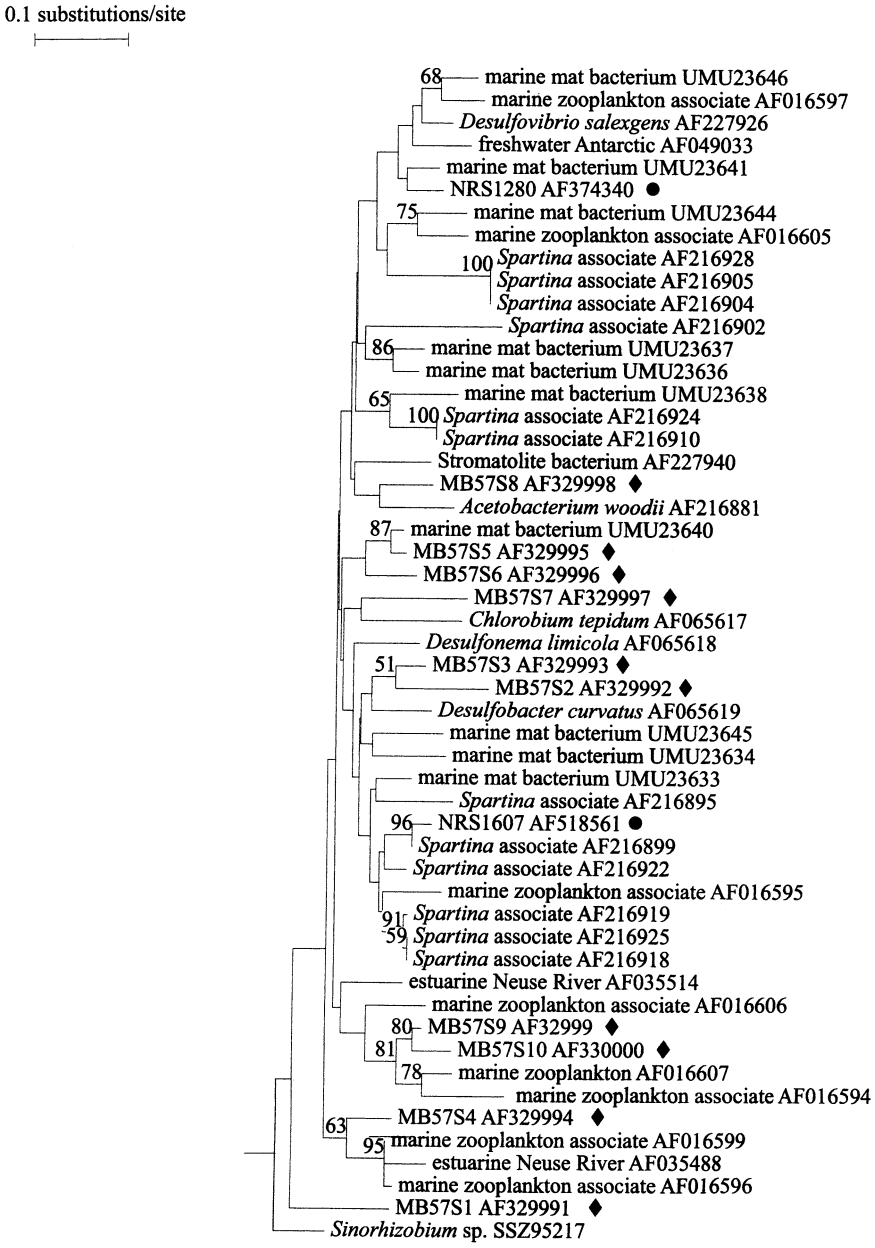

Fig. 1. Phylogenetic analysis of *nifH* sequences from Chesapeake Bay (♦) and Neuse River estuaries (●). Data sets were bootstrapped 500 times with values greater than 50% for neighbor-joining method indicated at appropriate nodes. (GenBank sequence ID indicated.) Tree is rooted to a *nifH*-like gene (*frxC*, chlorophyllide reductase X60490).

diazotrophic. While molybdate strongly inhibited nitrogenase activity in the 11–13 cm horizon of sediments from mid-Chesapeake Bay, sequences amplified from this depth interval did not appear to cluster with sequences from known diazotrophic sulfate-reducing bacteria. Sequences obtained from this depth clustered with a green sulfur bacterium (MB57S7) and a firmicute (MB57S8). One sequence, MB57S1, is deeply branching and does not cluster with known *nifH* sequences.

The clustering of sequences within each of the two sites, as opposed to between them, suggests the effectiveness of *nifH* phylogeny in identifying differences in the microbial assemblages of distinct sediment communities. The genotypes of the amplified sequences from the two sites were very distinct and this may reflect differences in physical and chemical characteristics between the two sites. Presumably, microbes that are most adapted to the biogeochemical conditions of each site are likely to be the most dominant at any given time. The amplified fragment of the *nifH* gene contains useful phylogenetic information that may ultimately be correlated with the physiological data obtained from manipulative experiments.

Obtaining diverse *nifH* sequences from the sediments of two estuarine environments also adds further credence to the proposition that the acetylene-reducing activity noted in such environments is indeed a result of nitroge-

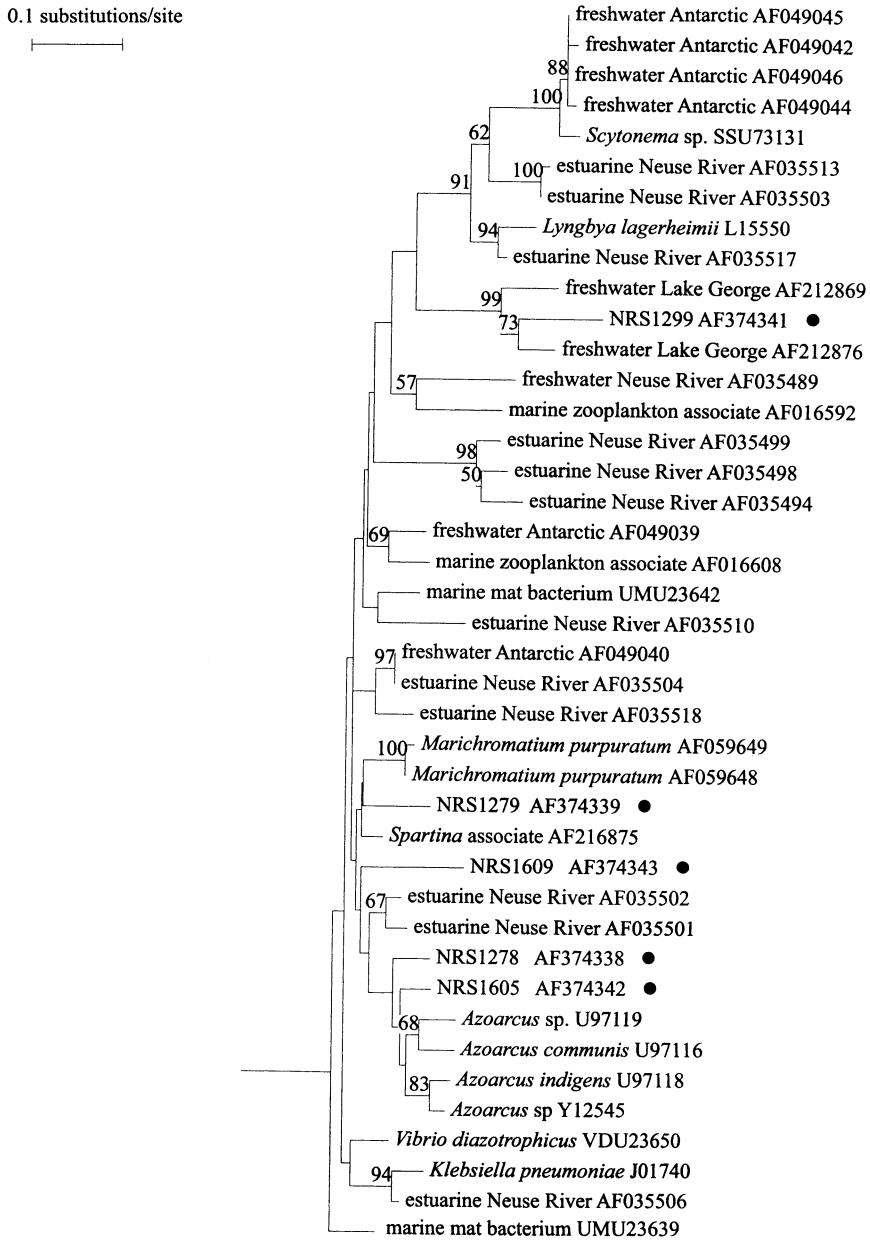

Fig. 2. Phylogenetic analysis of *nifH* sequences from Neuse River estuary (●). Data sets were bootstrapped 500 times with values greater than 50% for neighbor-joining method indicated at appropriate nodes. (GenBank sequence ID indicated.) Tree is rooted to a *nifH*-like gene (*frxC*, chlorophyllide reductase X60490).

nase activity. The high levels of NH_4^+ in these sediments [7] are above the concentration considered inhibitory to the expression of nitrogenase [15, 17]. However, the demonstration of low-level nitrogenase activity by the acetylene reduction assay suggests *nif* genes are being expressed to some extent. This is consistent with the findings of Yoch and Whiting [39], who demonstrated only partial inhibition of nitrogenase activity with the addition of NH_4^+ in sediments and rhizosphere of a *Spartina alterniflora* salt marsh. Identification of *nifH* transcripts would provide further corroboration for this as demonstrated by Zani et al. [41] in a low nutrient lake and, more recently, by Zehr et al. [45] in the open ocean.

Retention of the genetic potential for nitrogen fixation may provide the system with a mechanism to maintain a homeostasis by being able to respond to decreases in combined N (e.g., as a result of denitrification) by increasing N_2 fixation above the low levels observed. The ability of diazotrophs in systems with high ambient NH_4^+ concentrations to increase nitrogen fixation if combined N is removed from the system has been previously demonstrated [12]. The work of Piceno and Lovell [29] and Bagwell and Lovell [3] in the rhizosphere of marsh grass communities adds further evidence for the ability of natural diazotrophic assemblages to adapt to varying nutrient conditions.

The successful amplification of *nifH* genes from two different estuarine sediments demonstrates the genetic potential for nitrogen fixation in fixed nitrogen-rich sediment environments. Phylogenetic analysis of these *nifH* fragments places the sequences in clades of organisms with physiological characteristics consistent with the biogeochemistry of the study sites. These results demonstrate that the PCR amplification and sequencing of *nifH* genes can be useful for the detection and identification of diazotrophic organisms in environments that have historically been difficult to isolate and culture microbes from, such as anaerobic sediments.

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