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Using Molecular Tools to Elucidate Controls on Microbes Driving the Nitrogen Cycle in Marine Sediments

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USING MOLECULAR TOOLS TO ELUCIDATE
CONTROLS ON MICROBES DRIVING THE NITROGEN
CYCLE IN MARINE SEDIMENTS

BY

SHELLEY MARIE BROWN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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2013

DOCTOR OF PHILOSOPHY DISSERTATION
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ABSTRACT

Marine sediments harbor metabolically versatile bacteria whose activities can influence the cycle of nutrients on global scales. Microbial communities driving nitrogen (N) cycling are extremely diverse, thus making it difficult to identify the functional groups and elucidate controls on their activity. Denitrifiers in sediments remove significant amounts of N from the coastal ocean, while diazotrophs are typically considered inconsequential. Recently, N fixation has been shown to be a potentially important source of N in coastal sediments, however, the environmental drivers controlling this process are poorly understood. The goal of this dissertation was to identify and target the likely active denitrifiers and N fixers in coastal marine sediments through the analysis of genes expressed for proteins essential for denitrification, a nitrite reductase (*nirS*) and nitrogen fixation, a nitrogenase subunit (*nifH*). Subsequently, quantitative PCR and RT-PCR were used to follow the changes in abundance, distribution and *nifH* expression of the dominant diazotrophic groups in response to environmental conditions. Two groups of diazotrophs related to anaerobic sulfur/iron reducers and sulfate reducers dominated *nifH* expression in Narragansett Bay (RI, USA) sediments. Increased seawater temperature and severe hypoxia appear to be influencing the proliferation and activity of these two bacterial groups. Oxygen depletion also affects sediment porewater nutrients, indicating a shift in benthic microbial processes. In offshore sediments, *nifH* expression was related to UCYN-A, a unicellular cyanobacterium. These findings suggest that UCYN-A, a known tropical and subtropical open ocean symbiont, has a broader thermal tolerance than previously assumed and can survive in the benthos after the lifespan of its eukaryotic host.

Diazotrophic activity by these microbial communities in marine sediments is an unanticipated contribution of fixed N to coastal systems. Climate change may exacerbate the environmental conditions in which these microbes become active, consequently altering the global marine nitrogen cycle in unprecedented ways.

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PREFACE

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INTRODUCTION

Benthic Nitrogen Fixation in Coastal Systems

Benthic sediments in temperate coastal and estuarine ecosystems are generally considered areas of net nitrogen (N) loss through the removal of fixed N by denitrification (Nixon et al. 1996; Seitzinger et al. 1996). In these regions, denitrification typically exceeds rates of N fixation, the conversion of N₂ gas into ammonia. The result is a depletion of available N from the system (Seitzinger 1988). Therefore, inputs of N through N fixation are commonly regarded as an inconsequential contribution to most estuarine N budgets (Howarth et al. 1988b; Galloway et al. 2004).

Nearshore marine environments are characterized by high primary production of phytoplankton and rooted macrophytes, including seagrasses and salt marsh plants (Nixon et al. 1983). The availability of N in coastal marine systems limits primary production (Howarth 1988; Vitousek et al. 1991). To sustain high levels of growth, macrophytes require substantial sources of inorganic N (Patriquin 1972). While macrophytes largely recycle organic N entrained in the sediment (Iizumi et al. 1982; Dennison 1987; Caffrey et al. 1992; White et al. 1994), to combat the significant losses of N removed or denitrified, sources of new N are also essential to support their nutrient demands (Capone 1988). Consequently, resolving the importance N fixation in coastal benthic sediments has been of great interest to the scientific community.

Benthic N fixation has been extensively studied in areas associated with the photic zone, including sediments vegetated by macrophytes (Capone 1983; Capone 1988; McGlathery et al. 1998; Welsh 2000) and photosynthetic microbial mats

(Capone 1983; Paerl et al. 1996). Nitrogen fixation in non-vegetated, unlit sediments is just recently gaining more attention (Fulweiler et al. 2007; Bertics et al. 2010; Fulweiler et al. 2013). Many of the early studies documented N fixation by utilizing the acetylene reduction assay (ARA) to measure activity of the nitrogenase enzyme, the conserved protein complex catalyzing N fixation (eg. (Capone et al. 1982; Capone 1983; Paerl et al. 1996; Welsh et al. 1997)). More recent studies have used N₂/Ar flux measurements to document N fixation by observing a shift in the net balance of N fluxing into the sediment from N fixation as opposed to out of the sediment from denitrification (Gardner et al. 2006; Fulweiler et al. 2007; Fulweiler et al. 2013). However, these methods provide no information regarding the identity of the potentially active microbes driving N fixation in the environment, their diversity or the functional redundancy of this process in unvegetated sediments. Phylogenetic analysis of *nifH*, the gene encoding the iron protein component of the nitrogenase enzyme, has been widely used to study diazotrophs in a variety of environments reviewed in (Zehr et al. 2003; Jenkins et al. 2008; Gaby et al. 2011). Coupling molecular approaches and biogeochemical measurements can provide useful information regarding the ecology and functioning of benthic habitats.

Diazotrophic Activity By Sulfate Reducers Supports Macrophyte Growth

Intertidal zones of temperate coastal systems are vegetated by extensive meadows of rooted macrophytes including *Spartina alterniflora*, *Zostera marina*, and *Z. noltii*. The productivity of these meadows is largely supported by external inputs and by the recycling of remineralized nitrogen (Iizumi et al. 1982; Dennison 1987; Caffrey et al. 1992; White et al. 1994). Several studies have shown that the

concentrations of inorganic N in the sediment porewater are insufficient to meet the growth requirements of the macrophytes (Patriquin 1972; Short 1983; Moriarty et al. 1985). Heterotrophic N fixation in the rhizosphere provides a significant source of new N for plant growth, overcoming the limitation of N availability and, in turn, influences primary productivity of these ecosystems (Capone 1988).

High rates of N fixation have been recorded in sediments colonized by rooted macrophytes (Table 1). Rates ranged from 0.1 mg N m⁻² d⁻¹ to 7.3 mg N m⁻² d⁻¹ and were dependent on species, location and seasonality (Table 1). In the Bassion d'Arachon, France, N fixation contributed ~6-12% of the nitrogen requirement of the seagrass, *Z. noltii* (Welsh et al. 1996). Similar values were reported for *Z. marina* in Great South Bay, New York (Capone et al. 1982). Both studies also measured N fixation after the addition of sodium molybdate, a specific inhibitor of sulfate respiration. Severely reduced nitrogenase activity was detected in the rhizosphere, indicating sulfate reducing bacteria were important contributors to N fixation in the vegetated sediments (Capone et al. 1982; Welsh et al. 1996).

To gain a better understanding on the association between the nitrogen fixing bacteria and the macrophytes, several studies assessed the location of and controls on nitrogenase activity in the rhizosphere. Roots and rhizomes accounted for about 31% and 91% of rhizosphere nitrogenase activity in *Z. noltii* and *S. maritima*, respectively (Table 1) (Nielsen et al. 2001). In *Z. marina*, about 39% of the rhizosphere N fixation was associated with roots, while only 4% was associated with rhizomes (McGlathery et al. 1998). The roots and rhizomes of the *Zostera* seagrasses comprise a small portion of the rhizosphere biomass and, therefore, N fixation from the bacteria

colonizing the sediment is still significant (Nielsen et al. 2001). The combination of carbon availability and concentrations of ammonium appear to control N fixation in macrophyte-associated bacteria. The supply of organic substrate excreted from the plant can support heterotrophs, such as sulfate reducers, and has been shown to stimulate nitrogenase activity (Welsh et al. 1996; Nielsen et al. 2001). Several studies have debated the impact of sediment porewater ammonium on N fixation activity. In a *Z. noltii* meadow, about 30% of the nitrogenase activity remained after addition of 1mM ammonium chloride (Welsh et al. 1997), which contrasted results from a fjord in Denmark, in which the rhizosphere of *Z. marina* beds were not sensitive to ammonium additions (McGlathery et al. 1998).

Hundreds of diazotrophic bacteria were isolated from salt marsh rhizospheres and characterized both morphologically and physiologically (Bagwell et al. 1998). Representative species of *Enterobacter*, *Vibrio*, *Azotobacter*, *Spirilla*, *Pseudomonas* and *Rhizobia*, as well as strains with no clear taxonomic affiliations, were recovered (Bagwell et al. 1998). Culturing techniques, however, can be biased, so the natural diazotrophic communities inhabiting the rhizosphere of *S. alterniflora* were analyzed using a *nifH* fingerprinting method (Table 2) (Lovell et al. 2000). The diversity of N fixers was dominated by *nifH* sequences closely related to γ -*Proteobacteria*, including *Azotobacter chroococcum* and *Pseudomonas stutzeri*, and sulfate reducing anaerobes, such as *Desulfonema limicola* and *Desulfovibrio gigas* (Lovell et al. 2000). The same method was performed concurrently with measuring nitrogenase activity in sediments containing tall and short *S. alterniflora* stands (Piceno et al. 1999). Nitrogenase activity differed between these sites, but the composition of the diazotrophs remained

highly stable both spatially and temporally (Piceno et al. 1999). Conversely, another study of the same macrophyte detected seasonal variability of the rhizosphere diazotroph assemblages, in which several members of the anaerobic population were only detected during the winter months (Table 2) (Gamble et al. 2010). Only a small subset of the N fixing microbial community was identified to be actively expressing *nifH* in *S. alterniflora* rhizospheres (Table 2) (Brown et al. 2003). The diazotrophs were closely related to *Pseudomonas stutzeri*, *Vibrio diazotrophicus*, *Desulfovibrio africanus* and *D. gigas* (Brown et al. 2003). N fixation by macrophyte-associated bacteria helps contribute to the overall productivity of the plant communities by providing a significant source of new N to coastal ecosystems (Capone 1988; Welsh 2000).

Both Cyanobacteria and Sulfate Reducers Contribute to N Fixation in Microbial Mats

Marine benthic microbial mats frequently develop in the intertidal regions of estuaries and coastal embayments (Cohen et al. 1984; Cohen et al. 1989). The mats host metabolically diverse groups of microorganisms that contribute to critical steps in biogeochemical cycles (Cohen et al. 1984; Van Gemerden 1993). Temperate estuaries and coastal regions are typically considered N depleted (Vitousek et al. 1991), which in turn limits the primary productivity and growth of mat microbial communities (Paerl et al. 2000). N fixation by bacteria inhabitants provides a source of new N to aid in the development and persistence of the mat (Stal et al. 1985; Paerl et al. 2000). Nitrogenase activity was generally attributed to diazotrophic cyanobacteria, which are visually prominent and dominate the biomass and energy production of most

phototrophic mats (Paerl et al. 1991; Bebout et al. 1993). However, with the advent of molecular techniques, came the discovery that in addition to cyanobacteria, other microbes may be significant contributors of fixed N to the mats.

Microbial mats are composed of a rich assemblage of bacteria possessing the ability to fix N (Paerl 1990). By targeting the *nifH* gene, a high diversity of heterotrophic diazotrophs, many closely related to anaerobes including *Chromatium* (purple sulfur bacteria), *Desulfovibrio* (sulfate reducers) or *Clostridium* (strict anaerobes) spp., were detected in a North Carolina cyanobacterial mat (Table 2) (Zehr et al. 1995). The genetic potential to fix N shifted from diazotrophic cyanobacteria dominating the mat in the summer to heterotrophic N fixers in the fall and winter (Zehr et al. 1995). The seasonal shift indicates that the heterotrophs are likely responsible for fixing N during the cooler months. Phylogenetic analysis of *nifH* mRNA transcripts of the same site revealed that gene expression was limited to two groups related to non-heterocystous cyanobacteria and sulfate reducers, including *Desulfovibrio vulgaris*, *Desulfovibrio salexigens*, and *Desulfovibrio gigas* (Table 2) (Steppe et al. 2005). To further elucidate the N fixing potential of sulfate reducing bacteria residing in the mat, Steppe et al. (2002) inhibited sulfate reduction and confirmed that the sulfate reducers contributed to the majority of nighttime nitrogenase activity.

Similar results were reported from a study in Guerrero Negro, Baja California Sur, in which a *nifH* microarray was developed to examine the community composition and *nifH* expression of a microbial mat in response to nutrient loading (Moisander et al. 2006). Diazotroph diversity was high in both the control and the

nutrient amended mats, however *nifH* expression was only detected in the unaltered mat. A small subset of the diazotrophic community was actively expressing *nifH*, including three strains of cyanobacteria (related to *Cyanothece*, *Leptolyngbya* and *Halothece* spp.), two sulfate reducers (related to *Desulfovibrio vulgaris* and *Desulfovibrio salexigens*) and a green sulfur bacterium, *Prostecochloris aestuarii* (Table 2) (Moisander et al. 2006). Based on these molecular studies, the key diazotrophs potentially providing a source of N to the microbial mat are related to cyanobacteria and sulfate reducers.

High rates of N fixation in microbial mats have been reported for several temperate coastal systems (Table 1). Joye et al. (1994) reported rates as high as 79 mg N m⁻² d⁻¹. This fixed N by the mat microbial communities is locally essential to the development and high productivity of the mat. However, due to the restricted areal distribution of microbial mats and high rates of denitrification, their contribution to the total N budget in coastal marine ecosystems is minor (Joye et al. 1994; Herbert 1999).

Unprecedented N Fixation in Non-vegetated Sediments Driven By Sulfate Reducers

The benthos of most estuarine and coastal ecosystems consists primarily of non-vegetated sediments. These bare sediments are considered net sinks for N due to high rates of denitrification (Nixon et al. 1996; Seitzinger et al. 1996). In fact, during the 1970s, N fixation in the benthic sediments of Narragansett Bay, Rhode Island and Rhode River Estuary, Maryland accounted for <1% and <5% of the total annual influx of N into the systems, respectively (Marsho et al. 1975; Seitzinger 1987). Reported rates of N fixation during the 1970s and 1980s were usually less than 1 mg N m⁻² d⁻¹

(Table 1), with higher values detected in organically rich sediments such as those found in Waccasassa Estuary, Florida (Brooks et al. 1971). For temperate estuarine sediments, Capone (1983) calculated an annual N fixation rate of $0.4 \pm 0.07 \text{ g N m}^{-2} \text{ year}^{-1}$. Due to the low rates of N fixation in these benthic habitats, very few studies focused on N fixation as a source of N in unvegetated sediments.

During the summer of 2006, several sites in upper Narragansett Bay exhibited high rates of net N fixation (Fulweiler et al. 2007). The dramatic reversal in net sediment N flux was attributed to climate change induced oligotrophication of the bay (Fulweiler et al. 2007). Fulweiler et al. (2007) suggested N fixation in the estuarine sediments during those summer months added a net $(56-154) \times 10^6 \text{ g}$ of N to the bay over the annual cycle. To further investigate the impact of shifting phytoplankton bloom phenology, a biogeochemical-molecular approach was used to analyze the timing of organic matter deposition to mesocosms containing Narragansett Bay sediments (Fulweiler et al. 2013). Increased *nifH* expression occurred concomitantly with lower rates of denitrification in sediments starved of organic matter (Fulweiler et al. 2013). *nifH* mRNA transcripts were limited to two phylogenetic groups related to *Pelobacter carbinolicus* and *Desulfovibrio vulgaris*, anaerobes that can reduce sulfur and sulfate compounds, respectively (Table 2) (Fulweiler et al. 2013).

Several studies in other coastal ecosystems have also suggested sulfate reducing bacteria are responsible for the measured nitrogen fixation activity. N fixation has been documented as an important mechanism for adding N ($10.8 \pm 8.5 \text{ mg N m}^{-2} \text{ d}^{-1}$) to several Texas estuaries (Gardner et al. 2006). In Chesapeake Bay sediments, many *nifH* DNA sequences phylogenetically grouped with sulfate reducers,

such as *Desulfovibrio salexigens* and *Desulfobacter curvatus* (Table 2) (Burns et al. 2002). Additionally, nitrogenase activity decreased substantially when sulfate reduction was inhibited (Burns et al. 2002). Bertics et al. (2010) confirmed these results in bioturbated sediments off the coast of California. Nitrogenase activity decreased in the presence of sodium molybdate and DNA *nifH* sequences were closely related to various sulfur and sulfate reducers including *Desulfovibrio* and *Desulfobacter* spp. (Table 2) (Bertics et al. 2010). These recent reports indicate unvegetated sediments may be a significant source of N under certain environmental conditions.

Thesis Motivation and Outline

Unvegetated marine sediments are typically regarded as net sinks for N (Nixon et al. 1996; Seitzinger et al. 1996) and N fixation is thought to be a negligible process due to the high input of N into the system (Howarth et al. 1988a; Howarth et al. 1988b) and the process being repressed by combined N (Postgate 1982). Recently, benthic unvegetated sediments in several coastal ecosystems exhibited high rates of N fixation, challenging the denitrification-dominated paradigm (Table 1) (Gardner et al. 2006; Fulweiler et al. 2007; Bertics et al. 2010). The environmental factors driving this microbially mediated switch are not fully understood. The primary objective of this dissertation research is to identify and target the potentially active bacterial groups driving nitrogen fixation and denitrification in estuarine sediments and subsequently follow the changes in community composition and activity of these important populations of microorganisms under different environmental conditions. The response of these microbes may provide insight into the environmental controls

driving these N cycling processes and help us better understand the bacteria's influence on the ecosystem.

The capability to fix nitrogen or denitrify is widespread among diverse prokaryotic taxa (Young 1992; Zumft 1997). Analysis of expressed functional genes associated with nitrogen fixation (*nifH*) and denitrification (*nirS*) can be used to identify the most potentially active microbes driving these processes in the environment. Very few studies have used this method to study the biodiversity of likely active N cycling microbes in marine sediments. In Chapter 1, the expression of *nifH* and *nirS* were analyzed along the estuarine gradient of Narragansett Bay to an offshore continental shelf site over a temporal cycle. The dominant bacterial groups expressing *nifH* were related to anaerobic sulfur/iron reducers and sulfate reducers. The highest abundance and *nifH* expression of both groups was detected in upper Narragansett Bay sediments, which may be experiencing enhanced environmental disturbance due to warming seawater temperatures, seasonal hypoxia and the iron gradient exhibited in the upper bay.

At the offshore sites, *nifH* expression was closely related to a unicellular cyanobacterium, UCYN-A, recently renamed *Candidatus Atelocyanobacterium thalassa* (Thompson et al. 2012). UCYN-A is a known tropical and subtropical open ocean diazotrophic cyanobacterium (Zehr et al. 2001) that has a symbiotic association with a unicellular prymnesiophyte (Thompson et al. 2012). In Chapter 2, UCYN-A *nifH* transcripts were detected in the sediment (up to 6 cm in depth) when bottom water temperatures were 5-8°C, indicating that UCYN-A can survive after the lifespan of its eukaryotic host and also has a broader thermal tolerance than previously

believed. Diazotrophic activity from *A. thalassa* in sediments is an unexplored contribution of fixed N to coastal systems.

The results of Chapter 1 demonstrate that low dissolved oxygen conditions associated with hypoxia might be an important driver of sediment N fixation in coastal ecosystems. Chapter 3 further explores the impact of hypoxia on benthic sediments collected in upper Narragansett Bay. Sediment characteristics (eg. oxygen penetration and porewater nitrogen concentrations) varied in response to fluctuating oxygen concentrations in the water column, indicating a change in microbial activity in the sediment. For example, at some sites, when oxygen levels increased in the surface sediment, a greater concentration of porewater nitrite plus nitrate was observed suggesting nitrification (an oxygen requiring process) was stimulated. The greatest bulk *nifH* expression was detected in sediments usually impacted by hypoxia. Not surprisingly, the dominant groups expressing *nifH* in the sediment were related to iron/sulfur and sulfate reducing bacteria (previously identified in Chapter 1). The abundance and *nifH* expression of the dominant N fixing groups did not appear to respond to low oxygen conditions (2-3 mg/L DO). Perhaps closer to anoxic conditions are necessary to promote nitrogenase activity in these diazotropic groups.

The work from this dissertation demonstrates that anaerobes related to sulfate reducers and iron/sulfur respiring bacteria are the organisms that can add fixed N to non-vegetated marine sediments. In other coastal benthic habitats, N fixation provides a critical source of nutrients to support the growth and productivity of marine seagrasses, salt marsh plants and photosynthetic microbial mats (Capone 1988). Based on sulfate respiration inhibition assays and phylogenetic analyses, sulfate reducing

bacteria have been recognized as important active N fixers in these benthic environments, supporting our findings (Table 2). These anaerobic bacteria appear to be significant contributors to the fixed N pool in marine benthic ecosystems. However, very few studies have investigated the controls of N fixation in anaerobic bacteria, including sensitivity to combined N and oxygen tolerance. Regulation of anaerobic N fixation studies are needed to better understand the controls of this process in benthic habitats and to predict how coastal ecosystems will respond to future environmental changes.

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Table 1: N fixation rates recorded for temperate benthic coastal systems. All rates are presented in $\text{mg N m}^{-2}\text{d}^{-1}$, unless associated with specific parts of a rooted macrophyte (eg. roots or rhizomes), in which case N fixation rate is reported as $\text{nmol N cm}^{-3}\text{h}^{-1}$.

Coastal Benthic System	N fixation Rates	Reference
Vegetated by Macrophytes		
	($\text{mg N m}^{-2}\text{d}^{-1}$)	
<i>Zostera noltii</i> Bassin d'Arcachon, France	0.2-0.4 (Spring) 2.0-7.3 (Summer) 1.8-4.4 (Autumn) 0.1-0.2 (Winter)	Welsh et al. 1996
<i>Zostera marina</i> Limfjord, Denmark	1.5-2.6 (Spring) 4.2-6.0 (Summer) 3.1-5.5 (Autumn) 1.2-2.7 (Winter)	McGlathery et al. 1998
<i>Zostera marina</i> Great South Bay, New York	5.2 (Summer)	Capone et al. 1982
<i>Zostera marina</i> Vaucluse Shores, Virginia	3.9-6.5 (Summer)	Capone et al. 1982
	($\text{nmol N cm}^{-3}\text{h}^{-1}$)	
<i>Zostera noltii</i>	0.008 (Roots) 0.046 (Rhizomes) 0.117 (Sediments)	Nielsen et al. 2001
<i>Spartina maritima</i>	0.876 (Roots) 0.037 (Rhizomes) 0.087 (Sediments)	Nielsen et al. 2001
Marine Microbial Mat		
	($\text{mg N m}^{-2}\text{d}^{-1}$)	
Island of Mellum, Germany	2.19-4.11	Stal et al. 1984
Tomales Bay, California	6.0-79.0	Joye et al. 1994
Bird Shoal, North Carolina	4.47 ± 3.15	Steppe et al. 2005
Unvegetated Marine Sediment		
	($\text{mg N m}^{-2}\text{d}^{-1}$)	
Temperate Estuarine Sediments	1.1 ± 0.19	Capone 1983
Rhode River Estuary, Chesapeake Bay, Maryland	0.36	Marsho et al. 1975
Lune Estuary, England	0.38	Jones 1982
Narragansett Bay, Rhode Island	0.08	Seitzinger 1987
Waccasassa Estuary, Florida	1.02	Brooks et al. 1971
Chesapeake Bay, Maryland	1.1 ± 0.5	Burns et al. 2002
Four Estuaries in Texas	10.8 ± 8.5	Gardner et al. 2006
Catalina Harbor, California (1-10 cm)	10.1 ± 12.05	Bertics et al. 2010

Table 2: Temperate benthic diazotrophs identified through phylogenetic analysis of *nifH* genes.

Coastal Benthic System	Microbes	DNA or mRNA	Reference
Vegetated by Macrophytes			
<i>Spartina alterniflora</i> Rhizosphere	<i>Azotobacter chroococcum</i> <i>Pseudomonas stutzeri</i> <i>Desulfonema limicola</i> <i>Desulfovibrio gigas</i>	DNA	Lovell et al. 2000
<i>Spartina alterniflora</i> Rhizosphere North Inlet Salt Marsh, Georgetown, South Carolina	<i>Pseudomonas stutzeri</i> <i>Vibrio diazotrophicus</i> <i>Desulfovibrio africanus</i> <i>Desulfovibrio gigas</i>	mRNA	Brown et al. 2003
<i>Spartina alterniflora</i> Rhizosphere North Inlet Salt Marsh, Georgetown, South Carolina	Vibrionaceae clade <i>Klebsiella pneumoniae</i> <i>Sulfitobacter</i> sp. <i>Azoarcus</i> sp. <i>Desulfosporosinus</i> sp. <i>Desulfovibrio</i> spp. <i>Herbaspirillum seropedica</i>	DNA	Gamble et al. 2010
Marine Microbial Mat			
Bird Shoal, North Carolina	<i>Lyngbya lagerheimii</i> <i>Azotobacter</i> sp. <i>Klebsiella</i> sp. <i>Chromatium buderi</i> <i>Clostridium pasteurianum</i> <i>Desulfovibrio gigas</i>	DNA	Zehr et al. 1995
Bird Shoal, North Carolina	<i>Lyngbya lagerheimii</i> <i>Trichodesmium</i> sp. <i>Desulfovibrio vulgaris</i> <i>Desulfovibrio salexigens</i> <i>Desulfovibrio gigas</i>	mRNA	Steppe et al. 2005
Guerrero Negro, Baja California Sur	<i>Cyanothece</i> sp. <i>Leptolyngbya</i> sp. <i>Halothece</i> sp. <i>Desulfovibrio vulgaris</i> <i>Desulfovibrio salexigens</i> <i>Prostecochloris aestuarii</i>	mRNA	Moisander et al. 2006
Unvegetated Marine Sediment			
Chesapeake Bay, Maryland	<i>Desulfovibrio salexigens</i> <i>Desulfobacter curvatus</i> marine mat bacterium	DNA	Burns et al. 2002
Catalina Harbor, California	<i>Desulfuromonas acetoxidans</i> <i>Desulfobacter curvatus</i> <i>Desulfovibrio</i> spp.	DNA	Bertics et al. 2010
Narragansett Bay, Rhode Island	<i>Pelobacter carbinolicus</i> <i>Desulfovibrio vulgaris</i>	mRNA	Fulweiler et al. 2013

Manuscript 1

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Gene Expression to Identify and Follow Likely Active Diazotrophs in a Background
of Diverse Genetic Potential in Marine Sediments

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Abstract:

Nitrogen (N) cycling microbial communities in marine sediments are extremely diverse and it is unknown whether this diversity reflects extensive functional redundancy. Sedimentary denitrifiers remove significant amounts of N from the coastal ocean and diazotrophs are typically regarded as inconsequential. Recently, N fixation has been shown to be a potentially important source of N in estuarine and continental shelf sediments. Analysis of expressed genes for nitrite reductase (*nirS*) and a nitrogenase subunit (*nifH*) was used to identify the likely active denitrifiers and nitrogen fixers in surface sediments from different seasons in Narragansett Bay (RI, USA). The overall diversity of diazotrophs expressing *nifH* decreased along the estuarine gradient from the estuarine head to an offshore continental shelf site. Two groups of sequences related to anaerobic sulfur/iron reducers and sulfate reducers dominated libraries of expressed *nifH* genes. Quantitative PCR and RT-PCR data shows the highest abundance of both groups at a mid bay site (MNB), and the highest *nifH* expression at the head of the estuary (PRE), regardless of season. Warming seawater temperatures at PRE and MNB, along with seasonal hypoxia and higher concentrations of iron at site PRE, may influence the abundance and *nifH* expression of these two bacterial groups.

Introduction:

Estuaries and continental shelves are dynamic ecosystems that receive and process large inputs of anthropogenic added nutrients from human activity (Pinckney et al. 2001; Liu et al. 2010). Most of the nitrogen (N) is removed by denitrification in sediments in these coastal regions (Nixon et al. 1996; Seitzinger et al. 1996).

Denitrification is an anaerobic microbially mediated process in which oxidized forms of nitrogen are sequentially reduced to N_2 gas. This pathway is responsible for the major loss of fixed nitrogen in coastal margins, which in turn drives the global N deficit (Howarth et al. 1988a).

Biological nitrogen fixation, the conversion of N_2 gas into ammonia, is usually regarded as an inconsequential component in most estuarine nitrogen budgets (Howarth et al. 1988b; Galloway et al. 2004). However, N fixation is being considered increasingly important in specific benthic habitats, particularly in areas associated with the photic zone including photosynthetic microbial mats (Capone 1983; Paerl et al. 1996) and sediments vegetated by sea grasses (McGlathery et al. 1998; Herbert 1999) and salt marsh plants (Welsh et al. 1996; Herbert 1999). Due to the high rates of denitrification reported in estuarine systems (Seitzinger 1988; Nixon et al. 1996), little attention has been given to whether nitrogen fixation occurs in non-vegetated sediments. Recently, benthic sediments from the temperate estuary Narragansett Bay (RI, USA) were shown to exhibit a seasonal switch in nitrogen cycling with high rates of net N_2 fixation, challenging the denitrification-dominated paradigm (Fulweiler et al. 2007).

Functional genes encoding cellular proteins that mediate biogeochemical transformations not only provide insight into the ecology of a system, but also can be used to investigate the diversity of specific groups of microorganisms (eg. denitrifiers and nitrogen fixers) in the environment. The key intermediate step in the denitrification pathway, reduction of nitrite to nitric oxide, is catalyzed by the NirS and NirK proteins, two known forms of dissimilatory nitrite reductase. Bacteria harbor

copies of either *nirS* or *nirK* genes and both have been used as gene markers used for ecological studies to follow denitrifier community composition (Braker et al. 2001; Avrahami et al. 2003). *nirS* was targeted for this study as the gene is preferentially found in marine sediments through PCR-based methods, while *nirK* is detected more readily in soil (Braker et al. 2000). The study of diazotroph diversity has been largely based on the phylogenetic analysis of *nifH* (Zehr et al. 1989), the gene encoding the nitrogenase iron protein component of the conserved nitrogenase protein complex, an enzyme catalyzing nitrogen fixation in these microbes (Howard et al. 1996).

The capability to denitrify or fix nitrogen is distributed through diverse prokaryotic taxa throughout the bacteria and archaea (Young 1992; Zumft 1997). Several studies in Chesapeake Bay (MD and VA, USA) have sought to understand the mechanisms driving the distribution of denitrifiers (Bulow et al. 2008) and diazotrophs (Burns et al. 2002; Jenkins et al. 2004; Short et al. 2004; Steward et al. 2004; Moisander et al. 2007). These studies have highlighted that diverse communities of microbes containing the *nirS* or *nifH* gene are present in the estuarine ecosystem, but it remains unclear what factors control their diversity and what fraction of these microbes are metabolically active. In this study, we follow gene expression to identify the likely active groups driving denitrification and nitrogen fixation. Determining the functional groups can help elucidate the environmental controls regulating these two processes. Very few studies have used gene expression as a method to examine biodiversity of the most potentially active N cycling microbes. Only a few groups have detected *nirS* expression (Nogales et al. 2002; Bulow et al. 2008) and to our knowledge, there has been only one report of *nifH* expression in non-vegetated benthic

sediments (Fulweiler et al. 2013). Our purpose is to go beyond DNA diversity studies, targeting mRNA from sediments to understand the diversity of the assemblages of denitrifiers and nitrogen fixers expressing the *nirS* and *nifH* genes, respectively.

In this study, we examined the active *nirS*- and *nifH*-transcribing microbial populations to determine the likely functional denitrifiers and diazotrophs in benthic sediment samples collected along the estuarine gradient from the head of Narragansett Bay to an offshore continental shelf site. One of our aims was to determine if the expressed *nirS* and *nifH* sequence diversity patterns resembled the unique distribution of denitrifiers and nitrogen fixers in Chesapeake Bay, in which the diversity of *nirS* and *nifH* decreased along the estuarine gradient from the freshwater end to the more saline mouth (Moisander et al. 2007; Bulow et al. 2008). Nitrogen fixation in bare estuarine sediments is recently becoming recognized as an important process occurring in coastal systems (Fulweiler et al. 2007; Bertics et al. 2010; Bertics et al. 2012a; Bertics et al. 2012b; Fulweiler et al. 2013), so for the remainder of the study we focused on quantifying the transcriptional activity of bacterial populations actively expressing *nifH*. Predominant expressed *nifH* sequences were used to develop primers and probes for quantitative PCR to follow the changes in abundance, distribution and *nifH* expression of the microbial groups along the estuarine gradient over an annual temporal cycle. To investigate how the environment impacts the biodiversity of genetically active diazotrophs, we also examined potential mechanisms (eg. oxygen, temperature and salinity) driving shifts in these diazotroph communities in the benthic sediments.

Results:

Expression of functional genes associated with nitrogen fixation (*nifH*) and denitrification (*nirS*) were analyzed at four stations (PRE, MNB, RIS2 and MP1) along the estuarine gradient of Narragansett Bay to an offshore continental shelf site over a temporal cycle (Fig. 1, Table S1). (Refer to *Experimental Procedures* for more in-depth collection and site description.)

Phylogenetic Relationships of Expressed *nirS* and *nifH* Sequences

Expression of *nifH* was detected at all four sites throughout the temporal cycle (Fig. 2). The spatial distribution of *nifH* mRNA transcripts was variable along the sediment depth gradient and did not appear to be impacted by location or season of collection (Fig. 2). *nirS* expression was also observed at all four stations, however it was usually detected in the warmer sampling months (May through October) (Fig. 2). *nirS* expression was localized to the top 2 cm except at station MP1 (Fig. 2). The expression of *nirS* was rarely detected without concurrent *nifH* expression (Fig. 2).

Phylogenetic analysis of *nirS* mRNA transcript sequences shows that they are distributed throughout several diverse groups amongst *nirS* phylogeny (Fig. S1, Table S2). The majority of expressed *nirS* sequences group close to *Azoarcus toluolyticus*, a bacterium notable for its ability to both denitrify and fix nitrogen (Zhou et al. 1995) (Fig S1). Both spatially and seasonally, a major shift was not detected in the distribution of denitrifiers expressing *nirS*.

Phylogenetic analysis of the expressed *nifH* sequences from these sites shows that they are restricted to two main *nifH* phylogenetic groups (*nifH* Clusters I and III, as previously defined (Chien et al. 1996)) and group with known sulfate, sulfur and

iron reducing bacteria (Fig. 3, Table S3). The majority of expressed *nifH* sequences (61) are within group NB3 which has as its most closely related cultivated species *Pelobacter carbinolicus*, an anaerobe known to reduce sulfur and iron compounds (Lovley et al. 1995) (Fig. 3). The second largest group of expressed sequences, group NB7, contains 22 expressed *nifH* sequences with the most closely related cultivated bacteria being the sulfate reducers *Desulfovibrio salexigens* and *Desulfovibrio vulgaris* (Fig. 3). Even though the expressed *nifH* and *nirS* sequences we recovered are constrained to a few broad taxonomic groups, there is microdiversity detected among the different sites.

Diazotroph Diversity Shifts Along the Estuarine Gradient

The diversity of microbes expressing *nifH* in the sediment decreases along the estuarine gradient, from 10 groups identified at the head of Narragansett Bay (PRE) to 3 groups at RIS2 (Fig. 4). Even though we only sampled site MP1 once, the trend continues and 3 groups were detected to be expressing *nifH* at the most offshore station (Fig. 4). Group NB3, related to *Pelobacter carbinolicus* was detected at all four sites. The community composition of the remaining diazotroph shifts from being comprised by microbial groups with *nifH* sequences related to those from several different sulfate and sulfur reducers in the Upper Bay to a group of sequences related to an uncultivated marine cyanobacterium, (UCYN-A (Tripp et al. 2010)), recently renamed *Candidatus Atelocyanobacterium thalassa* (Thompson et al. 2012), at the offshore sites (Fig. 4). Seasonality and depth in the sediment column did not appear to impact the overall diversity of the bacterial populations expressing *nifH* at each site (Figs. S2 and S3).

QPCR Targeting Specific Diazotroph Groups

Changes in abundance, distribution and levels of expression of the two dominant microbial groups expressing *nifH*, groups NB3 and NB7, were determined by quantitative PCR. The greatest overall abundance of group NB3 was detected at site MNB at 3 to 4 cm in depth (Figs. 5a and 5c). Site PRE maintains the next highest levels of group NB3, with the lowest levels detected at the offshore sites RIS2 and MP1 (Figs. 5a and 5c). One-way ANOVA tests revealed the abundance of NB3 differed significantly among sites ($F(3,20)=20.98$, $p < 0.0001$), with the maximum difference between site MNB and the other three sites as determined by the Tukey-Kramer HSD test (Table S4). The highest *nifH* expression of group NB3 is observed at the head of the Bay (site PRE), with a peak from 4 to 6 cm in depth during June 2010 (Figs. 5b and 5d). Both the abundance and *nifH* expression of group NB3 are lowest at the offshore sites, RIS2 and MP1 (Fig. 5). Group NB7 followed a similar distribution in the depth profile, with the greatest abundance between 3 and 6 cm in depth at site MNB (Figs. 6a and 6c). The abundance of group NB7 differed significantly between sites ($F(3,20)=24.1$, $p < 0.0001$), with the greatest difference between the sites enclosed by land, PRE and MNB, versus the offshore locations, RIS2 and MP1 (Table S5). Highest *nifH* expression by group NB7 was also detected at lower depths at site PRE, with the exception of peak *nifH* transcripts detected at the sediment-water interface in October 2010 (Figs. 6b and 6d). Even though both groups NB3 and NB7 established highest abundances at site MNB, expression of *nifH* by these groups follows the estuarine gradient, with maximum levels observed at site PRE decreasing out to the continental shelf station, MP1 (Fig. 7). The expression of both microbial

groups is statistically significantly higher at site PRE compared to the other three sites (Tables S4 and S5). No statistical differences were detected in abundance or expression of groups NB3 and NB7 over the seasonal cycle or along the depth gradient at the sampling locations.

Discussion:

Recently, benthic sediments from several locations in upper Narragansett Bay, including sites PRE and MNB, were shown to exhibit a seasonal switch in nitrogen cycling with high rates of net nitrogen fixation (up to $-650 \mu\text{mol N}_2\text{-N m}^{-2} \text{h}^{-1}$) during the summer months (Fulweiler et al. 2007). These findings challenge the denitrification-dominated paradigm (Christensen et al. 1987; Hulth et al. 2005), in which nitrogen fixation was thought to be a negligible process occurring in coastal systems due to the high input of N into the system (Howarth et al. 1988a; Howarth et al. 1988b) and the process being repressed by combined N (Postgate 1982). Numerous studies have reported the importance of nitrogen fixation in coastal habitats, specifically in photosynthetic microbial mats and in sediments vegetated by salt marsh plants and seagrass (e.g. (Capone 1983; Paerl et al. 1996; Welsh et al. 1996; McGlathery et al. 1998; Herbert 1999). However, estuaries are still considered a net sink for nitrogen. More recently, nitrogen fixation (Bertics et al. 2010; Bertics et al. 2012a) as well as *nifH* expression (Fulweiler et al. 2013) has been detected in non-vegetated, bare sediments. We present gene expression data identifying and targeting the likely active microbes driving N fixation in a background of diverse genetic potential in marine sediments. Changing environmental conditions including elevated water temperatures due to climate change (Scavia et al. 2002) and increases in

eutrophication-induced hypoxia (Diaz et al. 2008; Zhang et al. 2010) may be driving active heterotrophic N fixation in coastal and shelf sediments.

Variable *nifH* and *nirS* Expression

We detected *nifH* expression at all sites and time points, however the expression varied throughout the depth profile with no apparent seasonal correlation. These results were not surprising as variable *nifH* expression has been recently reported in mesocosm experiments from sediments collected at site MNB (Fulweiler et al. 2013) and nitrogen fixation has been detected in sediments at depths >5cm (Bertics et al. 2010). *nirS* expression was likely only detected in the top 2 cm because in coastal sediments denitrification is typically coupled to nitrification (Seitzinger et al. 1984; Nowicki 1994), an oxygen requiring process and needs to occur in the surface sediments. *nirS* expression was detected in deeper sediments at Site MP1, an area that has not been well studied, which may be attributed to slightly increased rates of direct denitrification occurring on the continental shelf. For example, on the mid-Atlantic Bight, 9% of nitrogen removed was accounted for by direct denitrification (Laursen et al. 2002).

Fluctuating Environmental Conditions Promote Diazotroph Diversity

The diversity of major nitrogen cycling organisms, including denitrifiers, nitrifiers and nitrogen fixers, have been well studied in Chesapeake Bay by detecting the functional genes *nirS* (Bulow et al. 2008), *amoA* (Francis et al. 2003; Ward et al. 2007) and *nifH* (Moisander et al. 2007), respectively. The diversity pattern was similar for all genes studied, with the greatest diversity observed at the freshwater head of Chesapeake Bay decreasing to the mouth. For this study, we focused on how the

changing environmental variables along the down-bay gradient impacted the active microbes driving nitrogen fixation in Narragansett Bay sediments. We see a similar pattern with the potentially active nitrogen fixers, in which the highest diversity of microbes expressing *nifH* is detected at site PRE, near the head of the Bay and decreasing out to site MP1 on the continental shelf. Narragansett Bay, like Chesapeake Bay, exhibits an estuarine gradient with respect to temperature, salinity and nutrients (Kremer et al. 1978; Oviatt 2008). The northernmost area, site PRE, is a dynamic region with large fluctuations in temperature, salinity, oxygen and nutrients over a temporal cycle (Granger 1994). Because of these wide ranges in environmental conditions, microbes need to adapt to a continuously changing ecosystem. Conversely, the offshore sites (RIS2 and MP1) remain relatively stable because of exchange with the Atlantic Ocean and the deeper waters. For example, the bottom water at Rhode Island Sound ranges from 4.5 to 14 °C over the course of a year and salinity remains stable between 29.75 to 33.5 ppt (Codiga et al. 2010). The intermediate disturbance hypothesis (IDH) suggests that species diversity is maximized when ecological disturbance is neither too rare nor too frequent (Connell 1978; Huston 1979). At low levels of disturbance, more competitive organisms will dominate the ecosystem while at high levels of disturbance, organisms may not be able to adapt to their surroundings. IDH was originally developed for tropical rainforests and coral reefs (Connell 1978), and has recently been applied to plankton communities (Floder et al. 1999). We may be detecting the greatest diversity of diazotrophs expressing *nifH* at the head of Narragansett Bay because the microbes are competing to adapt to the intermediate

fluctuating environmental conditions, while at the more stable offshore sites, the benthos is dominated by the more competitive microbes.

Dominant Active Diazotrophs May be Influenced by Temperature, Oxygen and Metals

Sequence analysis revealed that *nifH* expression was dominated by two microbial groups (NB3 and NB7), related to the iron/sulfur reducer *Pelobacter carbinolicus* and the sulfate reducers *Desulfovibrio salexigens* and *D. vulgaris*, respectively. Interestingly, the majority of our *nifH* RNA sequences phylogenetically group with DNA sequences reported from sediments in coastal California and Eckernförde Bay, Baltic Sea (Bertics et al. 2010; Bertics et al. 2012a). In both studies, *nifH* sequence types were identified that were related to various sulfur and sulfate reducing bacteria, including *Desulfovibrio* spp. and *Desulfobacter* spp., which are microbes that have been shown to fix nitrogen in culture (Sisler et al. 1951; Widdel 1987). Based on acetylene reduction and sulfate reduction inhibition assays, Bertics et al. attributed the nitrogen fixation rates to sulfur and sulfate reducing bacteria (Bertics et al. 2010), corroborating our findings that these microbes are likely to be driving nitrogen fixation in these sediments. Our expressed *nifH* sequences also group with *nifH* RNA sequences recently reported from mesocosm experiments with sediment collected at site MNB (Fulweiler et al. 2013). Both microbial groups NB3 and NB7 are highly abundant in the sediment at sites PRE and MNB in upper Narragansett Bay (Figs 5c and 6c). Nitrogen fixation by these microbes could provide unanticipated inputs of nitrogen into ecosystems already stressed by eutrophication, including Narragansett Bay. Denitrification may not balance the anthropogenic inputs of N to

the extent previously believed, and the sediments could instead become a net source of N exacerbating the nutrient loading into the system.

Temperature and dissolved oxygen concentrations are potentially key drivers of growth and activity of NB3 and NB7 as these groups are related to mesophilic anaerobes. We detected the highest *nifH* expression during the summer months when the water temperature was the warmest (18 °C) at sites PRE and MNB. The water temperatures in Narragansett Bay can reach up to 24 °C during the summer (Kremer et al. 1978). Bottom water temperature at site RIS2 during July 2010 was 13 °C and at site MP1 during August 2011 only reached 8°C. We detect the lowest abundance and *nifH* expression of NB3 and NB7 at sites RIS2 and MP1, so the offshore regions may not provide an optimal temperature range for these microbial populations.

Low oxygen events may also be promoting these anaerobic diazotrophs to fix nitrogen. For the last several decades, episodic hypoxia has been documented in Narragansett Bay (Oviatt et al. 1984; Bergondo et al. 2005; Deacutis et al. 2006; Melrose et al. 2007; Deacutis 2008; Saarman et al. 2008). The severity of hypoxia generally decreases in intensity with distance from site PRE, in the Providence River Estuary, following the north-south gradient of nutrients, phytoplankton and freshwater influence (Oviatt et al. 2002; Prell et al. 2004; Melrose et al. 2007; Deacutis 2008; Oviatt 2008; Saarman et al. 2008). During the same summer when high rates of net nitrogen fixation were recorded at sites PRE and MNB (Fulweiler et al. 2007), there were several bouts of widespread hypoxia that severely impacted regions of upper Narragansett Bay (Codiga et al. 2009). The occurrence of these low oxygen events may be stimulating the growth and activity of groups NB3 and NB7. We observed the

highest expression of *nifH* at site PRE, which is an area that usually experiences severe hypoxia during the summer months (Saarman 2002). In some years, hypoxia can reach as far south as site MNB (Deacutis et al. 2006; Melrose et al. 2007). One possible explanation for the increase in abundance and expression at these northern sites is that hypoxic conditions caused by high rates of microbial respiration in the sediments may disrupt the link between coupled nitrification-denitrification, as the former is an oxygen requiring process. A shrinking oxic sediment layer and inhibition of the coupled N-removal pathway could thus expand the niche for sulfur and sulfate reducers to thrive.

In addition to elevated water temperatures and hypoxia, regions of upper Narragansett Bay are also exposed to high levels of metal contaminants and organic carbon due to anthropogenic input Nixon 1995, Murray 2007 (Nixon 1995; Rincón 2006; Murray et al. 2007). Concentrations of iron have been shown to match up with the mapped extent of hypoxia in the Bay (Prell et al. 2004; Rincón 2006). Both iron and organic carbon concentrations in the sediment decrease along the estuarine gradient (King et al. 1995; Murray et al. 2007). Several of the potentially active nitrogen fixers are related to heterotrophic anaerobes that have the ability to also reduce iron, including *Pelobacter carbinolicus* (Nealson et al. 1994; Lovley et al. 1995). The energy gained from respiring iron, a fairly energy yielding electron acceptor, and consuming organic carbon may be promoting the growth and activity of these diazotrophs in upper Narragansett Bay sediments.

In comparison to the sites PRE and MNB in Narragansett Bay, the offshore locations are more uniform in terms of seasonal temperature differences and in similar

rates of denitrification. We detected the lowest abundance and *nifH* expression of groups NB3 and NB7 at the offshore sites RIS2 and MP1. Environmental conditions, including temperature and dissolved oxygen concentrations, at these offshore sites may not be optimal for these potentially active diazotrophs to thrive in these regions. Bottom water temperatures are unlikely to rise to the optimal threshold for mesophiles, possibly impeding the growth and activity of these groups of diazotrophs. Along with lower temperatures, anoxic or hypoxic conditions have not been reported for waters in Rhode Island and Block Island Sounds. Net sediment denitrification rates recently recorded from several locations off of the coast of southern Rhode Island, including RIS2, were not significantly different between sites or over a temporal cycle, ranging from 20 to 75 $\mu\text{mol N}_2\text{-N m}^{-2} \text{h}^{-1}$ (Heiss et al. 2012) and are within the range of values reported from nearby continental shelf areas, including the Mid Atlantic Bight and South Atlantic Bight (Devol 1991; Devol et al. 1997). Conditions in upper Narragansett Bay may be more optimal for growth and activity of groups NB3 and NB7, while offshore sediments may not provide an appropriate niche for these microbes to thrive.

Conclusion:

Benthic nitrogen cycling processes are influenced by changes in environmental conditions, including temperature, dissolved oxygen concentrations, salinity and organic matter loading. Climate change is predicted to increase seawater temperatures (Scavia et al. 2002) and exacerbate eutrophication-driven hypoxia (Diaz et al. 2008; Zhang et al. 2010). Since the 1960s, the number of hypoxic zones has approximately doubled each decade (Diaz et al. 2008) and these expanding low oxygen events have

the potential to perturb the functioning of the nitrogen cycle in estuarine ecosystems. Narragansett Bay, like many coastal ecosystems, is exposed to elevated water temperatures and exhibits seasonal hypoxia (Diaz et al. 2008; Zhang et al. 2010). Microbes related to iron/sulfur and sulfate reducers that express *nifH* are highly abundant and have increased levels of *nifH* mRNA transcripts in Narragansett Bay sediments. These diazotroph communities may proliferate and increase activity in response to elevated water temperatures, episodic hypoxic events or high concentrations of organic carbon in the bay. Consequently, nitrogen fixation by these microorganisms in coastal sediments could provide unanticipated inputs of nitrogen into environments already stressed by eutrophication, significantly altering the nitrogen cycle in unprecedented ways.

Experimental Procedures:

Study Sites

We sampled for sediment at four sites in southern New England coastal waters from May 2010 to August 2011. Sites PRE (41°46.7', 71°22.8') and MNB (41°35.3', 71°22.4) are located within the temperate estuary, Narragansett Bay, Rhode Island. The offshore sites, RIS2 (41°17.1', 71°18.2') and MP1 (40°26.1', 70°28.9'), are located in Rhode Island Sound and on the continental shelf 110 km south of Cape Cod, Massachusetts, respectively. Sites PRE and MNB were sampled in June and October 2010 and January 2011. Site RIS2 was sampled in May, July, and October 2010 and January 2011. The most offshore site, MP1, was only sampled in August 2011 (Fig. 1, Table S1).

Field Methods

Intact sediment cores (10 cm inner diameter and 30.5 cm long) were collected at site PRE using a 5 m pull corer and at site MNB by SCUBA divers. At sites RIS2 and MP1, sediment cores were collected using a box corer (0.25 m²) and pre-mounted PVC cores. All cores were transported to and stored in the dark at field bottom-water temperature in a walk-in environmental chamber at the Graduate School of Oceanography at the University of Rhode Island. The cores were left uncapped with air gently bubbling through the overlying water for about 8-12 h prior to net N₂ flux incubations. For details regarding N₂ flux methods and offshore flux results, refer to Fulweiler et al. 2007 and Heiss et al. 2012.

Sub-sampling and Nucleic Acid Extractions

After the net N₂ flux incubations were completed, the cores were sub-sampled using a 60 mL syringe. The sub-cores were flash frozen in liquid N₂ and sectioned into 1 cm segments from the sediment water interface to 6 cm in depth. The frozen sediment cross-sections were cut up to yield 0.25 g and 0.5 g of wet sediment for DNA and RNA isolation, respectively. Total DNA was extracted using the MO Bio Powersoil DNA Isolation Kit (Carlsbad, CA, USA) and quantified using Invitrogen's Qubit dsDNA HS Assay Kit. All DNA samples were diluted to a concentration of 1 ng/uL for qPCR analysis. An optimal concentration of 1ng/uL was chosen based on saturation experiments. Total RNA was extracted using the MO Bio Powersoil RNA Isolation Kit (Carlsbad, CA, USA), however the kit was designed to extract RNA from 2 g of soil. To accommodate this reduction in reaction scale, a quarter of the volumes of the Bead, SR1, SR2, SR3 and SR4 solutions and phenol-chloroform-isoamyl

alcohol were used. After the RNA precipitation step, the dried pellet was resuspended with 100 μL of nuclease free water, 10 μL of 10X TURBO DNase buffer and 1 μL of TURBO DNase from the TURBO DNase-*free* Kit (Ambion, Austin, TX, USA) and incubated at 37°C for 30 min. To inactivate the reaction, 10 μL of DNase Inactivation reagent was added and incubated at room temperature for 5 min. The remaining RNA purification steps were carried out using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was quantified using Invitrogen's Qubit RNA Assay Kit and all RNA samples were diluted to 4 ng/ μL . cDNA copies of RNA were generated with Invitrogen's SuperScript First-Strand Synthesis System for RT-PCR. For all samples, 8 μL of DNase-treated RNA at a concentration of 4 ng/ μL was added to the reaction. Each reaction was primed with 1 μL of 2 μM outer reverse primers for both our genes of interest, *nifH3* and *nirS6R* (Tables S6 and S7). After the reverse transcriptase was added, the mixture was incubated at 50°C for 50 min. All the other steps followed the instructions of the manufacturer. For every sample, we also included controls that did not contain reverse transcriptase to confirm there was no DNA contamination in the subsequent PCR amplification.

Functional Gene Sequence Analysis

The *nifH* gene from environmental cDNA was isolated using nested PCR with degenerate outer primers *nifH4-nifH3* and inner primers *nifH1-nifH2* (Table S6). Both rounds of PCR consisted of an initial denaturation step of 2 min at 94°C, cycling steps that included: a denaturation step of 30 s at 94°C, an annealing step of 30 s at 50°C, and an extension step of 1 min at 72°C. All reactions had a final extension step of 7

min at 72°C. First round reactions had 25 cycles and the second round reactions had 30 cycles (Zehr et al. 1989; Kirshtein et al. 1991). *nirS* was amplified using the primer pair nirS1F-nirS6R (Table S7). After a 2 min initial denaturation step 94°C, a touchdown PCR was performed that consisted of a denaturation step of 30 s at 94°C, an annealing step of 30 s, and an extension step of 1 min at 72°C. During the first 11 cycles, the annealing temperature decreased 0.5°C every cycle starting at 56°C. For the last 25 cycles the annealing temperature was 54°C. A final extension step was performed for 7 min at 72°C (Braker et al. 1998; Braker et al. 2000).

After amplification, the PCR products were loaded on to a 1% agarose (wt/vol) TAE gel. Bands of the correct size were purified using the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen Valencia, CA, USA). The purified products were cloned into pGEM-T vectors (Promega, Madison, WI, USA), transformed into JM109 *E.coli* competent cells (Zymo Research, Irvine, CA, USA) and identified by blue-white screening. The plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and sequenced on the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the RI Genomic Sequencing Center at the University of Rhode Island. For every expressed *nifH* and *nirS* amplicon we collected 4-5 sequences.

Expressed environmental *nifH* sequences from sites PRE, MNB, RIS2 and MP1, accession numbers KF285284-KF285397, were combined with *nifH* sequences (23) from closely related cultivated species in GenBank (Benson et al. 2009) as determined by top BLASTn hits. DNA *nifH* sequences were translated into amino acid sequences in Geneious. Translated NifH protein sequences were aligned using the

multiple sequence alignment tool, MUSCLE (Edgar 2004) within the Geneious software package. A *nifH* maximum likelihood tree of aligned protein sequences was constructed in Geneious using PhyML with 1000 bootstrap replicates.

A *nirS* database was created by collecting all the *nirS* sequences from the National Center for Biotechnology Information (NCBI) (Benson et al. 2009) and importing the GenBank files into ARB (Ludwig et al. 2004). Translated NirS protein sequences were aligned using the multiple sequence alignment tool, MUSCLE (Edgar 2004) within the Geneious software package. A NirS protein maximum likelihood tree including the Narragansett Bay expressed *nirS* sequences, accession numbers KF285398-KF285429 was constructed using the PhyML algorithm in Geneious with 1000 bootstrap replicates.

Quantitative real-time PCR

Quantitative PCR was conducted on all environmental DNA and cDNA samples using the Roche's LightCycler 480 Probes Master Mix and were analyzed using Stratagene's Mx3005 qPCR System. Sets of degenerate qPCR primers and dual-labeled TaqMan probes were designed to target the *nifH* gene specifically related to *nifH* groups NB3 and NB7 (Fig. 3, Table S8). A standard curve was produced with triplicate 10-fold dilution series ranging from 1 ng to 1 ag of linearized plasmid containing a sequenced *nifH* clone from group NB3 and NB7, respectively. The qPCR reactions consisted of 10 uL of the Roche LightCycler 480 Probes Master mix, 5.7 uL of water, 2 uL of a primers/probe mix (at concentrations of 0.4 uM and 0.2 uM, respectively), and 0.3 uL of Stratagene Brilliant II qPCR reference dye (ROX). A saturation test was used to determine the optimal concentration of DNA and cDNA

template going into the reaction. It was determined that 2 uL of 1 ng/uL DNA sample or 2 uL of 1.5 ng/uL cDNA sample was added to the reaction totaling 20 uL. The qPCR thermocycling conditions for group NB3 were: 1 cycle of 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 seconds and 60°C for 1 minute. For group NB7, the qPCR thermocycling conditions were: 1 cycle of 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 seconds and 55°C for 1 minute. The standard curve was used to determine groups NB3 and NB7's absolute *nifH* gene copy or transcript copy number in the environmental samples.

Statistical Analyses

One-way analysis of variance (ANOVA) tests were conducted using JMP 10.0.2 to determine statistically significant differences among samples. If the p value was deemed significant (< 0.05), a Tukey-Kramer HSD post-hoc test was performed to distinguish statistical significance between samples compared.

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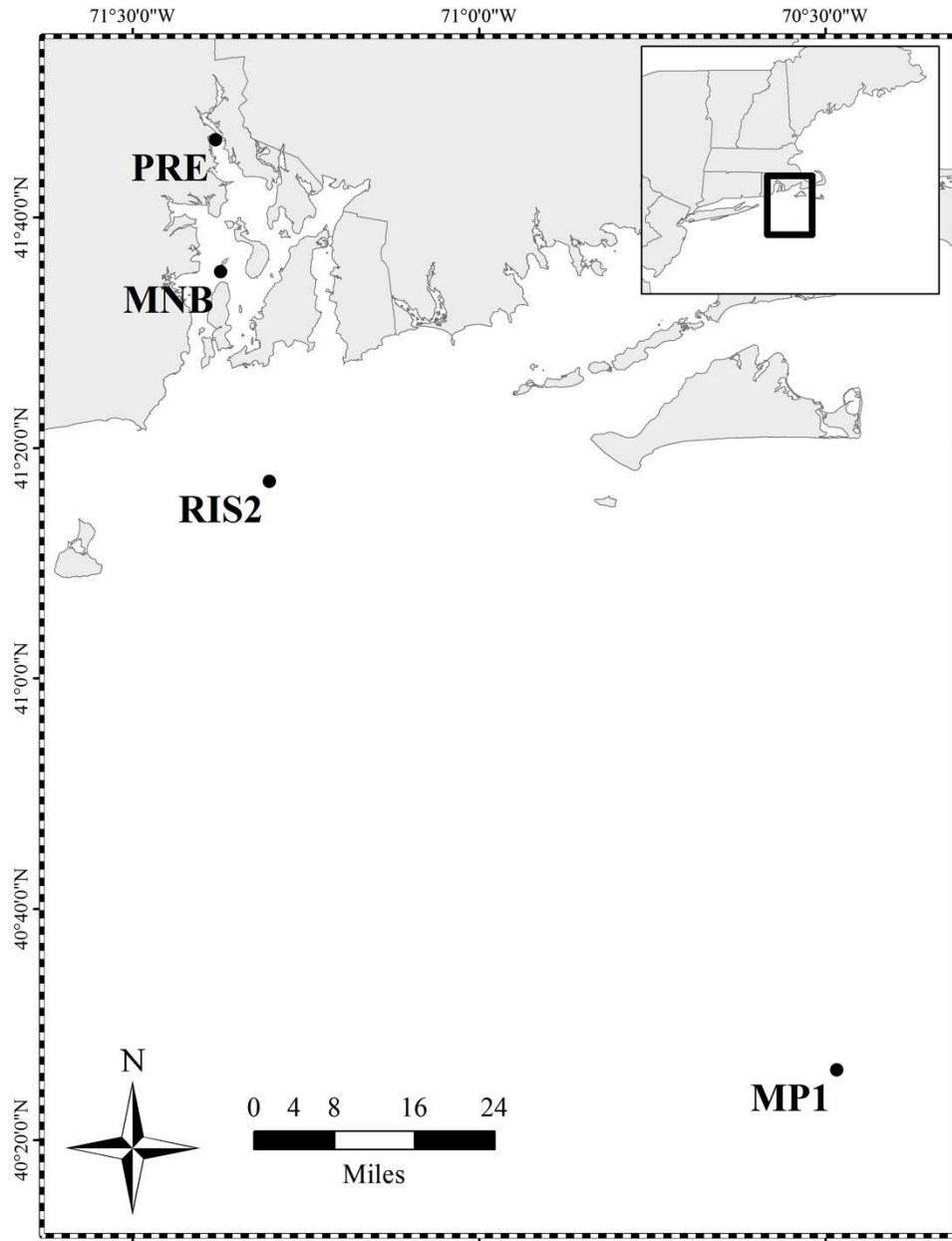


Figure 1: Map of Narragansett Bay and the southern coast of Rhode Island and Massachusetts. Black circles indicate sampling sites.

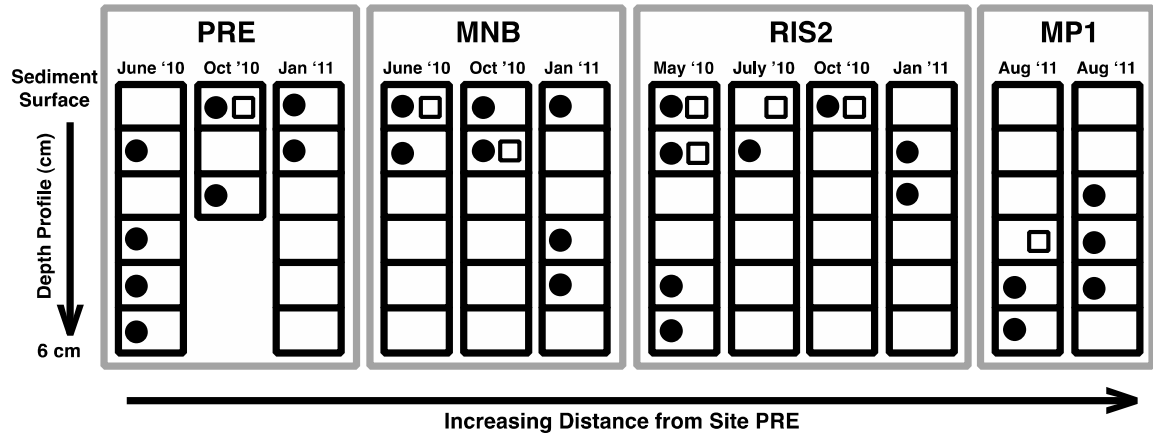


Figure 2: Downcore *nifH* (closed circles) and *nirS* (open squares) gene expression in 1 cm intervals in the sediment as a function of increasing distance from site PRE. Blank spaces indicate that no gene expression was detected. Sampling month is indicated above each profile.

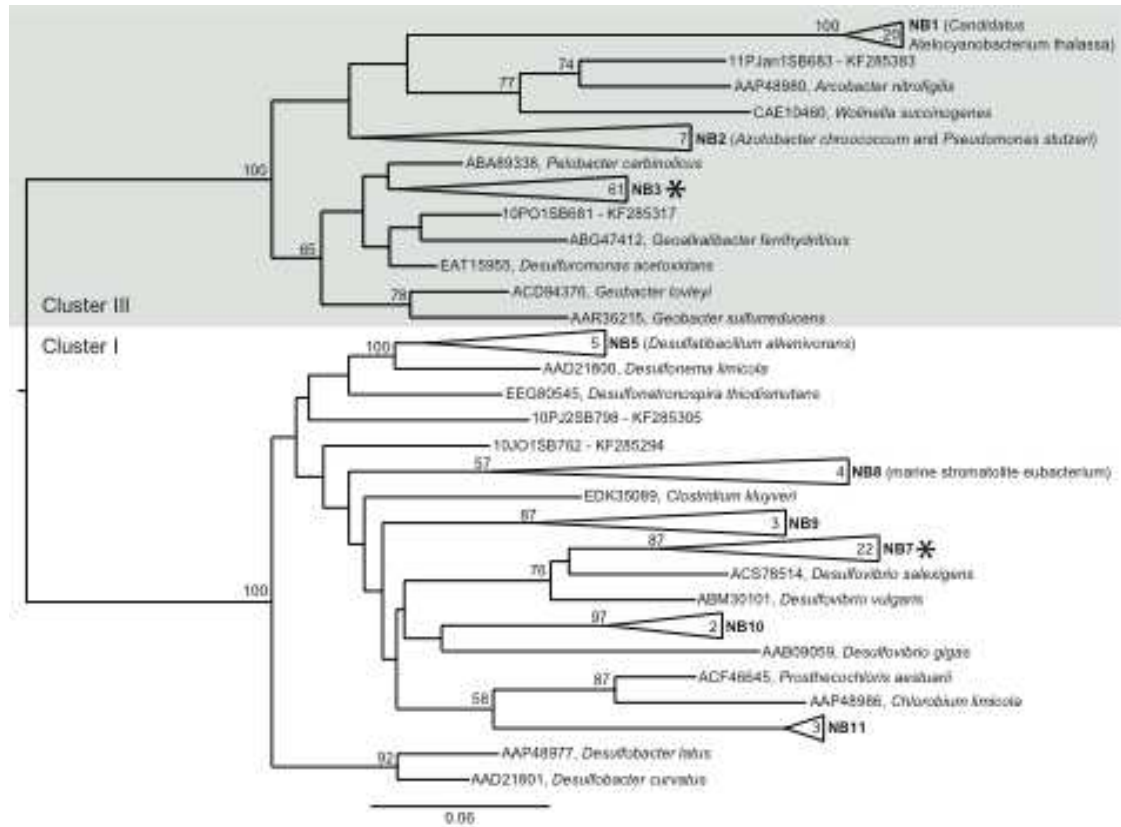


Figure 3: Maximum likelihood protein tree of expressed *nifH* sequences obtained from sediment samples and *nifH* sequences from cultivated representatives. *nifH* cluster designations are denoted according to Chien and Zinder (1996). Groups NB1-NB7 were previously described (Fulweiler et al. 2013), while groups NB9-NB11 are novel to this study. The number inside the group indicates the total number of sequences within the grouping. Table S3 describes all sequences within a collapsed group. Bootstrap values (1,000 replicates) > 50% are shown at the respective nodes. Asterisk indicates groups targeted for quantitative PCR.

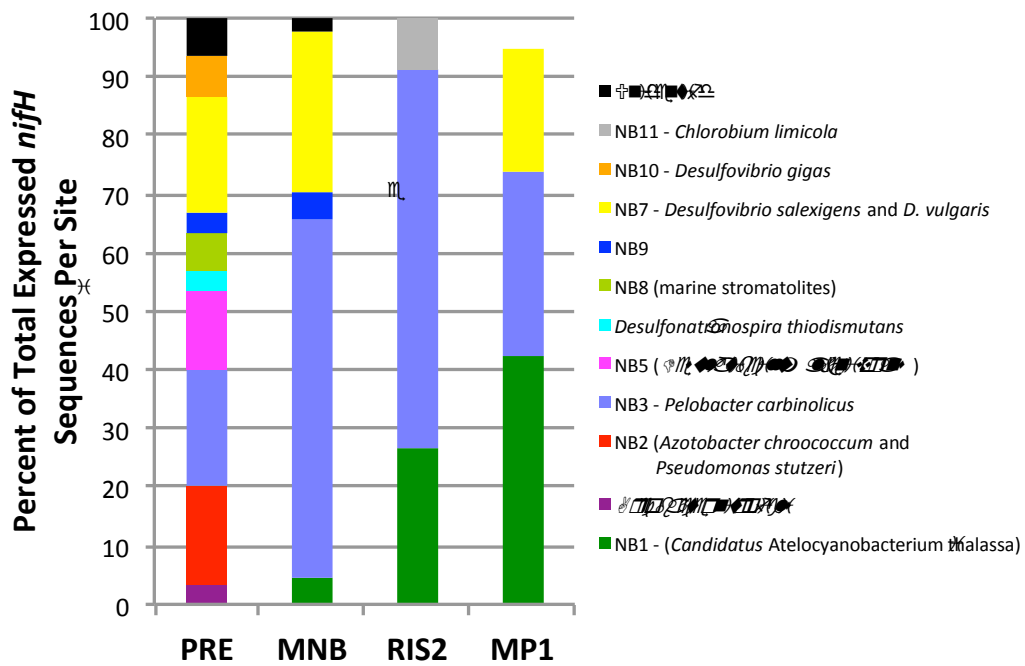


Figure 4: Percent of total expressed *nifH* sequences per site as a function of increasing distance from site PRE. Each color represents a cultivated species our environmental expressed sequences are related to as depicted in the *nifH* maximum likelihood tree (Fig. 3). Organisms listed in parentheses are contained within the grouping.

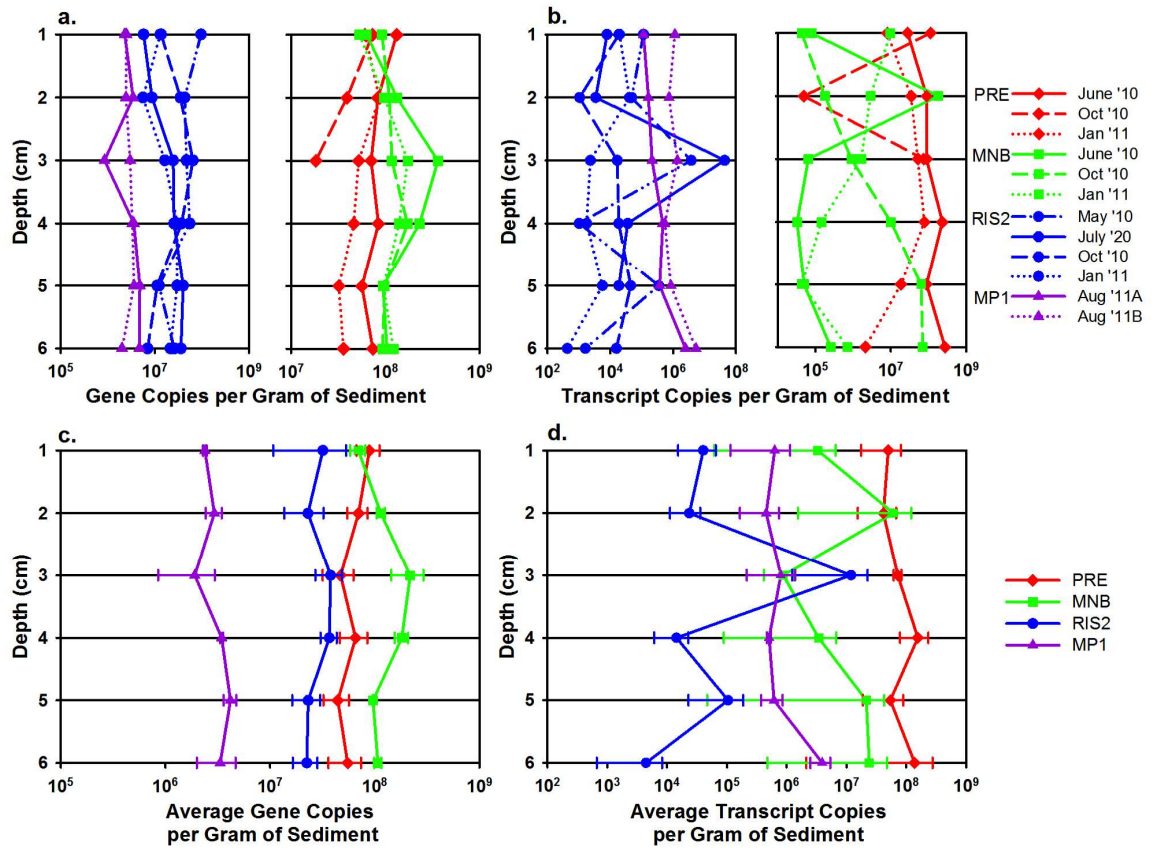


Figure 5: Downcore abundance and *nifH* expression of group NB3 (related to *P. carbinolicus*) enumerated by quantitative PCR in 1 cm intervals from sediment samples collected at four sites, PRE (red), MNB (green), RIS2 (blue) and MP1 (purple). NB3 a) abundance and b) *nifH* expression during each sampling month as indicated by different line styles. NB3 c) average abundance and d) average *nifH* expression over the sampling time points. Graphs are plotted on a log scale and the standard error of the mean indicated by the error bars.

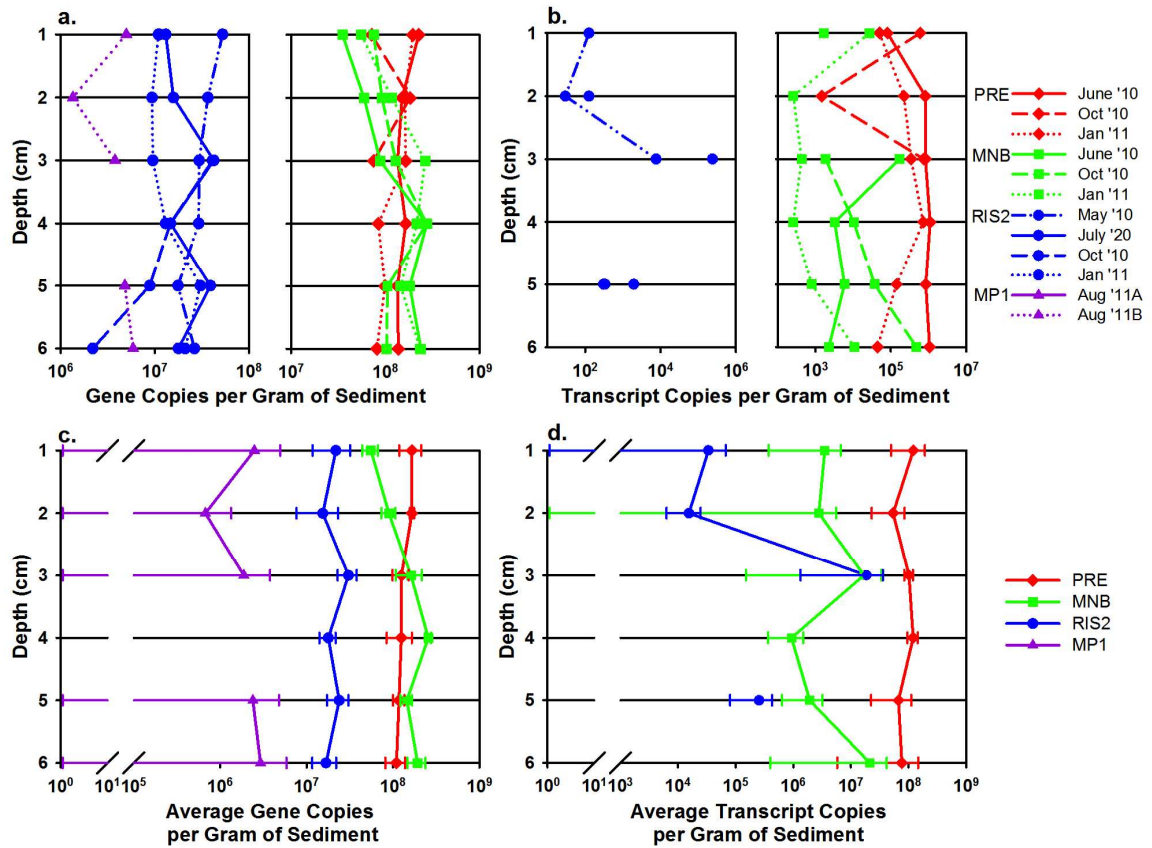


Figure 6: Downcore abundance and *nifH* expression of group NB7 (related to *D. salexigens* and *D. vulgaris*) enumerated by quantitative PCR in 1 cm intervals from sediment samples collected at four sites, PRE (red), MNB (green), RIS2 (blue) and MP1 (purple). NB7 a) abundance and b) *nifH* expression during each sampling month as indicated by different line styles. NB7 c) average abundance and d) average *nifH* expression over the sampling time points. Graphs are plotted on a log scale and the standard error of the mean indicated by the error bars.

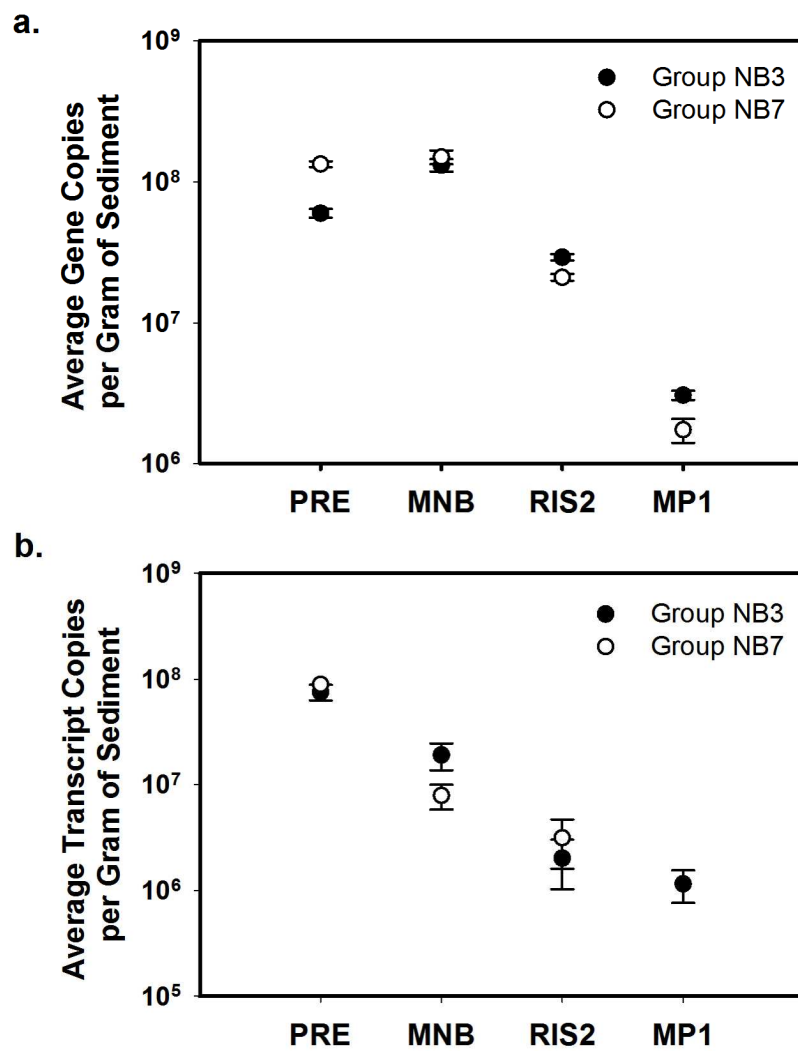
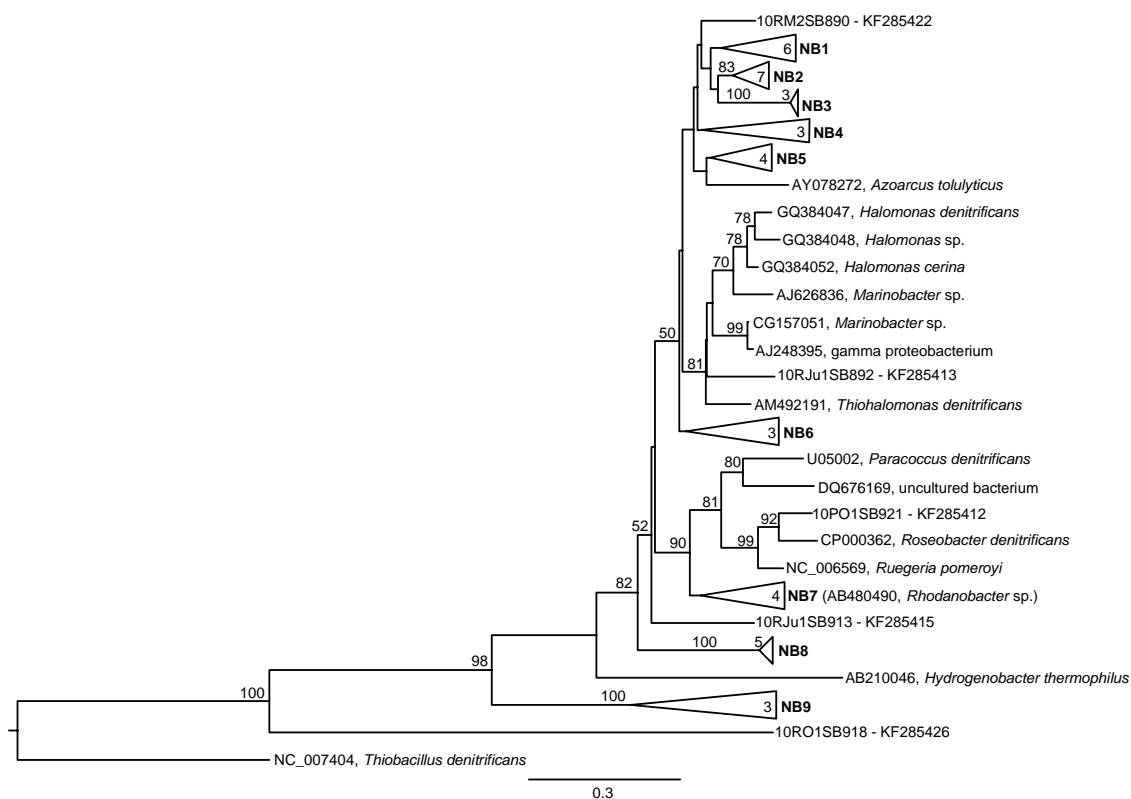
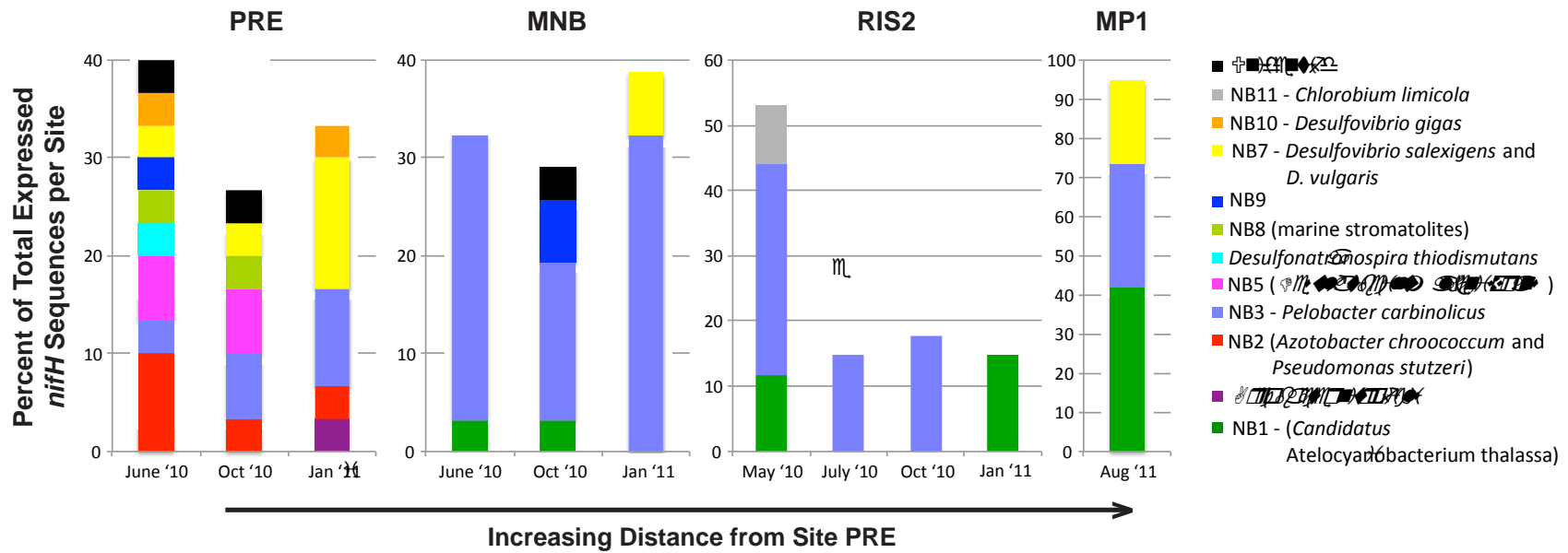


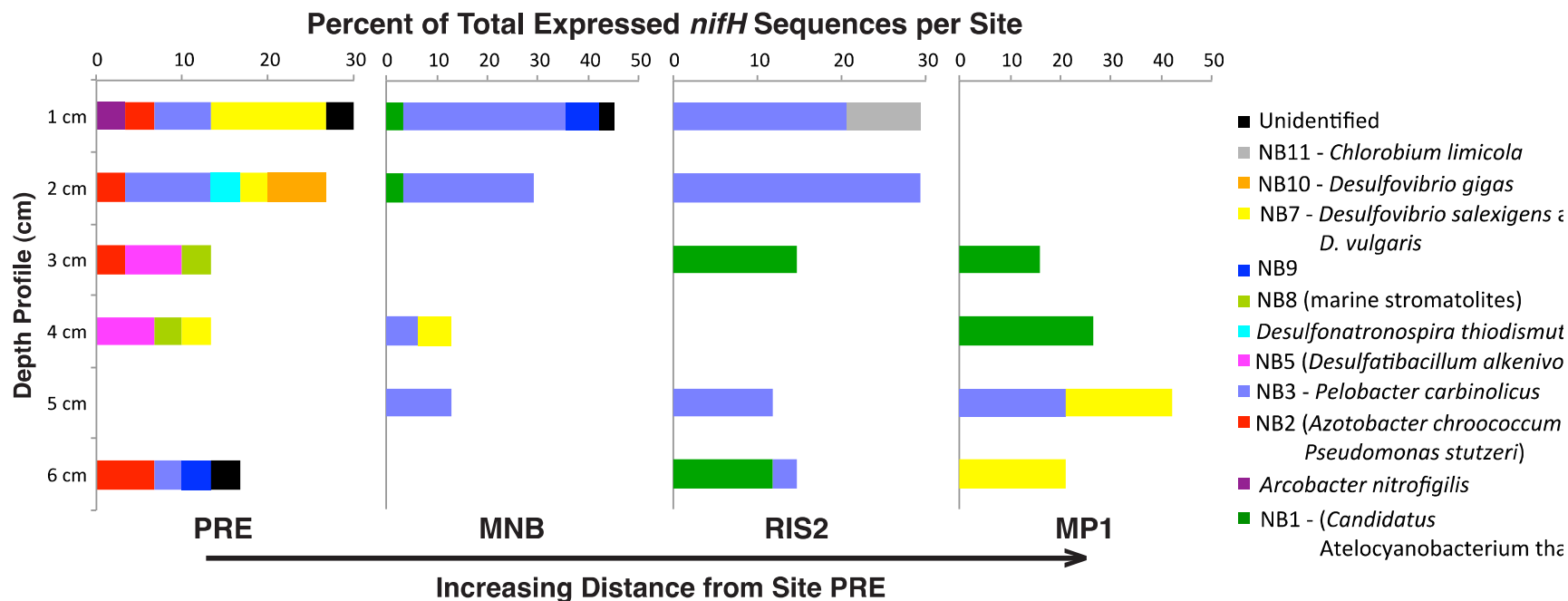
Figure 7: NB3 (closed circles) and NB7 (open circles) a) abundance and b) *nifH* expression integrated over depth and sampling time at the four sites as a function of increasing distance from site PRE. Graphs are plotted on a log scale and the standard error of the mean indicated by the error bars.



Supplementary Figure 1: Maximum likelihood protein tree of expressed *nirS* sequences from sediment samples and *nirS* sequences observed from cultured organisms. The number inside the group indicates the number of total sequences within the grouping. Table S3 describes all sequences within a collapsed group. Bootstrap values (1,000 replicates) > 50% are shown at respective nodes.



Supplementary Figure 2: Percent of total expressed *nifH* sequences per site as a function increasing distance from site PRE separated by month sampled. Each color represents a cultivated species our environmental expressed sequences are related to as depicted in the *nifH* Maximum Likelihood tree (Fig. 3). Y-axes have different scales between sites.



Supplementary Figure 3: Percent of total expressed *nifH* sequences per site as a function of depth and increasing distance from site PRE. Each color represents a cultivated species our environmental expressed sequences are related to as depicted in the *nifH* Maximum Likelihood tree (Fig. 3). X-axes have different scales between sites.

Supplementary Table 1: Coordinates of study sites and bottom water temperature during collection.

Site	Latitude	Longitude	Month Sampled	Temperature (°C)
PRE	41°46.7'	71°22.8'	June '10	18
			October '10	18
			January '10	2
MNB	41°35.3'	71°22.4'	June '10	18
			October '10	18
			January '10	2
RIS2	41°17.1'	71°18.2'	May '10	8
			July '10	13
			October '10	14
			January '11	5
MP1	40°26.1'	70°28.9'	August '11	8

Supplementary Table 2: List of *nirS* sequences (GenBank accession numbers) from mRNA in this study, closely related cultivated species and uncultivated species (Bulow et al. 2008) contained within groups NB1-NB9 (Fig. S1).

Group Name	Cultivated Species	<i>nirS</i> mRNA Sequences from this Study	Uncultivated Species
NB1		KF285404, KF285408, KF285410, KF285417-KF285419	
NB2		KF285400, KF285411, KF285429	DQ675891, DQ676160, DQ676224
NB3		KF285399, KF285401, KF285403	
NB4		KF285425, KF285427	DQ675734
NB5		KF285409	DQ675816, DQ676111, DQ675924
NB6		KF285414	DQ676007, DQ676217
NB7	AB480490	KF285421, KF285428	DQ676206
NB8		KF285402, KF285405-KF285407, KF285420	
NB9		KF285416, KF285423, KF285424	

Supplementary Table 3: List of *nifH* sequences (GenBank accession numbers) from mRNA in this study and closely related cultivated species contained within groups NB1-NB3, NB5 and NB7-NB11 (Fig. 3).

Group Name	Cultivated Species	<i>nifH</i> mRNA sequences from this study
NB1	ACJ53724	KF285290, KF285296, KF285342-KF285345, KF285364-KF285371, KF285393-KF285397
NB2	ACF22078, ABP79021	KF285304, KF285311, KF285313, KF285314, KF285319, KF285386
NB3		KF285284- KF285289, KF285291- KF285293, KF285295, KF285299- KF285302, KF285310, KF285315, KF285318, KF285323- KF285328, KF285332- KF285341, KF285346- KF285355, KF285357, KF285358, KF285360- KF285363, KF285376, KF285378-382, KF285389- KF285391, JN645433, JN645442, JN645443
NB5	ACL03220	KF285306, KF285308, KF285320, KF285321
NB7		KF285307, KF285316, KF285356, KF285359, KF285372-375, KF285384, KF285385, KF285387, KF285392, JN645432, JN645434- JN645441, JN645444
NB8	AAG23908, AAG23903	KF285309, KF285322
NB9		KF285297, KF285298, KF285312
NB10		KF285303, KF285388
NB11		KF285329- KF285331

Supplementary Table 4: Statistical analyses of group NB3 abundance and *nifH* expression using one-way ANOVA and Tukey-Kramer HSD post-hoc tests. F ratio (df_1 = degrees of freedom between groups, df_2 = degrees of freedom within groups) and *p* values are denoted. Asterisk (*) indicates statistically significant *p* values (< 0.05). If the one-way ANOVA test revealed statistical significance between samples compared, a Tukey-Kramer HSD post-hoc test was performed. Only the results with a *p* value < 0.05 are included underneath the corresponding ANOVA test.

Comparison	F (df_1, df_2) value	<i>p</i> value
Abundance by Site	F (3,20) = 20.9799	<0.0001*
MNB-MP1		<0.0001*
MNB-RIS2		<0.0001*
MNB-PRE		0.0032*
PRE-MP1		0.0128*
Abundance by Depth	F (5,18) = 0.2114	0.9532
Abundance by Season - PRE	F (2, 12) = 3.8401	0.0514
Abundance by Season - MNB	F (2, 15) = 1.0154	0.3858
Abundance by Season - RIS2	F (3, 20) = 2.1776	0.1224
Abundance by Season - MP1	N/A	N/A
Expression by Site	F (3,20) = 12.7419	<0.0001*
PRE-MP1		0.0002*
PRE-RIS2		0.0002*
PRE-MNB		0.0023*
Expression by Depth	F (5,18) = 0.2339	0.9425
Expression by Season - PRE	F (2, 12) = 3.3175	0.0713
Expression by Season - MNB	F (2, 15) = 0.5815	0.5712
Expression by Season - RIS2	F (3, 20) = 0.9376	0.4410
Expression by Season - MP1	N/A	N/A

Supplementary Table 5: Statistical analyses of group NB7 abundance and *nifH* expression using one-way ANOVA and Tukey-Kramer HSD post-hoc tests. F ratio (df_1 = degrees of freedom between groups, df_2 = degrees of freedom within groups) and *p* values are denoted. Asterisk (*) indicates statistically significant *p* values (< 0.05). If the one-way ANOVA test revealed statistical significance between samples compared, a Tukey-Kramer HSD post-hoc test was performed. Only the results with a *p* value < 0.05 are included underneath the corresponding ANOVA test.

Comparison	F (df_1, df_2) value	<i>p</i> value
Abundance by Site	F (3,20) = 24.1037	<0.0001*
MNB-MP1		<0.0001*
PRE-MP1		<0.0001*
MNB-RIS2		<0.0001*
PRE-RIS2		0.0003*
Abundance by Depth	F (5,18) = 0.0987	0.9911
Abundance by Season - PRE	F (2, 12) = 1.1606	0.3461
Abundance by Season - MNB	F (2, 15) = 0.3814	0.6894
Abundance by Season - RIS2	F (3, 20) = 2.9268	0.0588
Abundance by Season - MP1	N/A	N/A
Expression by Site	F (3,20) = 48.1908	<0.0001*
PRE-MP1		<0.0001*
PRE-RIS2		<0.0001*
PRE-MNB		<0.0001*
Expression by Depth	F (5,18) = 0.1267	0.9844
Expression by Season - PRE	F (2, 12) = 3.1323	0.00804
Expression by Season - MNB	F (2, 15) = 0.4345	0.6555
Expression by Season - RIS2	F (3, 20) = 0.9575	0.4319
Expression by Season - MP1	N/A	N/A

Supplementary Table 6: Primers and cycling conditions for PCR of *nifH* from environmental samples. All thermocycles included an initial 2 min denaturation at 94°C, and a final extension for 7 min at 72°C. *The outer reverse primer nifH3 was used to prime the RT reactions. **First round cycling conditions for RT products included 3 additional initial cycles with annealing steps at 44°C, 46°C, and 48°C.

Target	Sequence	Thermocycle	Cycles	Reference
Outer Fwd				
(nifH4)	5'-TTYTAYGGNAARGGNGG-3'	30" at 94°C	} 25**	Zehr 1989
Outer Rev		30" at 50°C		
(nifH3)*	5'-ATRTTRTTNGCNGCRTA-3'	60" at 72°C		
Inner Fwd				
(nifH1)	5'-TGYGAYCCNAARGCNGA-3'	30" at 94°C	} 30	Kirshtein 1991
Inner Rev		30" at 50°C		
(nifH2)	5'-ANDGCCATCATYTCNCC-3'	60" at 72°C		

Supplementary Table 7: Primers and cycling conditions for PCR of *nirS* from environmental samples. All thermocycles included an initial 2 min denaturation at 94°C, and a final extension for 7 min at 72°C. *For the first 11 cycles the temperature decreased 0.5°C every cycle.

Target	Sequence	Thermocycle	Cycles	Reference
Forward				
(nirS1F)	5'-CCTAYTGGCCGCCRCART-3'	30" at 94°C 30" at 56°C 60" at 72°C	11*	Braker 1998, 2000
Reverse				
(nirS6R)	5'-CGTTGAACTTRCCGGT-3'	30" at 94°C 30" at 50°C 60" at 72°C	25	

Supplementary Table 8: Primers, probe and cycling conditions for quantitative PCR targeting the *nifH* gene of group NB3 and NB7 (Fig. 3). The quantitative PCR cycling conditions for both target groups included an initial 10 minute denaturation at 95°C followed by 45 cycles of 95°C for 30 seconds and 60°C for 1 minute.

Target	Sequence
NB3	
Forward	5'-TGATCCTKCAYGCCAARG C -3'
Reverse	5'-GGYTACGGCGAYGTYATCTG-3'
Probe	5'-FAM-GGACAAGGTCCGYGARCTGGYACSGTWGAGGA-BHQ1-3'
NB7	
Forward	5'-TNGGYGGNYTGGCYCAGAARTC -3'
Reverse	5'-CCDCCNGAYTCMACACACCAKGT-3'
Probe	5'-FAM-YTDCGYGARGAAGGYGARGAYGTNGAACTYGA-BHQ1-3'

Manuscript 2

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Symbiotic Unicellular Cyanobacteria Actively Expressing *nifH* in Unlit Temperate Coastal Sediments

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Summary

Nitrogen (N) is often a limiting element for biological productivity in ocean ecosystems (Ryther et al. 1971). Diazotrophic cyanobacteria provide a source of fixed N to lit marine waters, alleviating the N deficit (Stal et al. 2008). A symbiotic unicellular cyanobacterium, UCYN-A, newly renamed *Candidatus Atelocyanobacterium thalassa* (Thompson et al. 2012), has recently been recognized as an important N fixer in global oligotrophic oceans (Zehr et al. 2007). Although an increase in discoveries of fixed N sources in marine ecosystems, including contributions from unicellular N-fixing cyanobacteria (Zehr et al. 2001), N fixation in coastal heterotrophic sediments (Fulweiler et al. 2007; Bertics et al. 2010) and cold seeps (Dekas et al. 2009), marine N fixation is still grossly underestimated, and therefore balancing the N budget has proved challenging (Brandes et al. 2002; Gruber 2004; Codispoti 2006). Here we report active expression of the nitrogenase iron component (*nifH*) of the conserved N fixing enzyme by UCYN-A in benthic sediments, with the highest *nifH* expression and abundance at offshore continental shelf sites when the bottom water temperatures were 5-8°C. UCYN-A, a known tropical and subtropical open ocean cyanobacterium, has a broader thermal tolerance than previously believed and are presumably active since mRNA transcripts are detected in the sediments. Once UCYN-A gets exported to the benthos, the physiology of the symbiotic cyanobacterium may allow it to thrive after the lifespan of its eukaryotic host. This diazotrophic activity is a new unexplored, unanticipated contribution of fixed N to sediment systems and challenges the N fixation paradigm.

Results and Discussion

Constraints on oceanic N fixation to oligotrophic tropical and subtropical waters was largely based on the temperature and growth requirements of the marine filamentous cyanobacterium *Trichodesmium* (Capone et al. 1997; White et al. 2007), which was believed to be the most abundant and active oceanic diazotroph (Capone et al. 1997) until the discovery of two unicellular cyanobacteria, *Candidatus Atelocyanobacterium thalassa*, also designated UCYN-A, and *Crocospaera watsonii* (Zehr et al. 2001; Montoya et al. 2004). Although first identified in the tropical open ocean at Hawaii Ocean Time-series (HOT) station ALOHA (Zehr et al. 2001) and widely distributed in oligotrophic tropical and subtropical waters (Zehr et al. 2001; Falcon et al. 2002; Church et al. 2005b; Moisander et al. 2010), recent evidence has shown that UCYN-A is found in abundance at colder temperatures, including higher latitudes and deeper in subsurface ocean waters (Moisander et al. 2010). Their domain has also expanded to include coastal regions (Mulholland et al. 2012) suggesting that oceanic N fixation may be more widespread than previously believed.

Unlike other cyanobacteria, UCYN-A lacks photosystem II, RuBisCo, and the tricarboxylic acid cycle among other crucial metabolic pathways (Zehr et al. 2008; Tripp et al. 2010). To support its carbon requirements in oligotrophic oceans, it has been proposed that UCYN-A is a symbiont of carbon fixing hosts (Tripp et al. 2010) and has a loose symbiotic association with a unicellular prymnesiophyte (Thompson et al. 2012). In exchange for fixed carbon for energy and biosynthesis, UCYN-A provides the host with fixed N (Thompson et al. 2012). Therefore, UCYN-A is a significant contributor of biologically fixed N to the environment, and could also play

a central role in the vertical downward flux of organic matter to the deep ocean if it is exported with its associated host.

Estuarine, continental shelf and other coastal margin sediments are responsible for 83% of biogeochemical cycling in the benthos, however these regions only make up only 9% of the total area of the seafloor (Jorgensen 1983). Denitrification, the sequential reduction of oxidized forms of nitrogen to N₂ gas by anaerobic bacteria, is responsible for the major loss of fixed N in coastal margins, therefore contributing to the unbalanced global N budget (Howarth et al. 1988). Due to the high inputs of anthropogenic added nutrients, estuaries and continental shelves are not typically considered N limited. Therefore, N fixation was considered to be an insignificant part of the benthic N cycle, unless associated with photosynthetic microbial mats (Capone 1983; Paerl et al. 1996), seagrass (McGlathery et al. 1998; Herbert 1999) and salt marsh plant (Welsh et al. 1996; Herbert 1999) vegetation in the photic zone. Only recently has N fixation by anaerobic microorganisms become recognized as an important process occurring in non-vegetated benthic sediments (Fulweiler et al. 2007; Bertics et al. 2010; Fulweiler et al. 2013).

Our goal was to use gene expression to determine if diazotroph activity was an underestimated component of the N cycle in marine heterotrophic sediments. N fixation is catalyzed by the conserved nitrogenase protein complex (Howard et al. 1996). *nifH*, the gene encoding the nitrogenase iron protein component, is a common marker for the phylogenetic analysis of diazotroph diversity (Zehr et al. 1989). UCYN-A nitrogenase expression has been linked to measured rates of N fixation (Zehr et al. 2007). Expression of the functional gene *nifH* was analyzed in sediments

collected at four sites (PRE, MNB, RIS2 and MP1) along the estuarine gradient of Narragansett Bay (RI, USA) to an offshore continental shelf site (Fig 1). Sites PRE, MNB and RIS2 were sampled over a seasonal cycle, while at site MP1, sediments were only collected in August 2011. Unexpectedly, *nifH* mRNA sequences related to UCYN-A were recovered in sediments at sites MNB, RIS2 and MP1 (Fig 2). At site MNB, expressed sequences were detected in the surface sediments (1-2 cm in depth), while the offshore sites contained expressed sequences up to 6 cm, the deepest subsection collected.

The UCYN-A related *nifH* sequences cluster into two sub-groups, designated A1 and A2 (Fig 2). Sub-group A1 contains mRNA sequences from all three sites and are identical to sequences collected from tropical and sub-tropical waters including station ALOHA, the South China Sea and the North Pacific sub-tropical gyre eddy (Fig 2). Sub-group A2 contains mRNA sequences from site MNB and RIS2 and are closely related to *nifH* sequences detected in temperate waters including the Western English Channel and the Mediterranean Sea (Fig 2). Although UCYN-A are globally distributed, unlike other marine bacteria including *Pelagibacter* and *Prochlorococcus*, populations of UCYN-A are homogenous (Tripp et al. 2010). Not surprisingly, sub-groups A1 and A2 shared a >98% pairwise sequence identity and with such low sequence diversity both UCYN-A sub-groups can be assessed with quantitative PCR.

Abundance, distribution and levels of *nifH* expression of UCYN-A in the sediments were determined by quantitative PCR targeting the *nifH* gene. The UCYN-A genome contains one copy of *nifH* and can be used as a proxy for abundance. The abundance of UCYN-A increased along the estuarine gradient of the Bay, with the

greatest number of gene copies detected at the most offshore site, MP1 (Fig 3a). At sites PRE, MNB and RIS2, the highest abundance occurred during the winter in January (Fig 3a). During July, UCYN-A was undetectable at site PRE and at very low levels at site MNB (Fig 3a). Diazotroph abundance and N fixation rates in surface North Atlantic coastal waters were measured between Cape Hatteras and Georges Bank. UCYN-A were the most abundant diazotroph measured along the coast and among the highest abundances ever recorded for the cyanobacterium (Carpenter et al. 2008; Moisander et al. 2010). At several regions just west of Georges Bank and in Block Island Sound, high rates of N fixation with concurrent high abundances of UCYN-A were measured (Mulholland et al. 2012), indicating that these microbes are prevalent and active in the water column near our offshore sites, RIS2 and MP1. About 53 km to the northeast of our sampling site MP1 (Fig 1), the highest areal rate of N fixation ($873.9 \text{ } \mu\text{mol N m}^{-2}\text{d}^{-1}$) was documented for North American coastal sites (Mulholland et al. 2012). Corroborating our results, the lowest abundance of UCYN-A was also measured in an estuary, at the mouth of Chesapeake Bay (Mulholland et al. 2012).

UCYN-A *nifH* expression followed a similar trend to abundance, with *nifH* transcript copies increasing from the head of the Bay to the offshore sites (Fig 3b). At site PRE, *nifH* expression was undetectable during all sampling time points and very low transcript copies were detected in October and January at site MNB (Fig 3b). Previous studies have observed *nifH* transcripts of UCYN-A at water temperatures as low as 12° to 19°C (Needoba et al. 2007; Short et al. 2007). At site MNB, although low levels, UCYN-A transcripts were detected in January when the bottom water

temperature was 2°C. The highest levels of *nifH* expression were detected at site MP1 in August and site RIS2 in January with bottom water temperatures at 8° and 5 °C, respectively (Fig 3b). Although the lowest recorded abundance at site RIS2, UCYN-A were most active in October with a gene expression to abundance ratio of 54.5 when the bottom water temperature was 14°C (Fig 3c). Since UCYN-A remains uncultivated, the boundaries of its temperature limit are not well understood, and may be lower than previously predicted. Regardless of season, the highest abundance and *nifH* expression of UCYN-A was detected at the offshore site RIS2 (Fig 4). Peak abundance and expression occurred at site MP1 (Fig 3a and b), but this location was only sampled in August 2011, so was disregarded in analysis. Due to UCYN-A's symbiotic lifestyle, the bacterium may be constrained by the geographical range of its eukaryotic partner.

UCYN-A has a loose extracellular association with a single-celled eukaryotic alga, identified as a prymnesiophyte closely related to *Braarudosphaera bigelowii* and *Chrysochromulina parkeae* (Thompson et al. 2012). Both eukaryotes, although morphologically distinct, appear to contain calcified scales (Gran and Braarud 1935, Saez 2004), which may help stabilize the fragile association. The distribution of *B. bigelowii* is restricted to nearshore regions (Hagino et al. 2009), typically in cold, low salinity waters (Bukry 1974). *C. parkeae* was first identified near South West England and Norway (Green et al. 1972); however, its range is not well-characterized. The global and widespread distribution of UCYN-A (from oligotrophic tropical oceans to temperate coastal regions), along with its loose partner association indicates that UCYN-A may have multiple prymnesiophyte hosts. During a two-year temporal study

of the Northeastern Atlantic Outer Continental Shelf, the abundance of coccoliths was greatest during the winter months and decreased in the summer and were replaced by diatoms southwest of Georges Bank (Aaron, J. 1980). These findings support our observations, in which we detect the highest abundance and expression of UCYN-A during January at site RIS2. UCYN-A's symbiotic partnership with potentially multiple calcifying prymnesiophytes has important implications for the broad thermal tolerance of the N fixing unicellular cyanobacteria and the global export of carbon and nitrogen into the deep ocean.

When the prymnesiophyte partner sinks to the benthos, presumably the loosely attached UCYN-A gets vertically transported to the sediments as well. UCYN-A lacks key genes for photosynthesis and carbon fixation (Tripp et al. 2010), and in the water column, obtains organic carbon from its host (Thompson et al. 2012). If UCYN-A is no longer associated with its partner in the sediment, its genome does contain genetic machinery to acquire essential nutrients from the environment, including multiple non-specific sugar transporters (Tripp et al. 2010). UCYN-A has a complete suite of enzymes for upper glycolysis and the pentose phosphate pathway to metabolize the sugars for energy and biosynthetic purposes (Tripp et al. 2010). The genome also encodes for trace metal transporters for iron, molybdenum and nickel which are essential for certain enzymatic function, including N fixation (Tripp et al. 2010). UCYN-A would be capable of surviving in the benthos without its partner due to the sources of organic matter available in the sediment (Rowe et al. 1988). *nifH* transcripts detected up to 6 cm in depth suggests that UCYN-A remains active in the sediment after the lifespan of its host. UCYN-A diazotrophic activity in coastal sediments

strengthens benthic-pelagic coupling and is an unprecedented source of fixed N to the marine system.

Methods Summary:

Sediment cores were collected at four sites (PRE, MNB, RIS2 and MP1) in southern New England coastal waters from May 2010 to August 2011 with bottom water temperatures ranging from 2 to 18°C. Sediment cores were sub-sampled with 60 mL syringes, flash frozen in liquid N₂ and sectioned into 1cm intervals to 6cm in depth. Total DNA and RNA were extracted from sediment samples as previously described (Fulweiler et al. 2013).

RNA was transcribed to cDNA and *nifH* mRNA transcripts were amplified from the cDNA using nested PCR (Zehr et al. 1989; Kirshtein et al. 1991). For every expressed *nifH* amplicon, 4-5 sequences were cloned. Expressed environmental *nifH* sequences from the four sites that were closely related to UCYN-A *nifH* (CP001842) were combined with the top 100 BLASTn hits. *nifH* nucleic acid sequences were aligned using the multiple sequence alignment tool, MUSCLE (Edgar 2004) within the Geneious software package. A *nifH* maximum likelihood tree of aligned nucleic acid sequences was constructed using PhyML in Geneious with 1000 bootstrap replicates. Quantitative PCR assays were performed on all environmental DNA and cDNA samples using primers targeting UCYN-A *nifH* (Church et al. 2005a).

Supplementary Information:

Study Sites and Field Collection

Intact sediment cores (10 cm inner diameter and 30.5 cm long) were collected at four sites in southern New England coastal waters. Sites PRE (41°46.7', 71°22.8')

and MNB (41°35.3', 71°22.4) are located within the temperate estuary, Narragansett Bay, Rhode Island. Cores were harvested using a 5 m pull corer at site PRE and at site MNB using SCUBA divers. The offshore sites, RIS2 (41°17.1', 71°18.2') and MP1 (40°26.1', 70°28.9'), are located in Rhode Island Sound and on the continental shelf 110 km south of Cape Cod, Massachusetts, respectively. Cores were collected using a box corer (0.25 m²) and pre-mounted PVC cores at the offshore sites. Sites PRE and MNB were sampled in June and October 2010 and January 2011. Site RIS2 was sampled in May, July, and October 2010 and January 2011. The most offshore site, MP1, was only sampled in August 2011 (Fig. 1, Table 1). All cores were transported to and stored in the dark at field bottom-water temperature in a walk-in environmental chamber at the Graduate School of Oceanography at the University of Rhode Island. The cores were left uncapped with air gently bubbling through the overlying water.

Sub-sampling and Nucleic Acid Extractions

The cores were sub-sampled using a 60 mL syringe and were subsequently flash frozen in liquid N₂. The sub-cores were sectioned into 1 cm segments from the sediment water interface to 6 cm in depth. The frozen sediment cross-sections were cut up to yield 0.25 g and 0.5 g of wet sediment for DNA and RNA isolation, respectively. The MO Bio Powersoil DNA Isolation Kit (Carlsbad, CA, USA) was used to extract total DNA from the sediment samples according to manufacturer's instructions. Subsequently, DNA was quantified using Invitrogen's Qubit dsDNA HS Assay Kit and all DNA samples were diluted to a concentration of 1 ng/uL for qPCR analysis. A concentration of 1ng/uL was chosen based on saturation experiments. Total RNA was extracted using the MO Bio Powersoil RNA Isolation Kit (Carlsbad,

CA, USA), however the kit was designed to extract RNA from 2 g of soil. A quarter of the volumes of the Bead, SR1, SR2, SR3 and SR4 solutions and phenol-chloroform-isoamyl alcohol were used to accommodate this reduction in reaction scale. After the RNA precipitation step, the dried pellet was resuspended with 100 μ L of nuclease free water, 10 μ L of 10X TURBO DNase buffer and 1 μ L of TURBO DNase from the TURBO DNase-free Kit (Ambion, Austin, TX, USA) and incubated at 37°C for 30 min. 10 μ L of DNase Inactivation reagent was added and incubated at room temperature for 5 min to inactivate the reaction. The remaining RNA purification steps were carried out using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was quantified using Invitrogen's Qubit RNA Assay Kit and all RNA samples were diluted to 4 ng/ μ L. cDNA copies of RNA were generated with Invitrogen's SuperScript First-Strand Synthesis System for RT-PCR. For all samples, 8 μ L of DNase-treated RNA at a concentration of 4 ng/ μ L was added to the reaction. Each reaction was primed with 1 μ L of 2 μ M outer reverse primer for our gene of interest, *nifH3*. After the reverse transcriptase was added, the mixture was incubated at 50°C for 50 min. All the other steps followed the instructions of the manufacturer. For every sample, we also included controls that did not contain reverse transcriptase to confirm there was no DNA contamination in the PCR amplification.

Functional Gene Sequence Analysis

nifH mRNA transcripts were amplified from environmental cDNA using nested PCR with degenerate outer primers *nifH4-nifH3* and inner primers *nifH1-nifH2* (Zehr et al. 1989; Kirshtein et al. 1991). Both rounds of PCR consisted of an initial

denaturation step of 2 min at 94°C, cycling steps that included: a denaturation step of 30 s at 94°C, an annealing step of 30 s at 50°C, and an extension step of 1 min at 72°C. All reactions had a final extension step of 7 min at 72°C. First round reactions had 25 cycles and the second round reactions had 30 cycles (Zehr et al. 1989; Kirshtein et al. 1991).

Amplified PCR products were loaded on to a 1% agarose (wt/vol) TAE gel and bands of the correct size were purified using the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen Valencia, CA, USA). Purified *nifH* products were cloned into pGEM-T vectors (Promega, Madison, WI, USA), transformed into JM109 *E.coli* competent cells (Zymo Research, Irvine, CA, USA) and identified by blue-white screening. The plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and sequenced on the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the RI Genomic Sequencing Center at the University of Rhode Island. For every expressed *nifH* amplicon, we cloned 4-5 sequences.

Expressed environmental *nifH* sequences from sites PRE, MNB, RIS2 and MP1, that grouped with UCYN-A *nifH* (CP001842), accession numbers KF285290, KF285296, KF285342-KF285345, KF285364-KF285371, KF285393-KF285397, were combined with the top 100 BLASTn hits in GenBank (Benson et al. 2009). All *nifH* nucleic acid sequences were aligned using the multiple sequence alignment tool, MUSCLE (Edgar 2004) within the Geneious software package. A *nifH* maximum likelihood tree of aligned nucleic acid sequences was constructed using PhyML in Geneious with 1000 bootstrap replicates.

Quantitative real-time PCR

Quantitative PCR assays targeting UCYN-A *nifH* were performed all on DNA and cDNA samples using the Roche's LightCycler 480 Probes Master Mix and were analyzed using Stratagene's Mx3005 qPCR System. Samples were amplified using primers, probe and thermocycling conditions previously described (Church et al. 2005a). The qPCR reactions consisted of 10 uL of the Roche LightCycler 480 Probes Master mix, 5.7 uL of water, 2 uL of a primers/probe mix (at concentrations of 0.4 uM and 0.2 uM, respectively), and 0.3 uL of reference dye (ROX). The qPCR cycling conditions were: 1 cycle of 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 seconds and 60°C for 1 minute. A standard curve was produced with triplicate 10-fold dilution series ranging from 1 ng to 1 ag of linearized plasmid containing a sequenced *nifH* clone from UCYN-A sub-group A1. The standard curve was used to determine absolute *nifH* gene copy or transcript copy number in the environmental samples.

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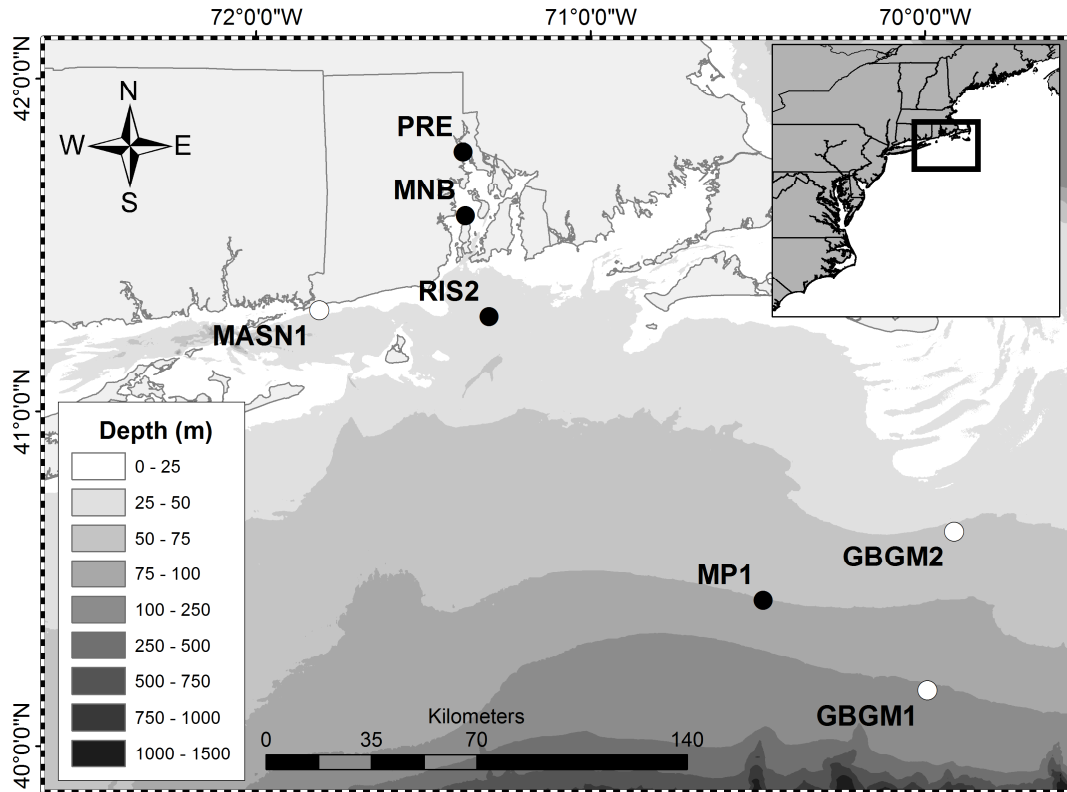


Figure 1. Map of sampling sites in Narragansett Bay and off the southern coast of Rhode Island and Massachusetts. Sampling sites from this study are represented by closed circles. Open circles indicate sites in which high rates of areal N fixation (GBGM1 = $208 \text{ } \mu\text{mol N m}^{-2}\text{d}^{-1}$, GBGM2 = $873.9 \text{ } \mu\text{mol N m}^{-2}\text{d}^{-1}$ and MASN1 = $152.3 \text{ } \mu\text{mol N m}^{-2}\text{d}^{-1}$) were documented (Mulholland 2012).

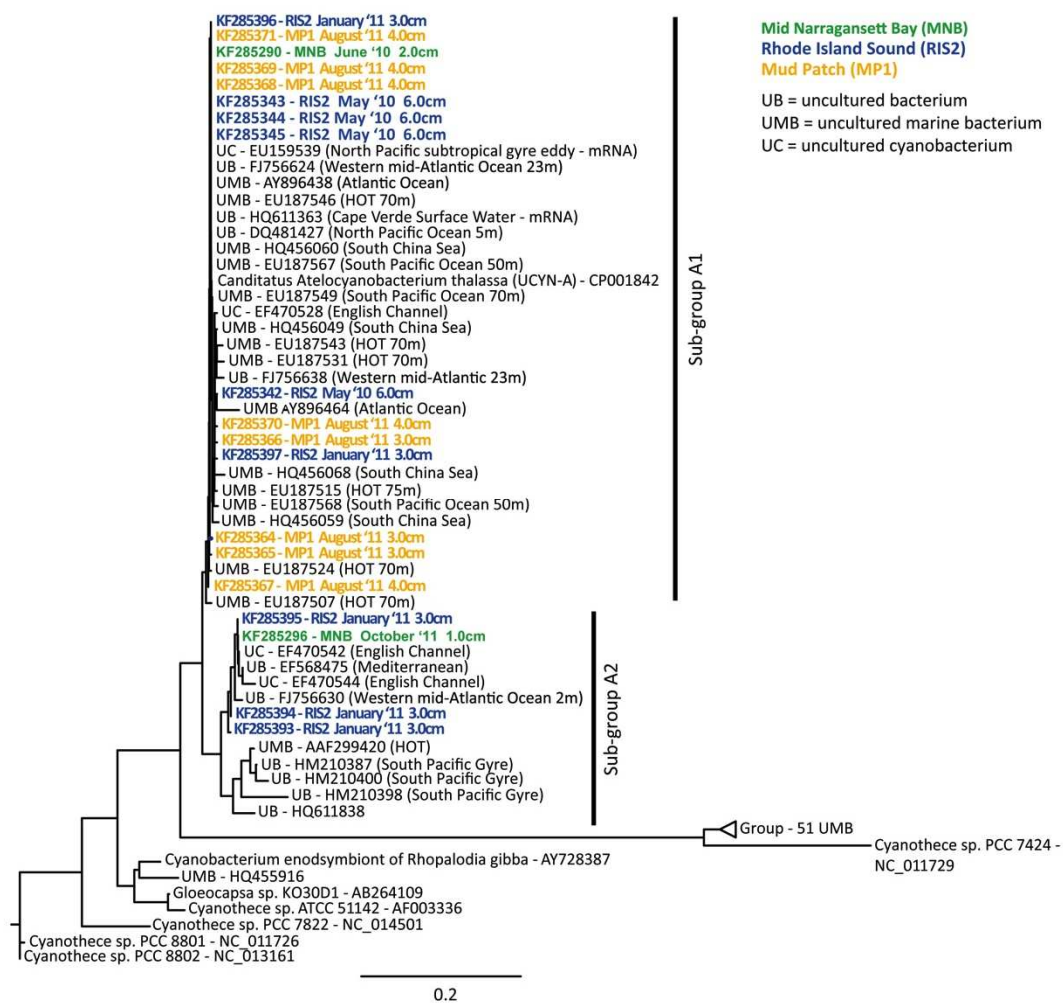


Figure 2. Maximum-likelihood nucleotide tree (PhyML) of expressed *nifH* sequences obtained from sediment samples and the top 100 BLASTn hits including *Candidatus Atelocyanobacterium thalassa nifH* gene sequence. *nifH* sequences from this study are highlighted in color according to site and sampling month and depth in the sediment are denoted. If there were two identical BLASTn sequences from the same location, only a representative sequence was shown. The two sub-groups A1 and A2 are indicated on the tree.

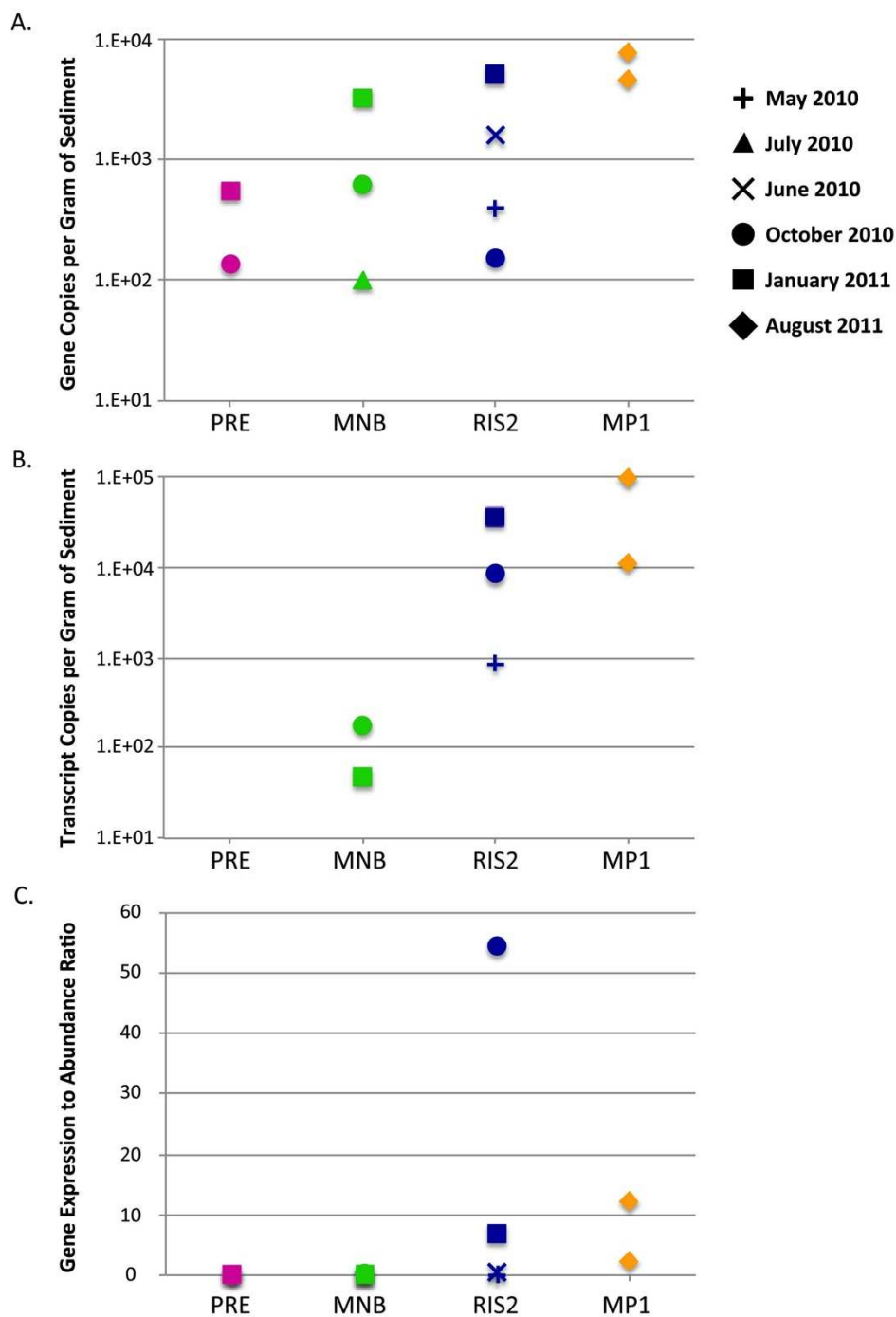


Figure 3. Abundance and *nifH* expression of UCYN-A along the estuarine gradient of the bay to the most offshore site, MP1. Each shape represents a month sediment samples were collected. Sites PRE (pink) and MNB (green) were sampled in June and October 2010 and January 2011. Site RIS2 (blue) was sampled in May, July and October 2010 and January 2011. Site MP1 (orange) was only sampled in August 2011. A. UCYN-A abundance, B. *nifH* expression, and C. gene expression to abundance ratio integrated over depth as a function of increasing distance from site PRE.

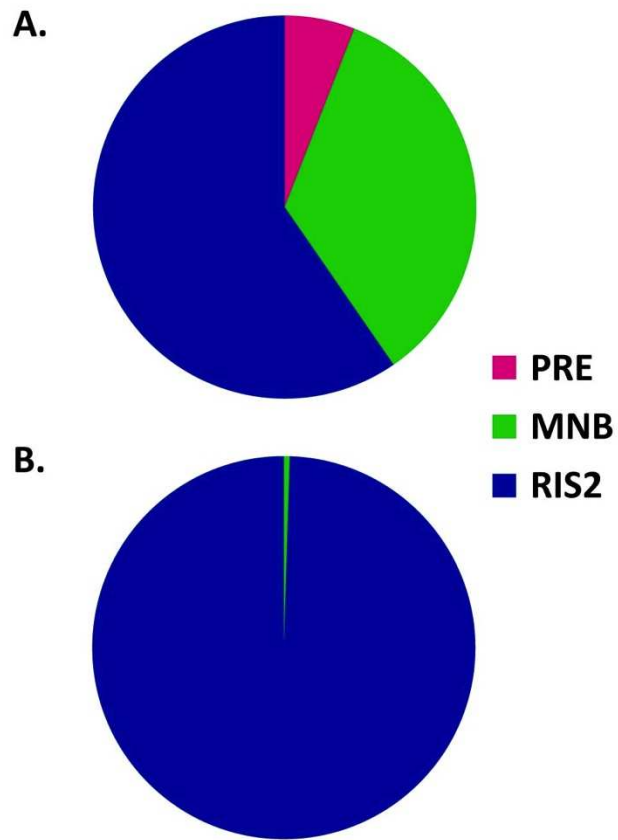


Figure 4. A. Average UCYN-A abundance. B. Average UCYN-A *nifH* expression. At sites PRE (pink) and MNB (green), June and October 2010 and January 2011 were averaged. At site RIS2 (blue), July and October 2010 and January 2011 were averaged. MP1 was not included as it was only sampled in August 2011.

Table 1. Bottom water temperatures of study sites during sediment collection.

Site	Month Sampled	Temperature (°C)
PRE	June '10	18
	October '10	18
	January '10	2
MNB	June '10	18
	October '10	18
	January '10	2
RIS2	May '10	8
	July '10	13
	October '10	14
	January '11	5
MP1	August '11	8

Manuscript 3

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Bottom-water Hypoxia Influences Sediment Microbial Nitrogen Transformations

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Abstract:

Episodic hypoxia is becoming a common occurrence in coastal systems globally, yet it remains unknown how water column oxygen depletion influences nitrogen (N) cycling processes in the sediment. Estuarine sediments are generally considered areas of net N loss through the coupled nitrification-denitrification. Conversely, inputs of N through N fixation are typically considered negligible. Recently, anaerobic diazotrophs have been shown to be a potentially important source of N in coastal benthic habitats. Sediments were collected at several sites prior to and after hypoxia in Narragansett Bay (RI, USA). Sets of pre-hypoxic cores were incubated under oxic and hypoxic conditions. Benthic sediment characteristics (e.g. C/N ratio, oxygen penetration and porewater N) were analyzed to determine the influence of oxygen depletion on sediment N cycling microbial activity. Concurrently, nitrogenase subunit (*nifH*) gene expression was used to identify the likely active nitrogen fixers in the surface sediments. The greatest bulk *nifH* expression was detected at the site most heavily impacted by hypoxia with anaerobic sulfur/iron and sulfate reducers dominating expression. After exposure to severe hypoxia, a disappearance of porewater nitrate was detected in sediments, indicating coupled nitrification-denitrification was possibly repressed. Oxygen depletion in the water column potentially influences natural sediment redox dependent N transformations and while also stimulating anaerobic N fixation.

Introduction:

Anthropogenic fertilization of coastal marine systems by excess nitrogen (N) can lead to undesirable impacts including eutrophication-induced hypoxia (Diaz et al.

2008; Rabalais et al. 2010; Zhang et al. 2010). Hypoxic zones form when river freshwater and excess nutrients from natural and anthropogenic sources enter coastal waters promoting phytoplankton blooms (Bricker et al. 2008). Eutrophication enhances deposition of organic matter to the benthos, which in turn promotes microbial decomposition and respiration (Meyer-Reil et al. 2000). Consequently, the increased demand for oxygen in the benthos coupled with water column stratification can deplete the system of dissolved oxygen (Rabalais et al. 2010). Hypoxia and anoxia are one of the most deleterious anthropogenic influences facing our marine environments (Diaz et al. 2008; Zhang et al. 2010).

Hypoxia is expanding in coastal regions globally (Diaz et al. 2008), yet the impact of oxygen-depletion on benthic N cycling is still poorly understood. In estuarine sediments, nitrogen is removed from the system through coupled nitrification-denitrification (Seitzinger 1988). Ammonia is first oxidized into nitrate by specialized groups of nitrifiers. Subsequently, denitrifiers anaerobically reduce the oxidized forms of nitrogen to N_2 gas. Hypoxia can indirectly inhibit denitrification through the suppression of nitrification, an oxygen requiring pathway (Childs et al. 2002), potentially repressing the loss of fixed N from coastal systems.

Benthic nitrogen fixation, the reduction of N_2 gas to biologically available ammonia, is typically considered an insignificant component in most estuarine nitrogen budgets (Howarth et al. 1988a; Galloway et al. 2004). However, biological N fixation is a critical source of nitrogen in specific benthic habitats, including sediments vegetated by rooted macrophytes (McGlathery et al. 1998; Herbert 1999; Welsh 2000) and photosynthetic microbial mats (Capone 1983; Paerl et al. 1996). More recently,

unvegetated coastal sediments have also been recognized as a periodic source of fixed N (Gardner et al. 2006; Fulweiler et al. 2007; Bertics et al. 2010).

Biological N fixation is catalyzed by the nitrogenase enzyme, which is highly conserved and distributed throughout diverse prokaryotic taxa (Young 1992). *nifH*, the gene encoding the nitrogenase iron protein subunit, is the gene marker largely used to study diazotroph diversity in various environments (Zehr et al. 2003; Jenkins et al. 2008; Gaby et al. 2011). Phylogenetic analysis of *nifH* DNA in the aforementioned benthic habitats, all revealed that anaerobes related to sulfate reducing bacteria were likely significant contributors of fixed N to the system (Lovell et al. 2000; Steppe et al. 2005; Bertics et al. 2010). Microbes respiring iron and sulfur compounds, along with sulfate reducers dominated active *nifH* gene expression in unvegetated sediments in Narragansett Bay, a temperate estuary in southern New England (Fulweiler et al. 2013; Brown et al. submitted). Because of the abundance and activity of anaerobic diazotrophs recovered from unvegetated sediments in Narragansett Bay, we hypothesize that a reduction in oxygen supply to the overlying water and surface sediments during hypoxic and anoxic events will stimulate N fixation by these anaerobic organisms. Hypoxia may perturb the functioning of coastal ecosystems by potentially inhibiting denitrification while also stimulating anaerobic nitrogen fixation. Exploring areas impacted by hypoxia may help resolve the global marine N budget discrepancy.

For the last several decades, episodic hypoxic events have been documented in Narragansett Bay (Oviatt et al. 1984; Bergondo et al. 2005; Deacutis et al. 2006; Melrose et al. 2007; Deacutis 2008; Saarman et al. 2008). Over the course of the 2013

summer season, water column oxygen concentrations, salinity and temperature were monitored at several sites in upper Narragansett Bay. Sediments were harvested at the end of May, prior to hypoxia and at the beginning of August, after a hypoxic event. Sets of pre-hypoxic sediment cores collected at the end of May were incubated in oxic or hypoxic conditions. To investigate the source and processing of organic matter deposited to the benthos, we measured total organic carbon and nitrogen in the sediments. Analysis of other benthic characteristics including oxygen penetration and porewater nutrients can provide insight into the microbial processes occurring in the surface sediments. Dominant *nifH*-transcribing microbial populations were identified and quantitative PCR was used to follow changes in abundance and *nifH* expression of these bacterial groups in response to fluctuating bottom water oxygen concentrations. The overall goal of this study was to determine (1) how hypoxia influences benthic microbial activities through analysis of sediment characteristics and (2) if changes in sediment characteristics reflects the activity of diazotrophs.

Results:

Water quality parameters, sediment characteristics and expression of *nifH*, the functional gene associated with nitrogen fixation, were analyzed during the 2013 summer season at three sites, Bullock Reach (BR), Sally Rock (SR) and Hope Island (HI) in Narragansett Bay, RI (Fig 1, Table 1). (Refer to *Experimental Procedures* for more in-depth collection and site description.)

May and August 2013 Field Measurements

Bottom Water Dissolved Oxygen

Sediment cores were harvested prior to hypoxia at the end of May when bottom water dissolved oxygen (DO) concentrations ranged from 7.39-9.09 mg/L (Fig 2, Table 2). Throughout much of July, sites BR and SR exhibited severe hypoxia (Fig 2). Near anoxic conditions (0.0 mg/L DO) were observed at site SR (Fig 2). Sediment cores were collected in early August after hypoxia (Fig 2). At site BR, the bottom water DO was 3.49 mg/L while the concentrations were a bit higher at sites SR and HI, 6.25 mg/L and 5.43 mg/L, respectively (Fig 2, Table 2).

Sediment Characteristics

Molar carbon to nitrogen (C/N) ratios of the sediment did not vary more than ± 0.33 from the average along depth profiles or between months sampled at each site (Fig 3). The average C/N ratio was 11.4 and 11.3 at sites BR and HI, respectively, with a slightly lower average C/N ratio of 10.4 at site SR. Weight percent total organic carbon (TOC) and weight percent total nitrogen (TN) of the sediment were much lower at site HI, with an average of 1.91% TOC and 0.20% TN compared with sites BR (3.62% TOC and 0.37% TN) and SR (3.34% TOC and 0.38% TN) (Fig 4).

Sediment downcore oxygen profiles differed across sites and months sampled (Fig 5). At all three sites in May and August, DO was depleted in the sediment by 0.5 cm in depth (Fig 5). The oxygen profiles for sediment collected in May and August at site BR were very similar (Fig 5a). Conversely, at site SR the oxygen percent saturation in the surface sediment in August was much lower and depleted more quickly compared to sediment collected prior to hypoxia in May (Fig 5b). At site HI, the sediment oxygen profiles in May and August follow a similar trend with oxygen penetrating slightly deeper (~0.5 mm) into the sediment in May (Fig 5c).

Nitrite plus nitrate in the sediment porewater varied between sites and months sampled (Figs 6a, 7a and 8a). The highest levels of nitrite plus nitrate were detected in the porewater of site BR (Fig 6). At the sediment water interface in May, the porewater nitrite plus nitrate concentration ranged between an average 0.6 μM to 5.6 μM depending on the site (Figs 6a, 7a, and 8a). In August, the concentration of nitrite plus nitrate was nearly undetectable along the entire sediment depth profile of all three sites (Figs 6a, 7a and 8a). Sediments from sites BR and SR exhibited similar porewater ammonium concentration profiles (Figs 6c and 7c), while slightly lower concentrations of ammonium were detected at site HI (Fig 8c). Between May and August at each site, the concentration of ammonium remained relatively stable (Fig 6c, 7c and 8c).

Oxic and Hypoxic Incubation Measurements

Bottom Water Dissolved Oxygen

Sets of pre-hypoxia cores collected in May were incubated under oxic and hypoxic conditions. The overlying bottom water DO concentrations of the oxic treatment cores were between 8.46-8.83 mg/L for all three sites (Fig 2, Table 2). For the hypoxic incubated cores, the DO concentrations of the water at the sediment water interface ranged from 1.5-3.3 mg/L (Fig 2, Table 2).

Sediment Characteristics

Sediment C/N ratios and weight percent TOC and TN of the incubated cores did not vary from the field cores with the exception of site SR. A slightly lower average C/N ratio of 10.1 at site SR was observed in the surface sediments (0-2 cm) of the oxic and hypoxic treatment cores (Fig 3). Also, an increase in weight percent TOC

and TN (3.51% and 0.41%, respectively) was detected in the surface sediments of site SR incubated cores compared sediment collected in May (Fig 4).

Downcore oxygen profiles of the incubated sediment cores varied depending on oxygen treatment. In all cores incubated under oxic conditions, the oxygen saturation at the sediment-water interface increased to ~100% and oxygen penetrated deeper into the sediments compared to the control cores collected in May (Fig 5). At site BR, the oxygen decreased more rapidly in the hypoxic incubated cores compared to sediment collected in May and August (Fig 5a). At site SR, similar oxygen profiles were observed in hypoxic treated sediment and sediment collected in August after hypoxia (Fig 5b). The oxygen profiles for sediment collected in May, August and exposed to hypoxic conditions were very similar for site HI (Fig 5c).

Sediment porewater nitrite plus nitrate from sites BR and SR differed between the incubated cores and sediments collected in May (Fig 6a,b and 7a,b). For site HI, little variation in nitrite plus nitrate concentrations was detected between May, oxic treatment and hypoxic treatment cores (Fig 8a,b). At both sites BR and SR, a spike in nitrite plus nitrate (up to 22 μM and 18 μM , respectively) was detected in the surface sediments of one set of oxic treatment cores (Figs 6b and 7b). At all three sites, porewater ammonium concentrations increased in both the oxic and hypoxic incubated cores compared to the May control cores (Figs 6d, 7d and 8d). The overall highest ammonium concentrations were detected in the hypoxic treatment cores (Fig 6d, 7d and 8d).

Phylogenetic Relationships of Expressed *nifH* Sequences

Expression of the *nifH* gene was observed at all three sites and was localized to the top 3 cm of sediment (Fig 9). The spatial distribution of *nifH* mRNA transcripts was variable between months sampled and treatments (Fig 9). The greatest *nifH* bulk expression was detected at site SR, particularly in the hypoxic treatment sediment (Fig 9).

Phylogenetic analysis of expressed *nifH* mRNA transcript sequences from the sediments revealed that they are limited to *nifH* Clusters I and III, as previously defined (Chien et al. 1996). Many expressed *nifH* sequences group with known sulfate, sulfur and iron reducing bacteria (Fig 10). Several of these phylogenetic groups (NB2, NB3, NB5, NB7 and NB10) have been previously reported from unvegetated sediments in the same estuary (Fulweiler et al. 2013; Brown et al. submitted). Three novel groups (NB12-NB14) were recovered in this study. Group NB3 contains the majority of expressed *nifH* sequences (41) and is most closely related to a sulfur and iron reducing anaerobe, *Pelobacter carbinolicus* (Lovley et al. 1995) (Fig 10). The second largest group of expressed *nifH* sequences (13), group NB7 also contains the sulfate reducers *Desulfovibrio salexigens* and *Desulfovibrio vulgaris* (Fig 10). At site HI, only groups NB3 and NB7 *nifH* mRNA transcripts were detected in the sediments (Fig 11). A higher diversity of microbes expressing *nifH* was identified at sites BR and SR (Fig 11).

Targeting Specific Diazotroph Groups with QPCR

Quantitative PCR was used to follow changes in abundance and levels of *nifH* expression of the two dominant microbial groups expressing *nifH*, groups NB3 and NB7 (Fig 10). The greatest abundance of group NB3 was observed at site HI at 1 cm

in depth in the hypoxic treatment sediments (Fig 12c). The distribution of group NB3 was similar at sites BR and SR, with a slight increase in abundance in the surface sediments of the oxic and hypoxic treatment cores at site SR (Fig 12a,b). Similar levels of NB3 *nifH* expression were detected at sites BR and SR (Fig 12d,e). Little to no *nifH* expression was observed between 3-5 cm in depth at site HI (Fig 12f). The overall abundance of group NB7 was greater than NB3, however, a difference between sites was not observed (Figs 12a-c and 13a-c). The lowest NB7 abundance was detected along the entire depth profile at site SR in May prior to hypoxia (Fig 13b). The expression of *nifH* by group NB7 followed a similar distribution to group NB3 (Fig 12d-e and 13d-e). No significant difference in NB3 and NB7 abundance and *nifH* expression was observed between months sampled and treatment.

Discussion:

Summer hypoxic events have been the subject of increasing concern in Narragansett Bay. Episodic hypoxia has been recorded in the bay for the last several decades (Oviatt et al. 1984; Bergondo et al. 2005; Deacutis et al. 2006; Melrose et al. 2007; Deacutis 2008; Saarman et al. 2008), and the summer of this study was no different. Severe hypoxia was documented for several weeks during July in Providence River Estuary and Greenwich Bay, locations of site BR and SR, respectively (Fig 2) (BART 2013; NBFSMN 2013). Additionally, near-hypoxic conditions reached as far south as site HI for part of July (Fig 2) (NBFSMN 2013). Interestingly, high rates of net nitrogen fixation were measured (up to $-650 \mu\text{mol N}_2\text{-N m}^{-2} \text{h}^{-1}$) at several sites in upper Narragansett Bay in 2006 (Fulweiler et al. 2007) during the same summer months that these regions were severely impacted by

widespread hypoxia (Codiga et al. 2009). We present sediment TOC, TN, oxygen and porewater nutrient profiles with concurrent gene expression data from *in situ* and incubated sediments under varying water column oxygen conditions. After severe hypoxia in upper Narragansett Bay, the concentration of porewater nitrite plus nitrate has disappeared in the sediment. In oxic incubated cores, an increase in nitrite plus nitrate was observed in the surface sediments, while an accumulation of ammonium was detected in cores exposed to hypoxic conditions. Hypoxic and anoxic conditions in the water column may in fact be driving a change in N cycling in coastal marine sediments.

Organic Matter Sources and Processing

The ratio of C/N can be a useful indicator of the source of organic matter preserved in sediments. Organic matter produced by marine algae and phytoplankton have C/N ratios of 6-9 (Bordovsky 1965), whereas terrestrial organic matter tends to have C/N ratios greater than 20 (Meyers et al. 2001). The C/N ratio of estuarine sediments, including sites BR, SR and HI, reflects a mixture of both marine and terrestrial sources. Site SR has a slightly lower C/N ratio compared to the other sites, indicating that marine algae may be a slightly more important source of organic matter to those sediments.

Measurements of total organic carbon (TOC) and total nitrogen (TN) can also provide evidence for the long-term impacts of hypoxia. The concentration of total C and N are indicators of primary productivity and represent the proportion of organic matter that remains after sedimentary remineralization. Increased TOC and TN concentrations have been attributed to several factors including, higher water column

productivity, greater terrestrial organic matter inputs or increased preservation in sediments due to hypoxic conditions (Gooday et al. 2009). In Chesapeake Bay sediments, several studies observed increases in percent TOC and TN corresponding to both higher primary productivity and periods of hypoxia (Cooper et al. 1991; Bratton et al. 2003). Higher concentrations of TOC and TN detected in sediments from sites BR and SR than in site HI sediments correlates with higher levels of primary productivity (Oviatt et al. 2002; Oviatt 2008) and more severe seasonal hypoxia (Melrose et al. 2007; Deacutis 2008; Saarman et al. 2008) observed at sites BR and SR in comparison to site HI. Site HI may have lower concentrations of TOC and TN due to differences in sediment composition (King et al. 2008). We did not observe a shift in C/N ratio or concentrations of total C and N in the sediments at any of our sites during the course of the sampling, including sites SR and BR that experienced severe hypoxia. It may be that one summer of sampling was not enough to detect a shift in the C/N ratio. However, differences in total C and total N concentrations between sites suggests that long-term hypoxia may impact organic matter preserved in the sediment.

Hypoxic Conditions Alter Sediment Porewater Nutrient Profiles

Redox dependent nitrogen transformations in the sediment are potentially influenced by low oxygen events occurring in the water column. Monitoring concentrations of porewater oxygenated nitrogen species (nitrite plus nitrate) and reduced species (ammonium) can provide insight into microbial N cycling activities occurring in the sediment. In comparison to sediment collected in May, concentrations of nitrite plus nitrate disappear to nearly undetectable levels throughout the entire sediment depth profile after hypoxia in early August. The depletion of nitrite plus

nitrate suggests nitrification has been repressed. Hydrogen sulfide, a respiratory poison (Wang et al. 1999) has been shown to specifically inhibit nitrification (Joye et al. 1995). Hypoxic waters in Chesapeake Bay have stimulated sulfate reduction and therefore heightened hydrogen sulfide levels (Kemp et al. 1990). Increased concentrations of hydrogen sulfide and low oxygen conditions associated with hypoxia are likely limiting nitrification.

Conversely, in the oxic incubated cores from sites BR and SR, increased oxygen penetration in the surface sediments appears to have stimulated nitrification activity as increased concentrations of nitrite plus nitrate were observed at the sediment water interface. Similar findings were detected in a Denmark estuary in which changes in oxygen penetration depth fueled nitrification (Rysgaard et al. 1995). Unlike the sediments collected after hypoxia in August, we did not observe depletion in nitrite plus nitrate in the hypoxic treatment cores. Perhaps in this short incubation, the low oxygen concentrations repress nitrification but the hypoxic incubation was not long enough to allow denitrification to completely reduce the stock of oxidized forms of nitrogen available in the sediment.

High concentrations of ammonium were observed in all sediment depth profiles, and fall within concentrations reported for other estuarine sediments (Bertics et al. 2010; Rao et al. 2011). Higher porewater ammonium concentrations were detected at sites BR and SR in comparison with site HI, possibly correlating with strong down bay gradients in water column inorganic nutrients and primary production (e.g (Oviatt 1980; Oviatt et al. 2002)).

An accumulation of porewater ammonium was observed in both oxic and hypoxic treatment cores at all three sites, with the highest concentrations detected in sediment incubated under hypoxic conditions. An increase in porewater ammonium was not detected in the *in situ* cores. The dissimilatory nitrate reduction to ammonium (DNRA) pathway retains available N in the system in the form of ammonium. In estuarine sediments, several studies have proposed that under low oxygen conditions, DNRA is favored over denitrification (Tiedje et al. 1983; Rysgaard et al. 1996) resulting in an increase in ammonium, supporting our findings in the hypoxic incubated sediments. However, we also observed an increase in porewater ammonium in the oxic treatment cores, but generally the highest concentration was detected below oxygen penetration (0.5 cm). Perhaps an increase in nitrate due to oxygen-stimulated nitrification in the oxic cores, promoted competition for nitrate by microbes involved in the denitrification and DNRA pathways resulting in an increase in ammonium compared to the May control cores.

Low oxygen concentrations in overlying bottom waters appear to, at least in part, influence sediment N cycling. For example, oxygen depletion and increased sulfate reduction associated with hypoxic conditions potentially inhibit nitrification, which has likely consequences for decreasing subsequent rates of denitrification. The repression of coupled nitrification-denitrification may open a niche for other N cycling microbes to thrive and become active.

Sulfur and Sulfate Reducers are the Dominate Diazotrophs in Benthic Habitats

Due to the high input of N into coastal systems (Howarth et al. 1988a; Howarth et al. 1988b) and N fixation being repressed by combined N (Postgate 1982), benthic

sediments were not consider a source of fixed N. However, in some marine environments, N fixation may not be as sensitive to dissolved inorganic N, as previously believed (Knapp 2012). *nifH* expression was detected in surface sediments with concentrations of ammonium generally $>40 \mu\text{M}$, indicating that these potentially active diazotrophs are not sensitive to high levels of combined N in the sediments. The greatest *nifH* expression was detected at the two sites most heavily impacted by seasonal hypoxia and with the highest concentrations of porewater nitrite plus nitrate and ammonium.

Similar to previous findings in other upper Narragansett Bay sediments (Fulweiler et al. 2013; Brown et al. submitted), the two microbial groups dominating *nifH* expression at all three sites are related to the iron/sulfur reducer *Pelobacter carbinolicus* (group NB3) and the sulfate reducers *Desulfovibrio salexiges* and *D. vulgaris* (group NB7). A few novel groups were detected at sites BR and SR. Our *nifH* RNA sequences phylogenetically group with *nifH* DNA sequences reported from unvegetated sediments in other coastal systems including Chesapeake Bay in Maryland, Catalina Harbor in California and Eckernförde Bay in the Baltic Sea (Burns et al. 2002; Bertics et al. 2010; Bertics et al. 2012), indicating these potentially active diazotrophs are widely distributed in coastal marine sediments.

Various sulfur and sulfate reducing bacteria are genetically capable of fixing N (Zehr et al. 2003) and have been shown to do so in culture (Sisler et al. 1951; Widdel 1987), suggesting these microbes may be potential sources of fixed N in benthic sediments. Phylogenetic analysis of the *nifH* gene in benthic habitats including sediments vegetated by macrophytes, microbial mats and unvegetated sediments have

identified sulfate reducers as being the dominant N fixer (Lovell et al. 2000; Brown et al. 2003; Steppe et al. 2005; Bertics et al. 2010; Fulweiler et al. 2013; Brown et al. submitted). Based on acetylene reduction and sulfate respiration inhibition assays, several studies have confirmed that sulfate reducers are responsible for the N fixation rates detected in these benthic habitats (Capone et al. 1982; Welsh et al. 1996; Steppe et al. 2002; Bertics et al. 2010).

Steady Abundance and *nifH* expression of the Dominant Diazotrophic Groups

We hypothesized low oxygen conditions associated with hypoxia would stimulate N fixation in the benthic sediments and we would detect this potential switch in N cycling with an increase in NB3 and NB7 *nifH* expression under low DO levels. However, the abundance and *nifH* expression of groups NB3 and NB7 did not appear to respond to changing bottom water oxygen concentrations. At each site, a steady abundance and *nifH* expression of both microbial groups was observed along the entire depth profile, with slight variation. Perhaps we did not capture an increase in *nifH* expression by these microbial groups because bottom water oxygen concentrations were too high. All sediments analyzed had an overlying DO concentration of >2 mg/L. The acetylene reduction assay was used to determine the rates of N fixation at the sites in this study as well as Greenwich Cove, near a wastewater treatment plant. Higher rates of N fixation were measured at Greenwich Cove with DO concentrations of <1 mg/L compared to sediments from sites BR, SR, and HI with DO concentrations ranging from 2-9 mg/L (Rodrigue Spinette, unpublished). Based on these results, our assumption seems likely. If we sampled

during anoxia in July or incubated the cores longer under lower oxygen conditions, we may have detected a greater change in *nifH* expression.

Alternatively, perhaps we are not following the most low oxygen responsive *nifH* expressing microbial group. The two phylogenetic groups (NB3 and NB7) targeted with qPCR are the most abundant and active *nifH* expressers under low oxygen (2-4 mg/L DO) and oxygenated conditions in the sediment. By cloning *nifH* transcripts in the surface of sediments exposed to near-anoxic conditions, we may identify novel groups with increased *nifH* expression under oxygen depletion.

Conclusions

Hypoxic regions are expanding and predicted to increase in the near future due to human activities and climate change (Diaz et al. 2008). However, little is known about how low oxygen waters influences nitrogen cycling dynamics in coastal marine sediments. The global N cycle is driven by microbial metabolism and oxygen depletion has the potential to perturb N transformations. In Narragansett Bay sediments, microbes expressing *nifH* are related to sulfur/iron and sulfate reducers. Close relatives of these microbes have been shown to actively fix N in other benthic habitats (Steppe et al. 2002; Bertics et al. 2010). Episodic hypoxic events in the bay not only may stimulate N fixing activity by these anaerobes, but if low oxygen conditions persistent over time, may also select for these diazotrophic microbial communities. An increase in N fixation in the sediments could establish a positive feedback loop, exacerbating hypoxic conditions. Understanding the impacts of low oxygen on N transformations in coastal sediments is crucial for predicting how these expanding hypoxic events will influence the marine N cycle globally.

Experimental Procedures:

Study Sites and Field Measurements

Sediments were sampled at three sites (BR, SR and HI), in the temperate estuary, Narragansett Bay, Rhode Island, USA in late May and early August 2013. All sites are located near Narragansett Bay Fixed-Site Monitoring Network (NBFSMN) buoys which record continuous measurements of dissolved oxygen (DO), chlorophyll, temperature and salinity (Fig 1, Table 1).

At each site, a YSI 6920 V2 was used to measure DO, temperature and salinity of the entire water column (Table 1). SCUBA divers harvested intact sediment cores (10 cm inner diameter and 30.5 cm long). In May, six cores were collected from each site. Two cores were sacrificed on day of collection and referred to as pre-hypoxic May cores. The four remaining cores were stored in the dark in an incubator at the University of Rhode Island at average *in situ* bottom water temperature (17°C). Two cores were capped to mimic hypoxic conditions (referred to as hypoxic treatment) and two cores were left uncapped with air gently bubbling through the overlying water (referred to as oxic treatment). Capped cores were monitored and when the DO dropped below ~3 mg/L of oxygen (7 days after collection), all four cores were sacrificed. In August, two cores were collected from each site and were sacrificed on day of collection and referred to as August control cores. Before sediment cores were sectioned, triplicate profiles of DO in the sediment surface of one core per treatment were measured with Unisense's oxygen microsensor. Calibration of the instrument and measurements were performed based on the manufacturer's protocol. Triplicate measurements were averaged for the sediment oxygen penetration profiles. Oxygen

percent saturation was measured in 250 μm intervals for the May, oxic and hypoxic cores and 500 μm intervals for the August cores.

Sediment Core Sectioning and Sub-sampling

Sediment cores were sectioned in 0.5 cm increments for the top 2 cm and then in 1 cm increments to 5 cm in depth. For each section, duplicate 0.25 mL and 0.5 mL samples were flash frozen for DNA and RNA isolation, respectively. A ~5 mL sub-sample was saved for carbon and nitrogen analysis. The remaining sediment was transferred into a 50 mL conical tube and centrifuged at 4,000 rpm for 5 min. The overlying water was saved for porewater analysis.

Carbon and Nitrogen Analysis

Samples were analyzed in 1cm increments, so sediment from the surface water interface to 1 cm in depth were pooled and sediment from 1 cm to 2 cm in depth were pooled. Samples were dried at 60°C for 18-24 hrs. The sediment was then homogenized using a clean mortar and pestle. ~10-15 mg of sediment was weighed into an ultra-clean tin capsule on a microbalance and then placed into a nickel sleeve. Sediment samples and acetanilide standards were analyzed with a Carlo Erba EA1108 CHN analyzer.

Porewater Nitrite plus Nitrate

Porewater nitrite and nitrate samples were reduced by a vanadium (III) solution to nitric oxide for chemiluminescence detection with a NO_x box as previously described (Hendrix et al. 1995). Standards were prepared from dilutions of potassium nitrate.

Porewater Ammonium

Porewater ammonium concentrations were measured colorimetrically using a modified version of the Koroleff method. Standards were prepared from dilutions of ammonium sulfate. In order, 20 μL of a 0.04% sodium nitroprusside and 3.8% phenol solution, 10 μL of a 48% trisodium citrate dehydrate solution and 20 μL of 1.5% hypochlorite solution were added to 450 μL of standard or sample. Solutions were mixed thoroughly between additions of each reagent. 200 μL of the standard or sample mixture was transferred to a Costar 96-well black plate with clear bottoms and incubated at room temperature for 90 min to allow for the reaction to complete. The absorbance of all standards and samples were measured at 630nm using a SpectraMax Plus384 Absorbance Microplate Reader in Rhode Island EPSCoR's Molecular Ecology Preparatory Laboratory.

Nucleic Acid Extractions

Total DNA was extracted using the MO Bio Powersoil DNA Isolation Kit (Carlsbad, CA, USA) and quantified using Invitrogen's Qubit dsDNA HS Assay Kit. All DNA samples were diluted to 1 ng/ μL for quantitative PCR analysis. Total RNA was extracted using the MO Bio Powersoil RNA Isolation Kit (Carlsbad, CA, USA). Detailed methods regarding RNA isolation and purification are previously described (Brown et al. submitted). Total RNA was quantified using Invitrogen's Qubit RNA Assay Kit and all RNA samples were diluted to 4 ng/ μL . Invitrogen's SuperScript First-Strand Synthesis System was used to generate cDNA copies of RNA for RT-PCR and quantitative RT-PCR. For all samples, 8 μL of DNase-treated RNA at a concentration of 4 ng/ μL was added to the reaction. Each reaction was primed with 1 μL of 2 μM outer reverse primer for our gene of interest, *nifH3*. After the reverse

transcriptase was added, the mixture was incubated at 50°C for 50 min. All the other steps followed the instructions of the manufacturer. For every sample, we also included controls that did not contain reverse transcriptase to confirm there was no DNA contamination in the subsequent PCR amplification.

Functional Gene Sequence Analysis

The *nifH* gene from environmental cDNA was isolated using nested PCR with degenerate outer primers nifH4-nifH3 and inner primers nifH1-nifH2 (Zehr et al. 1989; Kirshtein et al. 1991). Both rounds of PCR consisted of an initial denaturation step of 2 min at 94°C, cycling steps that included: a denaturation step of 30 s at 94°C, an annealing step of 30 s at 50°C, and an extension step of 1 min at 72°C. All reactions had a final extension step of 7 min at 72°C. First round reactions had 25 cycles and the second round reactions had 30 cycles (Zehr et al. 1989; Kirshtein et al. 1991).

Amplified *nifH* PCR products were loaded on to a 1% agarose (wt/vol) TAE gel and purified using QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen Valencia, CA, USA). The purified products were cloned into pGEM-T vectors (Promega, Madison, WI, USA). Plasmids were transformed into JM109 *E.coli* competent cells (Zymo Research, Irvine, CA, USA) and identified by blue-white screening. The plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and sequenced on the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the RI Genomic Sequencing Center at the University of Rhode Island. For every expressed *nifH* amplicon, we collected 4 sequences.

Expressed environmental *nifH* sequences from sites BR, SR and HI, were combined with *nifH* sequences (23) from closely related cultivated species in GenBank (Benson et al. 2009) as determined by top BLASTn hits. All *nifH* DNA sequences were translated into NifH protein sequences and aligned using the multiple sequence alignment tool, MUSCLE (Edgar 2004) within the Geneious software package. A *nifH* maximum likelihood tree of aligned protein sequences was constructed in Geneious using PhyML with 1000 bootstrap replicates.

Quantitative Real-Time PCR

All environmental DNA and cDNA samples were analyzed by quantitative PCR using Roche's LightCycler 480 Probes Master Mix and were measured using Stratagene's Mx3005 qPCR System. Sets of degenerate qPCR primers and dual-labeled TaqMan probes were previously designed to target the *nifH* gene specifically related to *nifH* groups NB3 and NB7 (Fig 10) (Brown and Jenkins, submitted). Both probes were labeled with a fluorophore FAM on the 5' end and BHQ1 quencher on the 3' end and reactions were run separately. A standard curve was produced with triplicate 10-fold dilution series ranging from linearized plasmid containing a sequenced *nifH* clone from group NB3 and NB7, respectively. The qPCR reactions consisted of 10 μ L of the Roche LightCycler 480 Probes Master mix, 5.7 μ L of water, 2 μ L of a primers/probe mix (at concentrations of 0.4 μ M and 0.2 μ M, respectively), and 0.3 μ L of Stratagene Brilliant II qPCR reference dye (ROX). A saturation test was used to determine the optimal concentration of DNA and cDNA template going into the reaction. It was determined that 2 μ L of 1 ng/ μ L DNA sample or 2 μ L of 1.5 ng/ μ L cDNA sample was added to the reaction totaling 20 μ L. The qPCR

thermocycling conditions were identical for both targeted groups, except for the annealing temperature: 1 cycle of 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 seconds and 60°C (NB3) or 55°C (NB7) for 1 minute. The standard curve was used to determine groups NB3 and NB7's absolute *nifH* gene copy or transcript copy number in the environmental samples.

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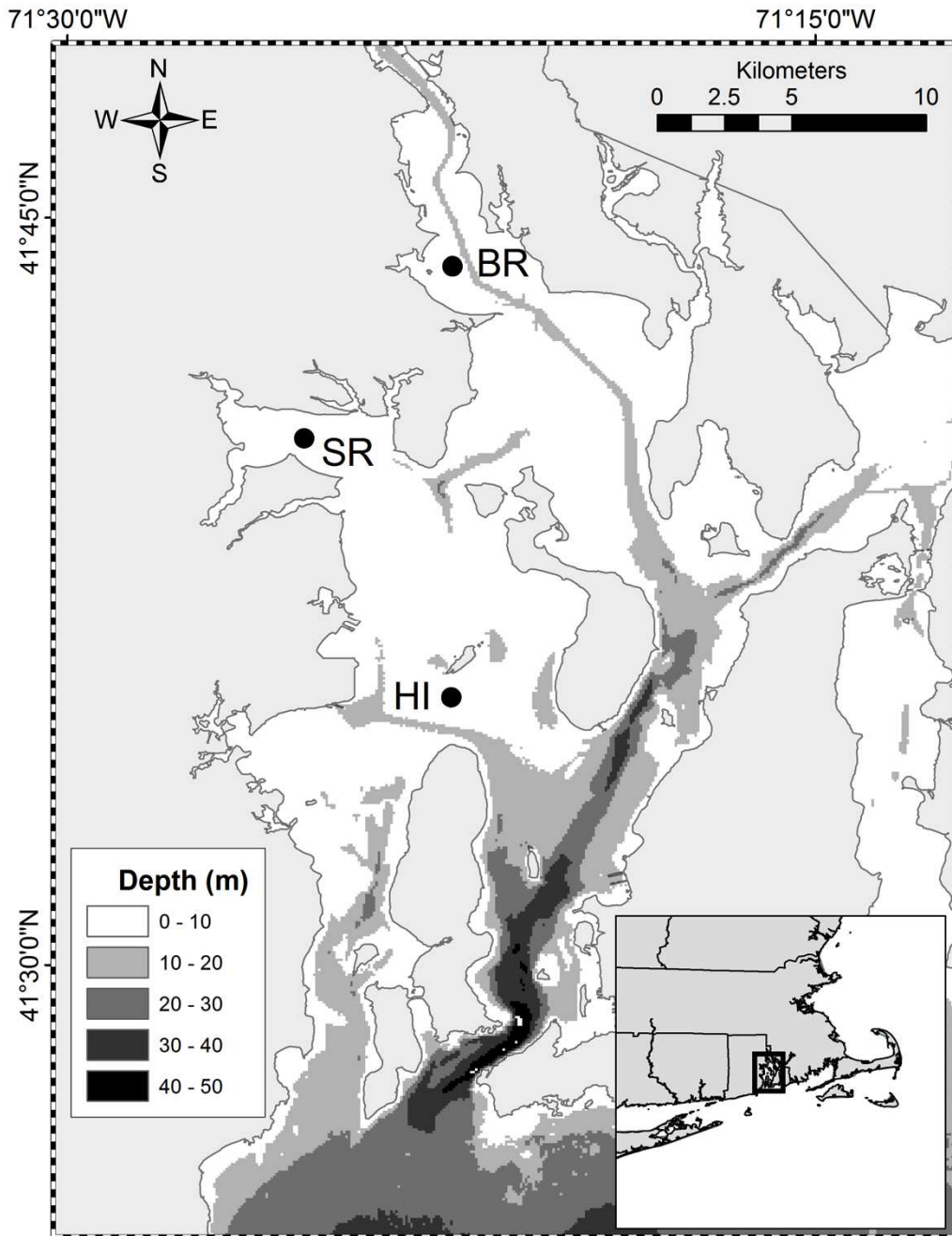


Figure 1: Map of sampling sites (black circles) in upper Narragansett Bay, Rhode Island.

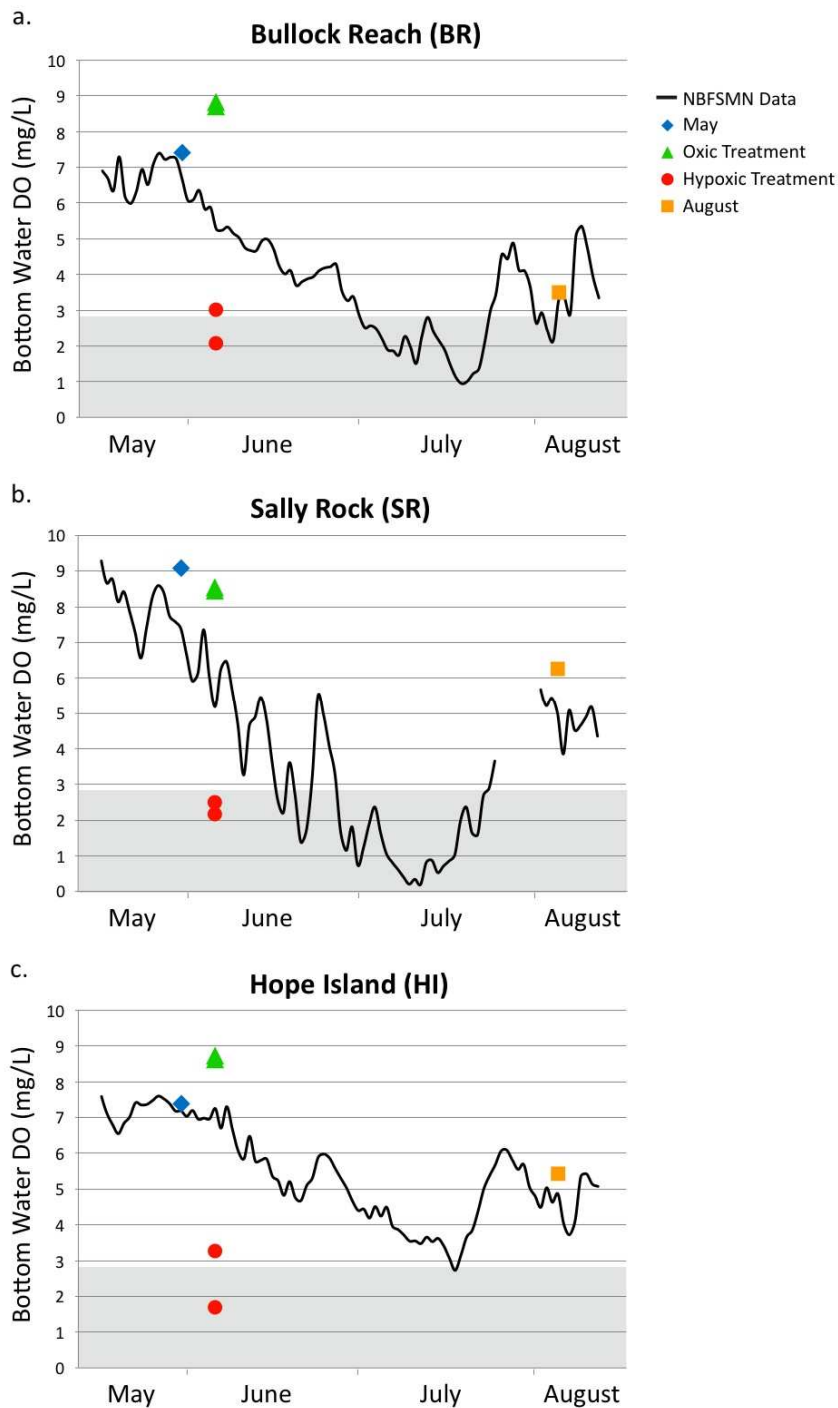


Figure 2: Bottom dissolved oxygen concentrations at sites a) BR, b) SR and c) HI. The black line indicates the daily average of continuous measurements of bottom DO from the end of May through early August (Narragansett Bay Fixed-Site Monitoring Network (NBFSMN), data provided by Heather Stoffel). Gray box indicates hypoxia ($DO < 2.9\text{ mg/L}$) as designated by RI Department of Environmental Management.

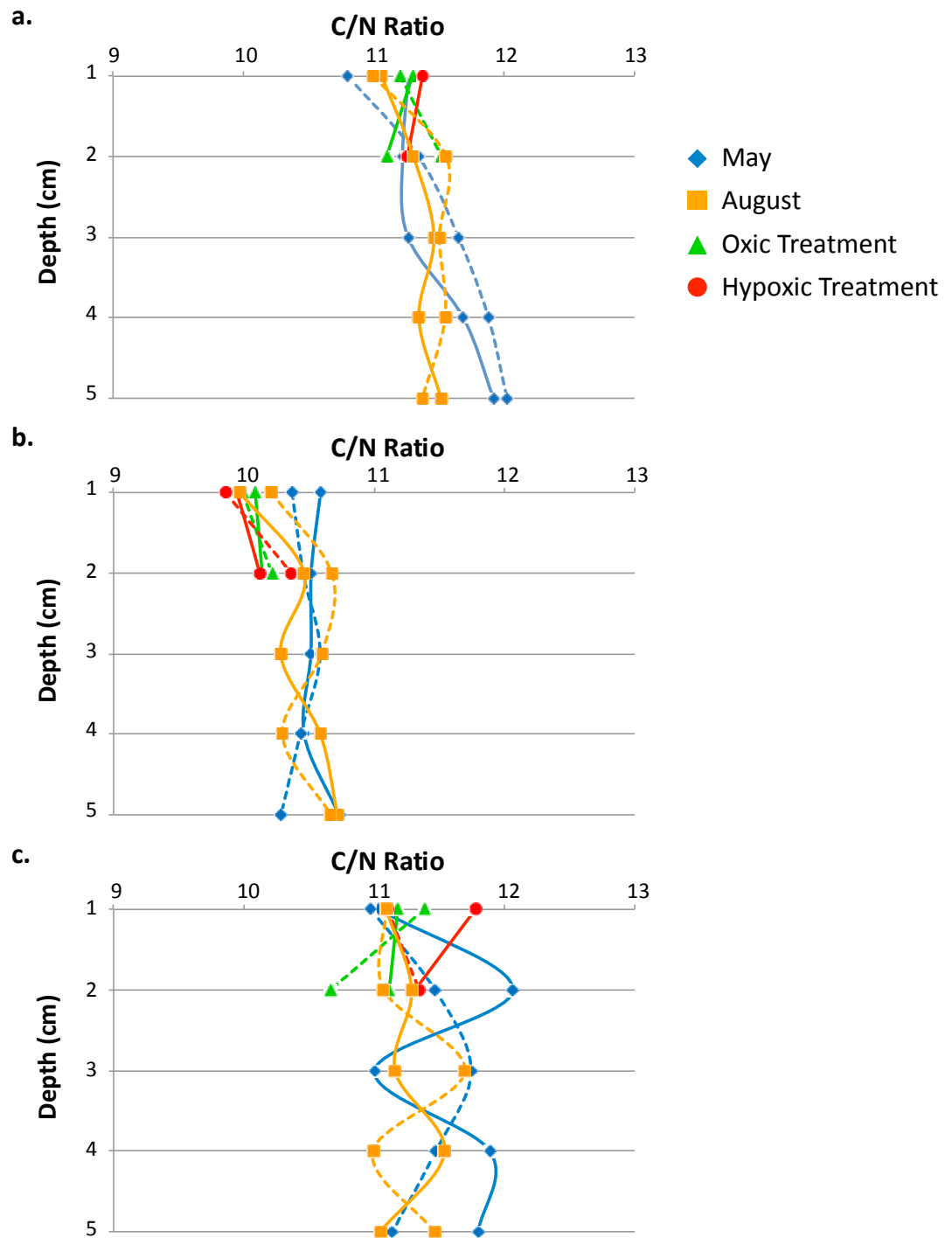


Figure 3: Downcore carbon to nitrogen (C/N) ratios in 1 cm intervals from sites a) BR, b) SR and c) HI. Dashed lines indicate replicate core measurements.

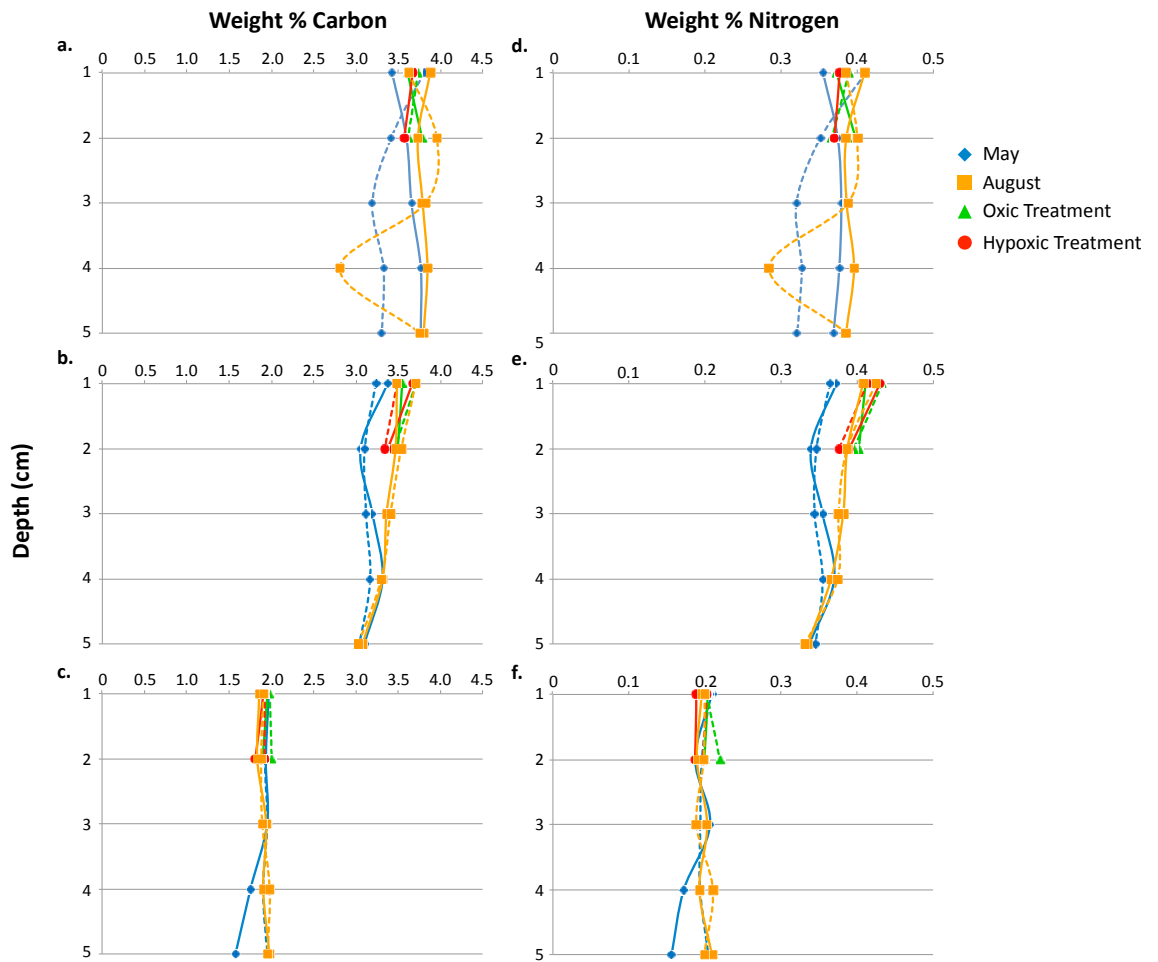


Figure 4: Downcore weight percent carbon (a-c) and weight percent nitrogen (d-e) in 1 cm intervals at sites BR (a,d), SR (b,e) and HI (c,f). Dashed lines indicate replicate core measurements.

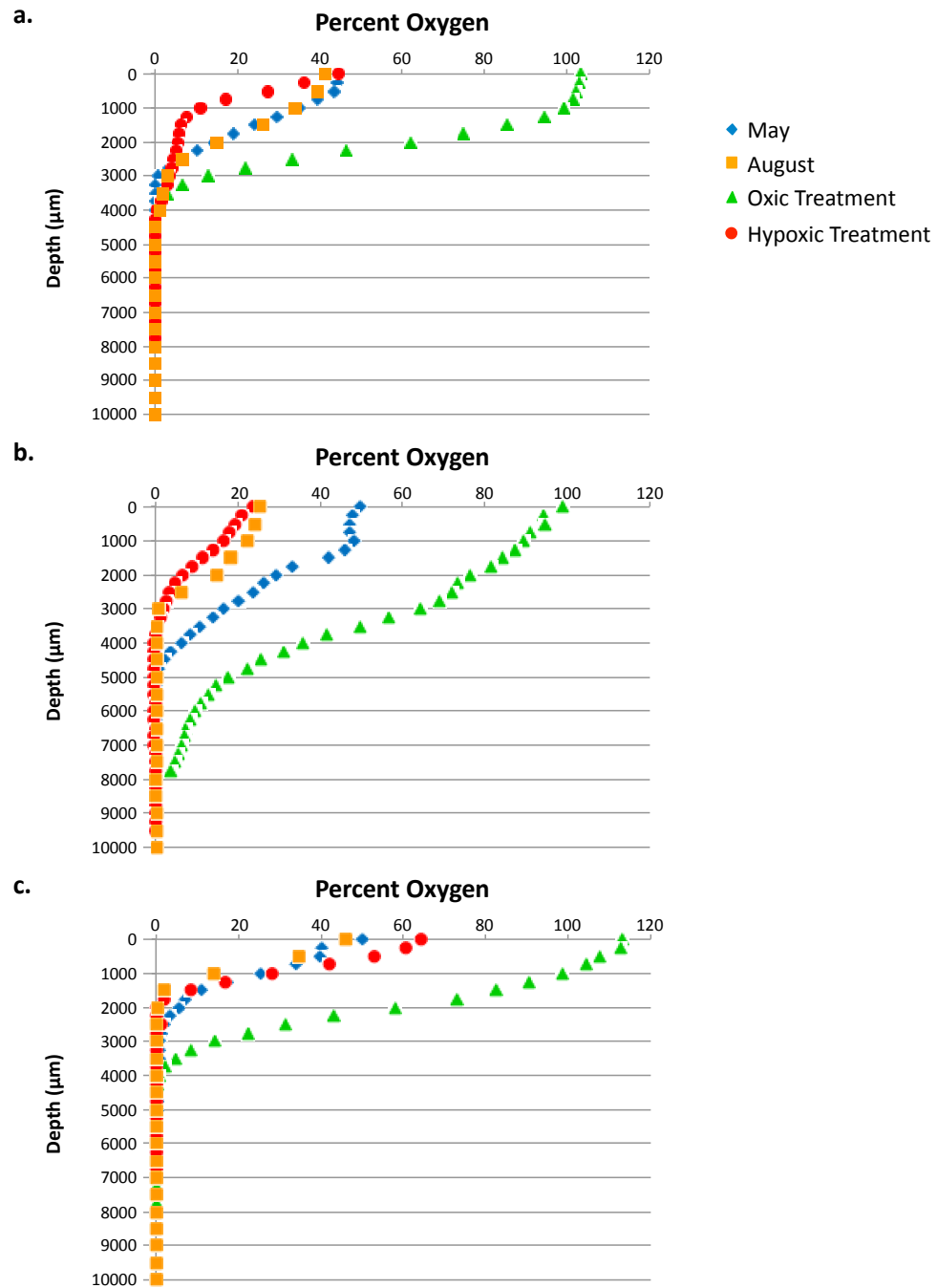


Figure 5: Profiles of sediment oxygen percent saturation at sites a) BR, b) SR and c) HI. Measurements were made in triplicate.

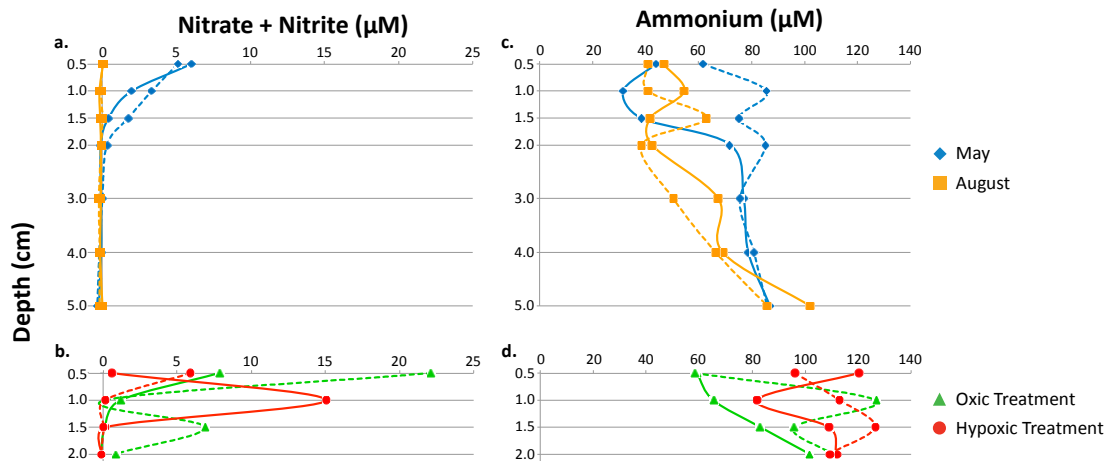


Figure 6: Site BR sediment porewater nitrite plus nitrate (a,b) and ammonium (c,d) profiles for May (a,c), August (a,c), oxic treatment (b,d) and hypoxic treatment (b,d). Dashed lines indicate replicate core measurements. Only the top 2 cm of sediment were analyzed for the oxic and hypoxic treatment cores.

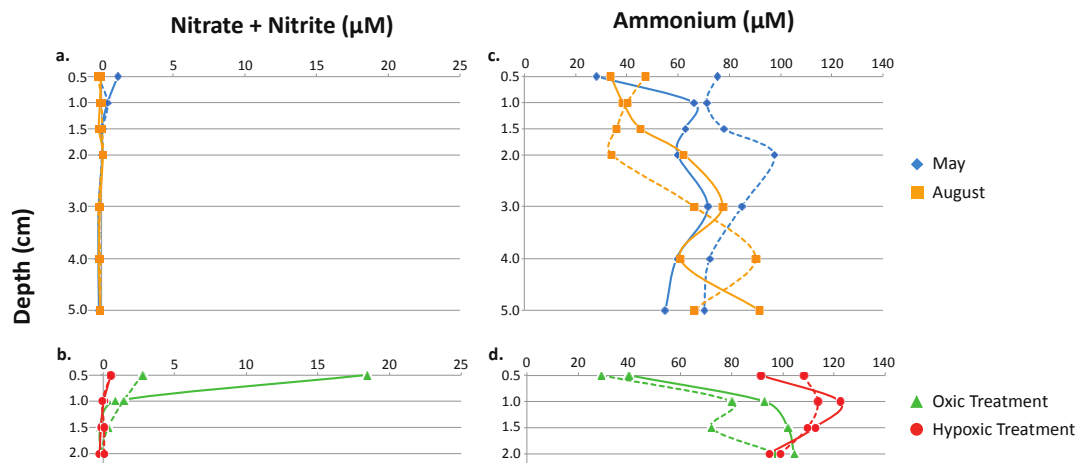


Figure 7: Site SR sediment porewater nitrite plus nitrate (a,b) and ammonium (c,d) profiles for May (a,c), August (a,c), oxic treatment (b,d) and hypoxic treatment (b,d). Dashed lines indicate replicate core measurements. Only the top 2 cm of sediment were analyzed for the oxic and hypoxic treatment cores.

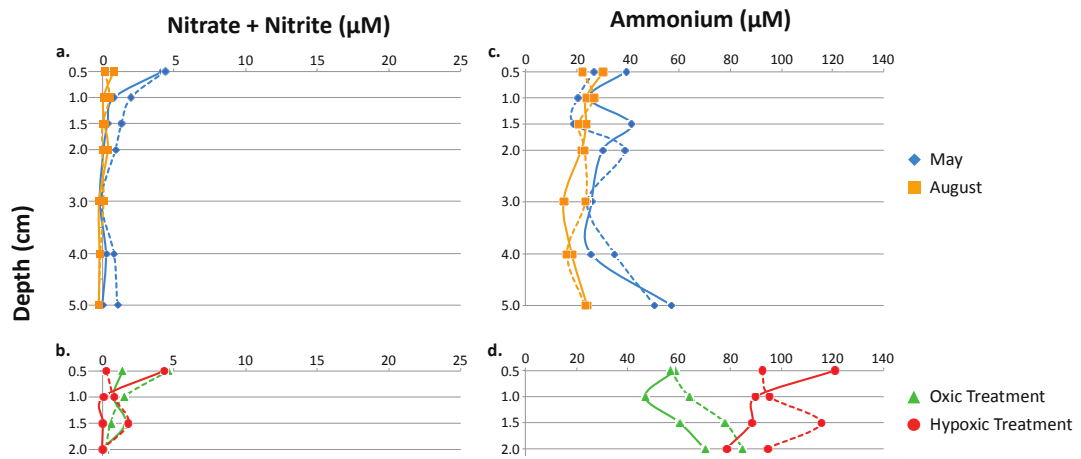


Figure 8: Site HI sediment porewater nitrite plus nitrate (a,b) and ammonium (c,d) profiles for May (a,c), August (a,c), oxic treatment (b,d) and hypoxic treatment (b,d). Dashed lines indicate replicate core measurements. Only the top 2 cm of sediment were analyzed for the oxic and hypoxic treatment cores.

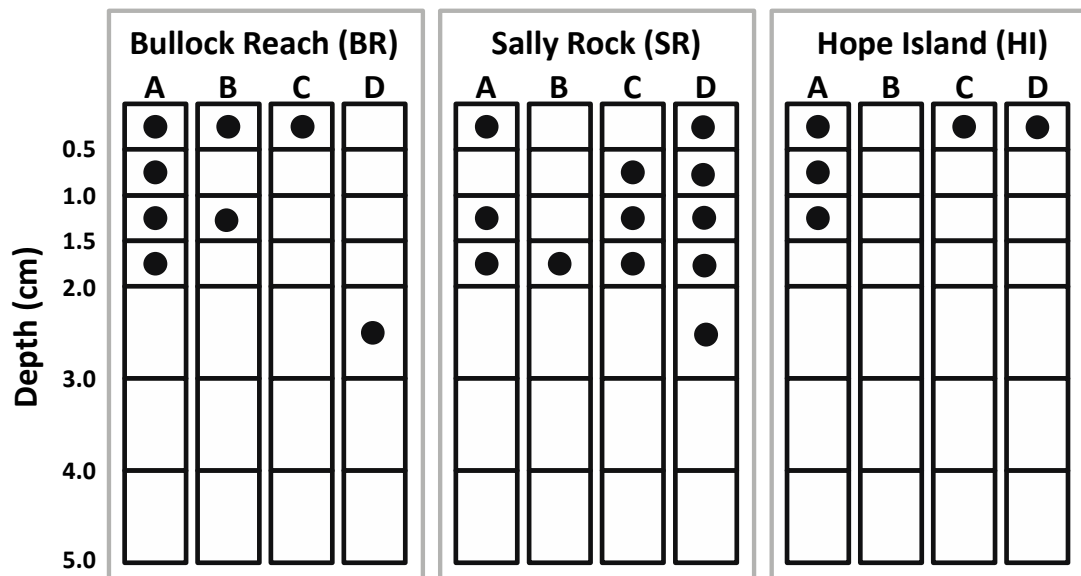


Figure 9: Downcore *nifH* gene expression (closed circles) at sites BR, SR and HI. Blank spaces indicate that no gene expression was detected. Sampling month or treatment denoted above each profile (A= May, B= August, C= oxic treatment and D= hypoxic treatment).

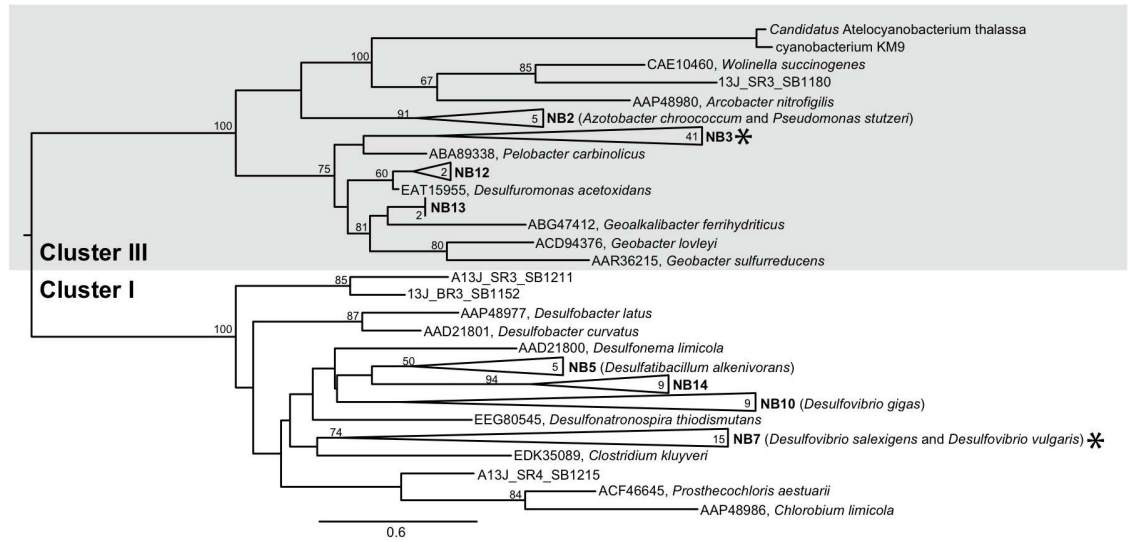


Figure 10: Maximum likelihood (PhyML) protein tree of expressed *nifH* sequences obtained from sediment samples and *nifH* sequences from closely related cultivated representatives. *nifH* cluster designations are denoted according to Chien and Zinder (1996). Groups NB2, NB3, NB5, NB7 and NB10 were previously described (Fulweiler et al. 2013 and Brown et al. submitted), while groups NB12-14 are novel to this study. The number inside the group indicates the total number of sequences within the grouping. Bootstrap values (1,000 replicates) > 50% are shown at the respective nodes. Asterisk indicates groups targeted for quantitative PCR.

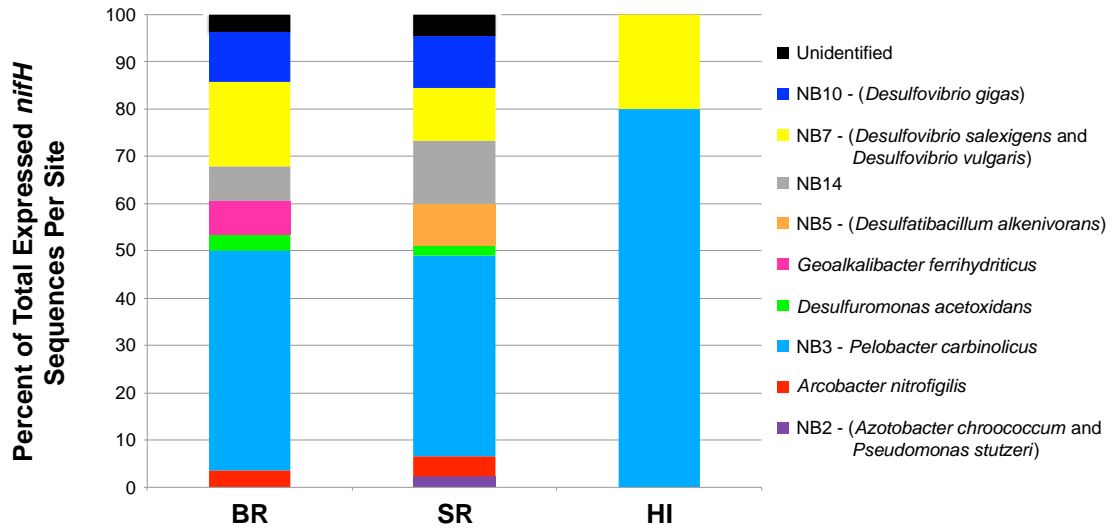


Figure 11: Percent of total expressed *nifH* sequences per site. Each color represents a cultivated species our environmental expressed sequences are related to as depicted in the *nifH* maximum likelihood tree (Fig. 3). Organisms listed in parentheses are contained within the grouping.

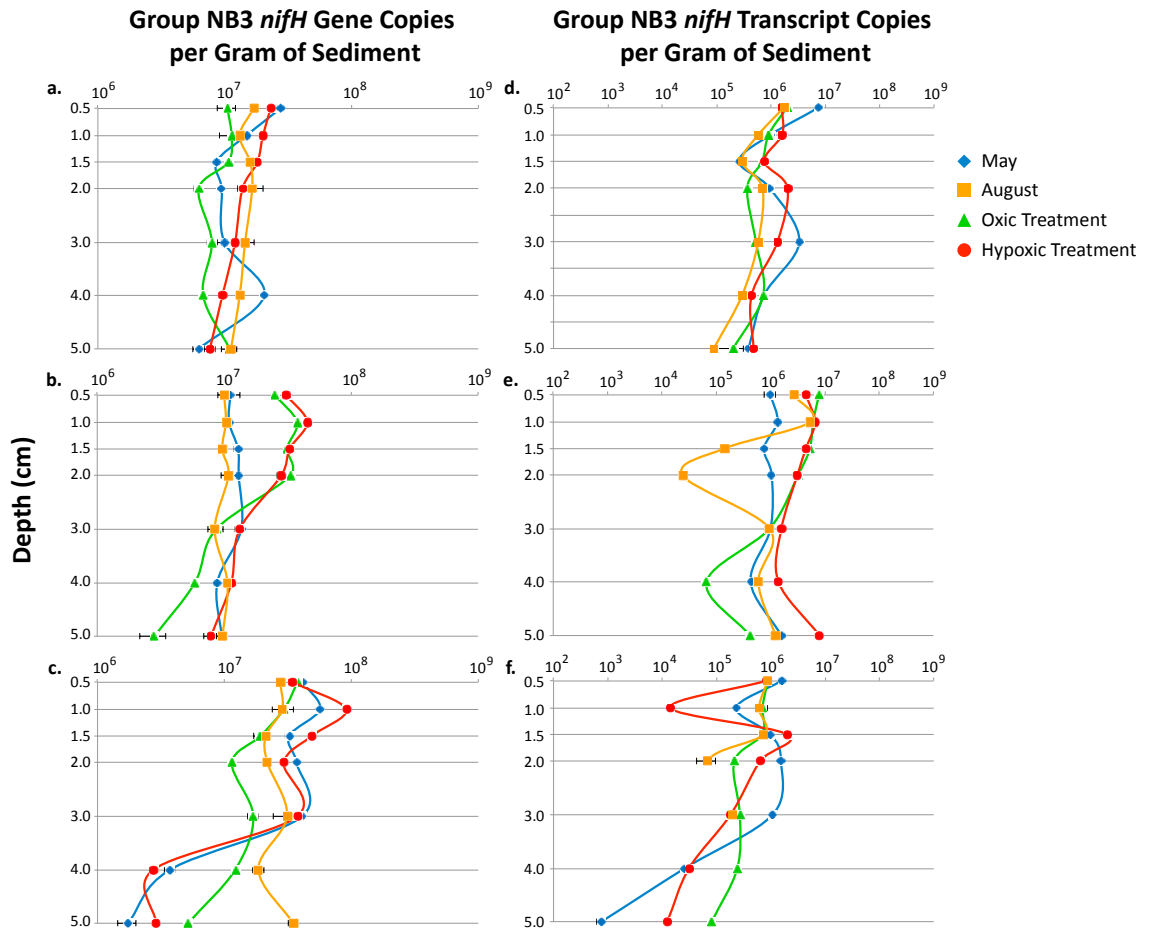


Figure 12: Group NB3 (related to *P. carbinolicus*) downcore abundance (a-c) and *nifH* expression (d-f) of enumerated by quantitative PCR from sediment samples collect at sites BR (a,d), SR (b,e) and HI (c,f). Graphs are plotted on a log scale.

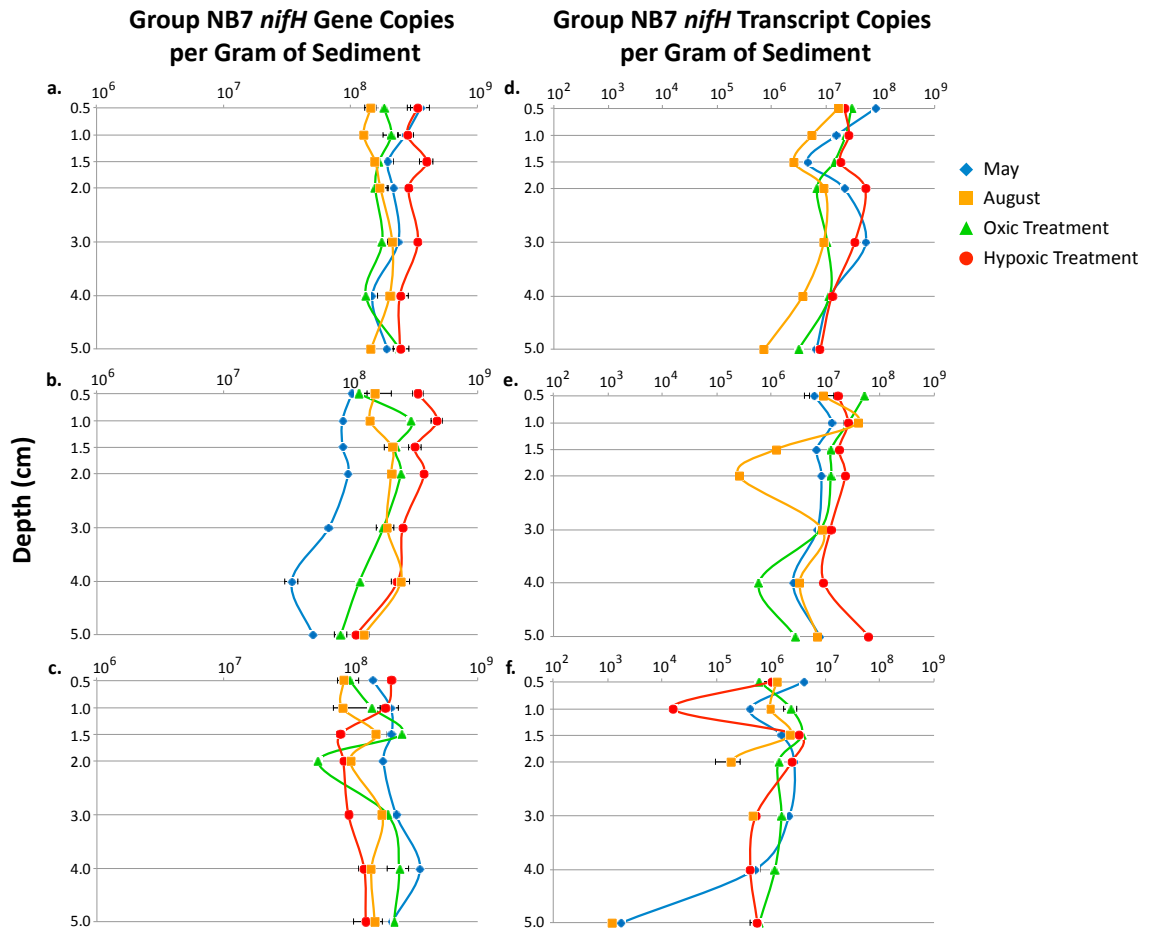


Figure 13: Group NB7 (related to *D. sallexigens* and *D. vulgaris*) downcore abundance (a-c) and *nifH* expression (d-f) of enumerated by quantitative PCR from sediment samples collect at sites BR (a,d), SR (b,e) and HI (c,f). Graphs are plotted on a log scale.

Table 1: Coordinates of study sites and *in situ* bottom water temperature and salinity during sampling.

Site	Coordinates (Latitude Longitude)	Bottom Water Temperature (°C)	Bottom Water Salinity (ppt)
Bullock Reach (BR)	41 43.985°N 71 22.062°W		
May		15.42	27.92
August		21.71	24.90
Sally Rock (SR)	41 40.613°N 71 25.222°W		
May		19.73	28.00
August		23.00	27.65
Hope Island (HI)	41.35.556°N 71 22.218°W		
May		14.52	29.92
August		20.00	29.41

Table 2: Bottom water dissolved oxygen (DO) concentrations during collection and incubations. May and August values are reported as the average DO concentration

collected with the NBFSMN buoys a week prior to sampling. Standard deviations are denoted. Values in parentheses are DO concentrations collected at time of sampling. For the oxic and hypoxic treatments, values for individual cores on day of sampling are reported.

Site	Bottom Water DO (mg/L)
Bullock Reach (BR)	
May	7.06 ±0.3 (7.42)*
Oxic Treatment	8.83* and 8.71
Hypoxic Treatment	3.02* and 2.08
August	3.02 ±0.7 (3.49)*
Sally Rock (SR)	
May	7.92 ±0.5 (9.09)*
Oxic Treatment	8.46* and 8.54
Hypoxic Treatment	2.18* and 2.49
August	5.32 ±0.3 (6.25)*
Hope Island (HI)	
May	7.39 ±0.2 (7.39)*
Oxic Treatment	8.63* and 8.74
Hypoxic Treatment	3.27* and 1.69
August	4.94 ±0.4 (5.43)*

*Sediments analyzed for microbial analysis

CONCLUSION

Marine nitrogen cycling is complex and the transformations between different chemical forms of N are driven by extremely diverse microbial communities (Zehr et al. 2002; Gruber 2004). Functional genes encoding cellular proteins that mediate these biogeochemical processes can provide insight into the functioning of an ecosystem. Additionally, phylogenetic analysis of these genes can be used to investigate the diversity of specific groups of microorganisms (eg. denitrifiers and nitrogen fixers) in the environment, however the analysis does not provide information on what fraction of these microbes are metabolically active. This dissertation aimed to use a gene expression approach to identify and follow the likely active microbes responsible for the removal and input of N to coastal marine sediments under different environmental conditions.

During the summer of 2006, benthic sediments in Narragansett Bay exhibited a seasonal switch in N cycling with high rates of net N fixation (Fulweiler et al. 2007). We proposed that timing of organic matter deposition to the benthos might be important in determining which process (denitrification vs. nitrogen fixation) dominates (Fulweiler et al. 2013). Using mesocosms with sediment from the same site, we analyzed expression of functional genes associated with nitrogen fixation (*nifH*) and denitrification (*nirS*) in response to varying the timing of organic matter deposition. We discovered that both processes were occurring simultaneously (Fulweiler et al. 2013). Phylogenetic evidence suggested that sulfur/iron respiring microbes and sulfate reducers were responsible for the nitrogen fixation activity

(Fulweiler et al. 2013). These findings led us to investigate the spatial extent of heterotrophic sediment N fixation in temperate regions.

Expression of *nifH* and *nirS* were analyzed along the estuarine gradient of Narragansett Bay to an offshore continental shelf site over a temporal cycle. The biodiversity of N fixers expressing *nifH* decreased along the gradient of the bay. The freshwater head of the bay experiences fluctuating conditions (e.g. oxygen, temperature, salinity and nutrients) (Granger 1994), whereas the offshore sites remain relatively stable throughout the year (Codiga et al. 2010). Exposure to varying environmental factors may be driving diversity in the upper bay sediments as microbes are competing to adapt to a continuously changing ecosystem, while at the more stable offshore sites, the benthos is dominated by the more competitive microbes. Again, the dominant likely active diazotrophs in upper bay sediments were related to anaerobic sulfur/iron and sulfate reducing bacteria. Warming seawater temperatures, low oxygen events and high organic carbon content in the sediment appear to stimulate growth and activity of these microbial groups as the highest abundance and *nifH* expression was detected in the upper bay sediments. Conversely, at the offshore sites, *nifH* expression was dominated by a unicellular cyanobacterium, *Candidatus Atelocyanobacterium thalassa* (UCYN-A) (Zehr et al. 2001).

The genome of UCYN-A revealed that this cyanobacterium cannot fix carbon, but does have the capability to fix N (Tripp et al. 2010). In fact, UCYN-A lacks critical metabolic pathways including photosystem II, RuBisCo, and the tricarboxylic acid cycle, yet contains the complete suite of nitrogenase genes (Tripp et al. 2010). UCYN-A was recently recognized as being a symbiont of carbon fixing eukaryotic

hosts and was reported to have a loose symbiotic association with a unicellular prymnesiophyte (Thompson et al. 2012). In return for fixed carbon, UCYN-A provides the host with fixed N (Thompson et al. 2012). Recovering *nifH* mRNA transcripts related to UCYN-A in temperate coastal sediments was surprising because to date this microbe has only been documented to be an important N fixer in tropical and subtropical oligotrophic oceans (Zehr et al. 2001; Falcon et al. 2002; Church et al. 2005; Zehr et al. 2007; Moisander et al. 2010). We detected the highest UCYN-A abundance and *nifH* expression in sediments when bottom water temperatures were 5-8 °C suggesting this microbe has a broader thermal tolerance and may be more widespread than previously believed. This microbe may also have a wider range of host associations than currently understood. Presumably, when the prymnesiophyte partner sinks to the benthos, loosely attached UCYN-A gets exported to the sediments as well. UCYN-A's genome suggests that the microbe is physiologically capable of thriving in the sediments after the lifespan of its host. In fact, we detected mRNA transcripts from UCYN-A as deep as 6 cm, indicating UCYN-A remains active in sediments long after burial of any eukaryotic hosts it may be associated with. These findings from Chapter 2 have implications for benthic-pelagic coupling and N cycling in continental shelf sediments.

The results of Chapter 1, demonstrating that bacteria that live anaerobically are the major groups of active N fixers in estuarine sediments, led us to further investigate the influence of hypoxia on microbial activities in estuarine sediments. Hypoxia is increasingly becoming a common occurrence in Narragansett Bay (Saarman 2008), yet little is known about how oxygen depletion in the water column impacts N cycling in

the sediments. Based on analysis of porewater nutrients, it appears that an increase in oxygen in surface sediments stimulates nitrification at some sites, while hypoxic conditions repress nitrification and perhaps stimulate nitrogen fixation. The greatest bulk *nifH* expression was detected at sites with the highest organic carbon content in the sediment and most heavily impacted by hypoxia. Similarly to Chapter 1, the dominant diazotrophs expressing *nifH* were related to iron/sulfur and sulfate reducers. The abundance and *nifH* expression of the dominant N fixing groups did not vary in response to fluctuating oxygen concentrations; however, at the times we sampled, the dissolved oxygen was near the EPA's hypoxia threshold of 2.3 mg/L (USEPA 2000) during sediment collection. Perhaps conditions closer to complete anoxia are needed to stimulate nitrogenase activity in these diazotrophic communities.

In conclusion, this thesis has provided evidence that anaerobes related to iron/sulfur respiring bacteria and sulfate reducers can be a source of fixed N in benthic coastal sediments. We propose that increased water temperatures, hypoxia and sediment organic carbon concentrations are key drivers promoting their activity. Although these microbes are highly abundant in marine sediments, their contribution to the N budget was considered negligible. Therefore, little is known regarding controls on N fixation activity in these anaerobic bacteria. To better predict how an ecosystem, like Narragansett Bay, may respond to future environmental changes, regulating factors including sensitivity to combined N and oxygen tolerance need to be evaluated in these microbes, both in the laboratory and in the environment. Determining how microbial community composition and activity respond to different

environmental factors and in turn influence N cycling can be used to help improve ecosystem-based management.

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APPENDICES

Molecular Biology Terms for Oceanographers

cDNA – complementary DNA: single-stranded DNA synthesized from messenger RNA (mRNA) template in a reaction catalyzed by the enzyme reverse transcriptase

PCR – polymerase chain reaction: a biochemical technique that allows a specific DNA sequence to be exponentially amplified by separating the DNA into two strands and incubating with oligonucleotide primers and DNA polymerase under cycles of varying temperatures

RT-PCR – reverse transcriptase polymerase chain reaction: a variant of the polymerase chain reaction in which messenger RNA (mRNA) expression is detected by amplifying complementary DNA (cDNA)

qPCR – quantitative polymerase chain reaction (also called real-time polymerase chain reaction): a method that allows a targeted DNA sequence to be amplified and simultaneously quantified based on incorporation of a fluorescent reporter dye; fluorescent signal increases proportionally to the amount of amplified product synthesized

qRT-PCR – quantitative polymerase chain reaction: a variant of the quantitative polymerase chain reaction which allows mRNA expression to be measured by amplifying and simultaneously quantifying targeted RNA