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Evidence and a conceptual model for the co-occurrence of nitrogen fixation and denitrification in heterotrophic marine sediments

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ABSTRACT: Marine waters are often nitrogen (N) limited. Denitrification, the microbial conversion of nitrate to dinitrogen (N_2) gas, is responsible for significant N removal from the coastal ocean. In contrast, nitrogen fixation, the microbial transformation of N_2 to ammonium, is typically regarded as an inconsequential N source. The imbalance between these 2 processes is responsible, at least in part, for N limitation in the coastal ocean. Organic matter quality and quantity has been shown to determine rates of these critical N cycling processes. We hypothesized that the timing of organic matter deposition to the benthos might also be important in determining which process dominates. We tested this hypothesis using a coupled biogeochemical-molecular approach. We report directly measured net sediment denitrification rates and corresponding expression of *nirS*, a gene in the denitrification pathway, with the simultaneous expression of *nifH*, a gene associated with nitrogen fixation. The timing of organic matter deposition determined the magnitude of the net sediment N_2 fluxes. Highest rates of denitrification occurred soon after deposition, and the lowest rates occurred over 200 d after the last deposition event concomitant with increased *nifH* expression. Phylogenetic evidence suggests that sulfur and sulfate reducers are responsible for the nitrogen fixation. Globally, warming water temperatures, changes in light, and reduced nutrient loads through management intervention have been linked to decreases and/or altered phenology of water column productivity. Based on a conceptual model developed here, we suggest that in these systems, heterotrophic sediment nitrogen fixation may become an important component of the nitrogen budget.

KEY WORDS: Denitrification · Heterotrophic nitrogen fixation · Marine sediments · *nifH* expression · Direct sediment N_2 fluxes

INTRODUCTION

Canonical denitrification is the microbially mediated process that converts nitrate to dinitrogen (N_2) gas, thus removing it from the nitrogen (N) pool that is available to most photosynthetic organisms. The opposing process, nitrogen fixation, converts N_2 gas to biologically available ammonium. The working paradigm is that the imbalance between denitrification and nitrogen fixation helps promote N limitation in marine systems (Howarth et al. 1988a,b). Various environmental factors, including sediment oxygen concentrations and substrate availability, regulate these 2 processes and often in the same direction (Cornwell et al. 1999). Of particular importance is the link between sediment N_2 fluxes and organic matter, which has been shown to be positively correlated with both denitrification (Seitzinger & Giblin 1996) and nitrogen fixation (Capone & Budin 1982). However, it is not simply the amount of organic matter

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that regulates these processes but a complicated interplay between organic matter quantity and quality (Fulweiler et al. 2007).

Due to energetic constraints and the typically abundant concentrations of nitrogen in marine systems, it was long thought that heterotrophic nitrogen fixation was likely an unimportant process (Howarth et al. 1988a,b). However, over the last decade this paradigm has begun to shift with observations of direct N_2 uptake in a variety of marine systems. For example, significant rates of directly measured nitrogen fixation have been recorded in temperate and subtropical estuaries (e.g. Fulweiler et al. 2007, Gardner et al. 2006, Gardner & McCarthy 2009). In addition, it has been known for some time that nitrogen fixation is a significant source of nitrogen in some salt marsh (e.g. Jones 1974, Teal et al. 1979, Jones 1982) and seagrass (e.g. McGlathery et al. 1998, Cole & McGlathery 2012) systems.

We previously reported a dramatic seasonal switch in N cycling with high rates of net heterotrophic sediment nitrogen fixation in July and August for a temperate New England estuary (41.3° N, 71.1° W; Narragansett Bay, RI, USA; Fulweiler et al. 2007). Using field observations and a large mesocosm experiment, we linked these rates of nitrogen fixation to a climate induced oligotrophication of the bay brought on by long-term warming (Nixon et al. 2004, Oviatt 2004) and the loss of the winter-spring diatom bloom (Nixon et al. 2009). But as observed for a variety of systems (Farnsworth et al. 1995, Walther et al. 2002, Kahru et al. 2011), climate change in this estuary has also altered the phenology or seasonal sequencing of phytoplankton blooms, leading to more phytoplankton production in the summer and fall and decreased organic deposition on the bottom (Nixon et al. 2009). Thus, we hypothesized that shifts in phytoplankton bloom phenology and the subsequent changes to organic matter deposition to the sediments might also be important in determining whether nitrogen fixation or denitrification dominates.

Using a coupled biogeochemical-molecular approach we tested this hypothesis by altering the timing of phytoplankton deposition in 9 large (4 m^2) mesocosm tanks designed to replicate the bottom of the estuary. Our purpose here is to report on the co-occurrence of denitrification and nitrogen fixation in heterotrophic sediments, and on the dynamic response of these sediments in relation to time since organic matter deposition. In addition, we present a conceptual model hypothesizing how and why these 2 opposing processes may take place simultaneously.

MATERIALS AND METHODS

Mesocosm experimental details

Sediments were collected from a site located in mid-Narragansett Bay (41° 35.3′ N, 71° 22.3′ W) on 29 November 2006 where benthic metabolism and net N_2 fluxes have been measured at various time intervals since the late 1970s (e.g. Nixon et al. 1976, Seitzinger et al. 1980, Fulweiler et al. 2007, Fulweiler & Nixon 2009). This site is completely heterotrophic, approximately 7 m deep (mean low water; mlw), with a 1.1 m tidal range, and receives less than 1–2% of surface light. Sediments were collected using a large box corer (0.25 m^2) to a depth of approximately 30 cm and placed into trays; approximately 4 box cores were needed to fill each tray, and we took great care to preserve their vertical structure. The sediments were transported to the Graduate School of Oceanography at the University of Rhode Island, and placed into one of 9 large (4 m^2) mesocosm tanks maintained in the dark with 65 cm of overlying water. The overlying water was slowly mixed and exchanged 10% each day with water from the lower West Passage of the bay. The mesocosms mimicked the *in situ* mid-bay site temperatures (~1 to 21°C annual range) and the environmental chambers for N_2 gas flux measurements were maintained at the field temperature at the time of collection. Annual salinity range was ~27 to 32. Surface water column nutrient concentrations for mid Narragansett Bay averaged 4.3 µmol l^{-1} over the 2007 annual cycle (www.gso.uri.edu/phytoplankton/). Nitrate was highest during the winter (12.3 µmol l^{-1}) and was undetectable during the spring, summer, and fall. In contrast, ammonium and nitrite were highest in the fall and winter (8.3 and 13.8 µmol l^{-1}, respectively) and undetectable in the spring and summer (www.gso.uri.edu/phytoplankton/).

This mesocosm experiment was designed to quantify how the temporal addition of organic matter to the benthos affected sediment N_2 fluxes. The amount of organic matter delivered to the benthos from water column phytoplankton production varies both spatially and temporally. Percentages of organic matter reaching the sediment range from 20–30% in the Gulf of Mexico to approximately 25% off the coast of New England and off the Amazon (Seitzinger & Giblin 1996). In general, a larger proportion of winter-spring blooms is deposited to the bottom than of summer blooms (Keller et al. 2001). The amount of organic matter deposition can also depend on water column depth. Based on our previous experiments

(Fulweiler et al. 2007) and literature values (e.g. Seitzinger & Giblin 1996) we assumed a deposition of 25% (or 50 g C m^{-2}) of the winter-spring bloom as observed in Narragansett Bay during the 1970s. The 9 tanks were randomly assigned to a timing treatment and were subsequently amended with a total of 50 g C m^{-2} in equal daily aliquots over a 2 wk period in the beginning of either January, April, or June 2007. Carbon was added in the form of commercially available spray-dried phytoplankton with a molar C:N of 10.5. We did not include for comparison mesocosms with no organic matter additions as the infrastructure was not available for this experiment and our goal was to test the impact of timing rather than starvation versus deposition (which we tested previously; Fulweiler et al. 2007, 2008).

Sediment N_2 gas fluxes

In early July (17°C) and again in September (22°C) 2007, sediment cores (10 cm inner diameter, 30.5 cm long) were collected from each of the 9 mesocosms with a pull corer (n = 9, 3 cores from each treatment). The sediment cores were rapidly transported to a water bath in a dark environmental chamber at the Graduate School of Oceanography at the University of Rhode Island. The cores were left uncapped with air bubbling the overlying water for 8 to 12 h. The water overlying each core was carefully replaced with filtered (1 µm) lower Narragansett Bay (salinity ~32) water before each incubation. The cores were then sealed with a gas-tight lid (with no air headspace; mean water headspace was 1.2 l) and replicate water samples for N_2/Ar (Kana et al. 1994) analysis were collected at 5 points over the course of an incubation and preserved with $HgCl_2$. Incubations lasted between 7 and 8 h allowing for at least a 2 mg O_2 l^{-1} drop in the overlying water, but the sediment cores were not allowed to go hypoxic. Each water sample was analyzed for dissolved gas concentration using a quadrupole membrane inlet mass spectrometer (MIMS, Bay Instruments) (Kana et al. 1994). The N_2/Ar technique is actually a measure of net N_2 production or consumption (gross denitrification minus gross nitrogen fixation). Over the course of the incubation, the change in N_2 concentration was determined from the change in the measured N_2/Ar multiplied by the Ar concentration at air saturation. N_2 flux across the sediment-water interface was determined for each of the cores using a 5-point linear regression, and rates were then prorated for the volume and area of the core.

Sub-sampling and nucleic acid extractions

After the net N_2 flux incubations were completed, we sub-sampled 2 of 3 cores. From each sediment core, 4 replicate sub-cores (~4 to 8 cm length, 0.25 cm diameter) were collected: 2 for DNA extractions and 2 for RNA extractions. The sub-cores were flash frozen in liquid N_2, and sub-sectioned into 0.5 cm segments from the sediment water interface to 3 cm in depth. Total DNA was extracted using the Powersoil DNA Isolation Kit (MO Bio). For RNA isolation, replicate sub-core samples from each depth were pooled (approximately 0.5 g of wet sediment) to average biological heterogeneity across the larger core and to increase biomass and RNA yield. Total RNA was extracted using the Powersoil RNA Isolation Kit (MO Bio); 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 10× TURBO DNase buffer and 1 µl of TURBO DNase from the TURBO DNase-*free* Kit (Ambion) 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) according to the manufacturer's instructions. cDNA copies of RNA were generated with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). For all samples, 8 µl of DNase-treated RNA at a concentration of 4 ng $µl^{-1}$ 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 (Zehr & McReynolds 1989) (5′ATR TTR TTN GCN GCR TA3′) and nirS6R (Braker et al. 1998) (5′CGT TGA ACT TRC CGG T3′). 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 DNA or cDNA was isolated using nested PCR with degenerate outer primers nifH4 (5′TTY TAY GGN AAR GGN GG3′) and

nifH3 (5′ATR TTR TTN GCN GCR TA3′), and inner primers nifH1 (5′TGY GAY CCN AAR GCN GA3′) and nifH2 (5′AND GCC ATC ATY TCN CC3′) (Zehr & McReynolds 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 & McReynolds 1989, Kirshtein et al. 1991). *nirS* was amplified using the primer pair nirS1F (5′CCT AYT GGC CGC CRC ART3′) and nirS6R (5′CGT TGA ACT TRC CGG T3′) (Braker et al. 1998, 2000). 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, 2000).

To obtain relative quantities of *nifH* amplification, we determined the intensity of *nifH* amplification bands with the manual band quantification function in GeneTools (SynGene). We normalized the intensity of every detectable band on the gels to that of a 100 ng band on HyperLadder II (Bioline Tauton) for a relative quantification value. Based on a distribution curve of the relative values, samples were then assigned low (<200 ng), medium (200 to 400 ng) or high (>400 ng) expression levels. For every expressed *nifH* and *nirS* amplicon we collected 3 or 4 sequences.

Expressed *nifH* sequences from Narragansett Bay, accession numbers JN645307–JN645499, were combined with the *nifH* sequences (4505) selected as unique sequence representatives by J. P. Zehr (pers. comm.) from the NifH database encompassing all currently available sequences in GenBank (Benson et al. 2009) as of September 2009. 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 JN645500–JN645545, was constructed using the PhyML algorithm in Geneious with 1000 bootstrap replicates.

RESULTS

Sediment N_2 fluxes over the course of this experiment ranged from 27 to 65 µmol N_2-N m^{-2} h^{-1}. There was no statistical difference ($t(13) = 2.16$, $p = 0.33$) between the mean net N_2 fluxes measured at 17°C and the mean N_2 fluxes measured at 22°C. However, the relationship between days since last organic matter deposition and the magnitude of the net sediment N_2 fluxes was strongly correlated ($R^2 = 0.87$, $p = 0.0002$). The highest rates of denitrification occurred soon after deposition and the lowest rates occurred over 200 d after the last organic matter deposition (Fig. 1).

In two of the triplicate cores measured for direct N_2 fluxes, we identified and followed the presence and expression of functional genes associated with nitrogen fixation (*nifH*) and denitrification (*nirS*). Both *nifH* and *nirS* genes were detected in all sediment samples analyzed from the sediment-water interface

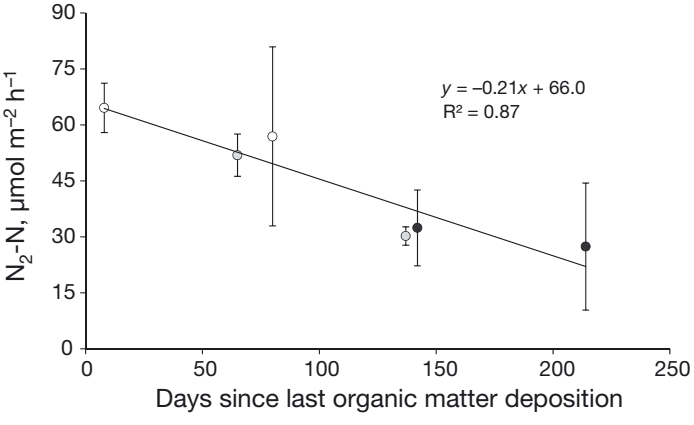

Fig. 1. Net N_2-N fluxes across the sediment-water interface as a function of number of days since last organic matter deposition. Sediments were collected from the mid-bay historic site (41° 35.3′ N, 71° 22.3′ W). Triplicate tanks received 50 g C m^{-2} at 3 different times in 2007: January (open circles), April (shaded circles), and June (closed circles). Net N_2-N fluxes were measured in July and September 2007 (mean ± SE, n = 3). Both the slope ($F = 27.1159$, $p = 0.0065$) and intercept ($p = 0.0002$) of the regression are significant

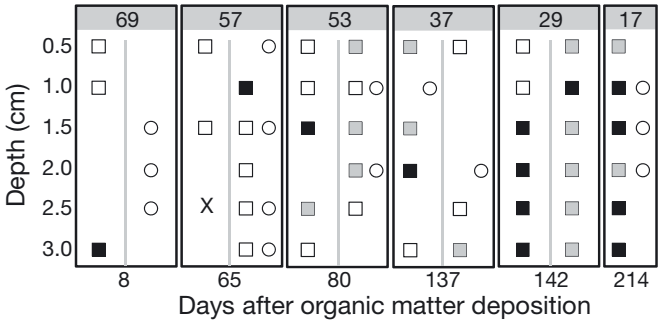

Fig. 2. Normalized *nifH* (squares) and *nirS* (circles) expression data from mesocosm sediment samples analyzed by gel electrophoresis. Duplicate cores are shown for each treatment and are aligned in order of days since organic matter deposition. Mean net N_2 flux (N_2-N μmol m^{-2} h^{-1}) is shown in the shaded bar. For the January treatment (Day 214), RNA yields were below detection, thus only one profile is shown. Expression levels of *nifH* are designated as low, medium, or high (open, shaded, or closed squares, respectively) from normalized gel image analysis. *nirS* amplification was low in most cores, and samples from which we obtained clones are indicated by open circles. Blank spaces: no gene expression detected; X: no sample available

to a depth of 3.0 cm (data not shown). While *nifH* mRNA transcripts were detected in every treatment, the spatial distribution of *nifH* expression changed with treatment. Specifically, in sediments recently enriched with organic matter, high *nifH* expression was only found at the 3.0 cm depth in one of the replicate cores. In contrast, sediments starved of organic matter had expression throughout the sediment column from the surface to 3.0 cm depth. In fact, the majority of *nifH* expression was seen in the treatment tanks 142 to 214 d after organic matter addition (Fig. 2). This *nifH* expression correlates with our direct N_2 flux measurements which also show the lowest net N_2 fluxes during this time.

We observed no temporal or vertical trend in *nirS* expression signals (Fig. 2). We did detect *nirS* expression in some of the same samples and, importantly, at the same depths in which we also observed *nifH* expression (Fig. 2).

Expressed *nifH* sequences from Narragansett Bay sediments are restricted to 2 phylogenetic groups within *nifH* Clusters I and III, as previously defined (Chien & Zinder 1996), and group with known sulfur and sulfate reducing bacteria (Fig. 3). The majority of

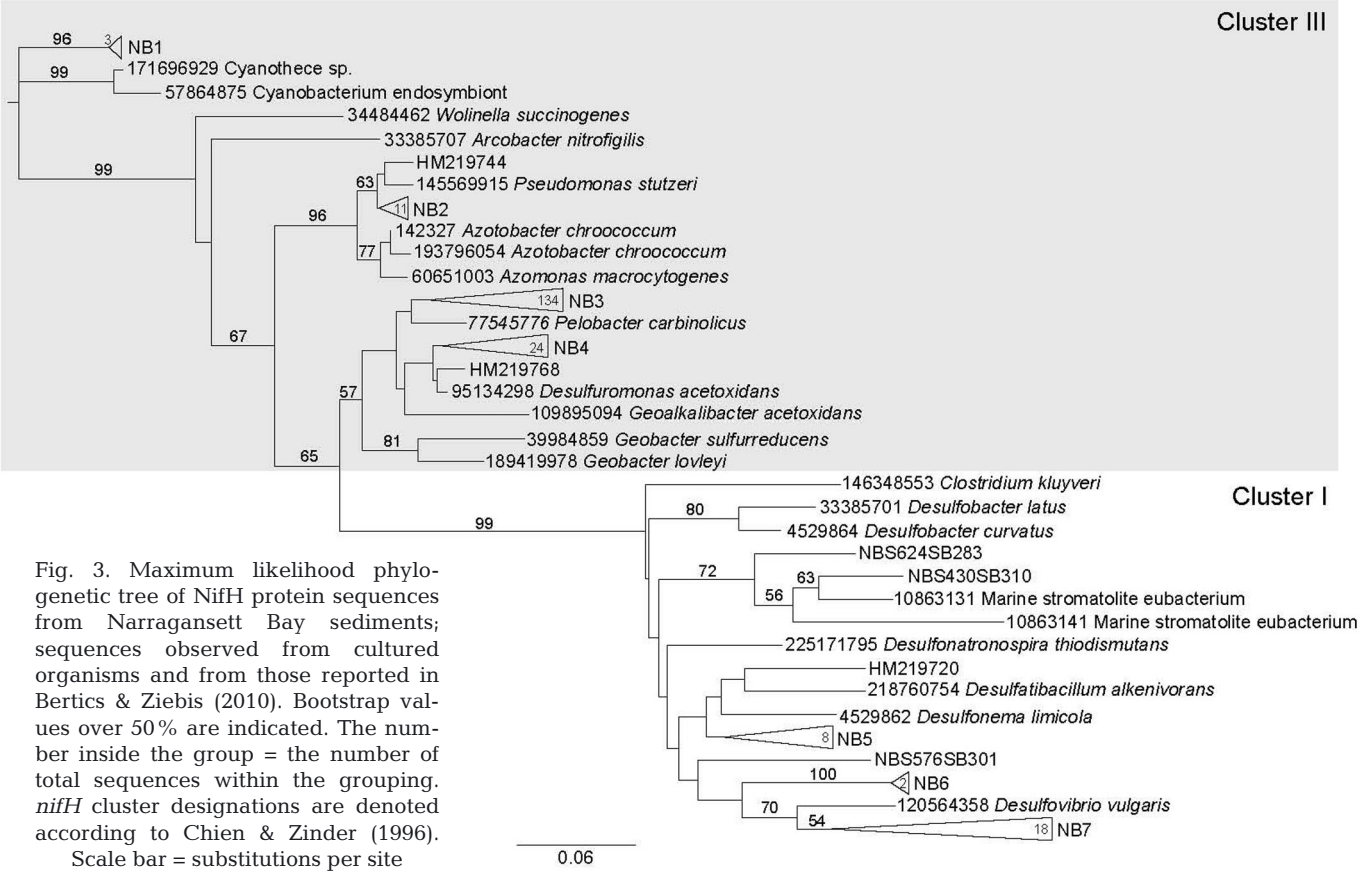

Fig. 3. Maximum likelihood phylogenetic tree of NifH protein sequences from Narragansett Bay sediments; sequences observed from cultured organisms and from those reported in Bertics & Ziebis (2010). Bootstrap values over 50% are indicated. The number inside the group = the number of total sequences within the grouping. *nifH* cluster designations are denoted according to Chien & Zinder (1996). Scale bar = substitutions per site

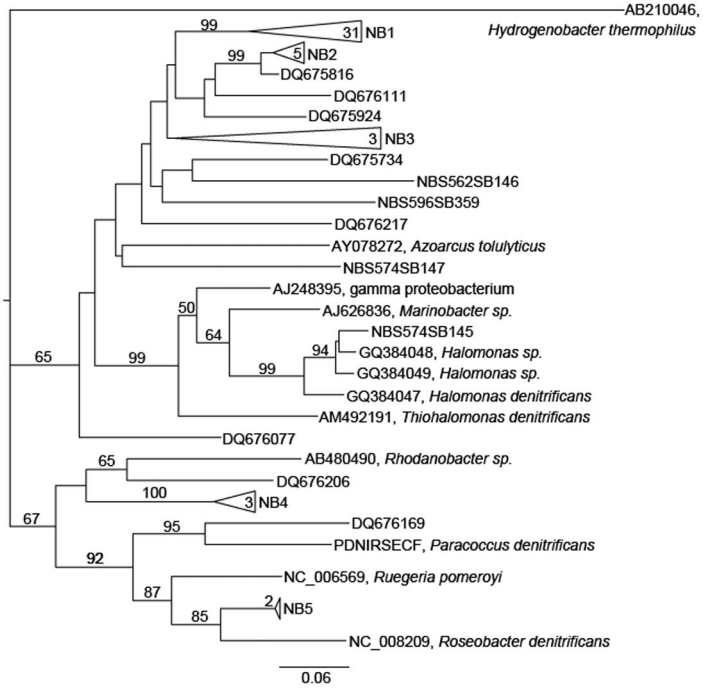

Fig. 4. Maximum likelihood phylogenetic tree of NirS protein sequences from Narragansett Bay and closely related cultured species with bootstrap values. The number inside (or directly next to) the group = number of total sequences within the grouping. Scale bar = substitutions per site

expressed *nifH* sequences (134) are within group NB3 which is most closely related to *Pelobacter carbinolicus,* a sulfur reducer (Lovley et al. 1995) (Fig. 3). The second largest expressed sequence group, NB7, within Cluster I contains 18 expressed *nifH* sequences and the most closely related bacterium is the sulfate reducer *Desulfovibrio vulgaris* (Fig. 3). Expressed *nirS* sequences were found in 5 main phylogenetic groups, none of which contain any cultivated species (Fig. 4).

DISCUSSION

N_2 fluxes

The experiment we report here was motivated by our observations of a switch in sediment N_2 fluxes from net denitrification to net nitrogen fixation in Narragansett Bay in the summer of 2006 (Fulweiler et al. 2007). In our previous work, we linked the switch from net denitrification to net nitrogen fixation to a decrease in water column productivity over the last 30 yr and the subsequent decline in the amount of organic matter loading to the benthos. Based on these field observations, we ran a mesocosm experiment which showed that the amount of organic matter deposited to the benthos determined the net sediment N_2 flux (Fulweiler et al. 2007). We found that sediments that received organic matter had a positive sediment N_2 flux or net denitrification rate of 245 µmol N_2-N m^{-2} h^{-1}. In contrast, sediments that did not receive any organic matter exhibited net nitrogen fixation rates of −289 µmol N_2-N m^{-2} h^{-1}. In addition, when sediment microcosms exhibiting net nitrogen fixation were given organic matter they switched to net denitrification (Fulweiler et al. 2007, Fulweiler et al. 2008). However, Narragansett Bay and other coastal systems are also experiencing changes in the phenology or seasonal sequencing of phytoplankton blooms. Changes to the timing of phytoplankton blooms may alter both the quantity and quality of organic matter loading to the benthos (Nixon et al. 2009). The purpose of this second large mesocosm experiment was to determine how sediment N_2 fluxes changed over time since the last organic matter deposition. We hypothesized that sediment N_2 production would decrease as number of days since the last organic matter deposition event increased. Further, we anticipated that nitrogen fixation would become a more important process over this time and that we would observe net nitrogen fixation rates and/or an increase in the expression of *nifH*.

As we hypothesized, there was a significant decrease in sediment N_2 fluxes as a function of time since organic matter deposition. Because we measured N_2 fluxes at 2 separate temperatures (17 and 22°C) it is tempting to attribute the observed relationship to temperature. However, there was no significant difference between the mean net N_2 flux of the 9 cores across all treatments at 17°C vs. 22°C. Further, extensive measurements at the same site where these sediments were collected in Narragansett Bay have shown that sediment N_2 fluxes are not correlated with temperature (Seitzinger et al. 1984, Fulweiler & Nixon 2012). In fact, the relationship between temperature and both denitrification and nitrogen fixation appears to be complicated and variable, with no clear pattern across systems. In some cases, rates of denitrification increase with temperature (e.g. Boston Harbor and Massachusetts Bay; Nowicki et al. 1997) while in others they decrease (e.g. Chesapeake Bay; Kemp et al. 1990). Similarly, some have reported an increase in nitrogen fixation with temperature (e.g. Jones 1982) while others have

found the opposite (e.g. Fernandez et al. 2011). While we cannot discount the role of temperature completely, it appears that it is not the main driver of the N_2 flux signal we observed.

We did find a significant relationship between number of days since organic matter deposition and net sediment N_2 fluxes. These experimental results confirm our previous work that found that denitrification responds rapidly and positively to organic matter deposition (Fulweiler et al. 2007, 2008). If we extrapolate the relationship between time since organic matter deposition and sediment N_2 fluxes beyond the data, we can estimate a point when the sediments would switch from a net sink of N (denitrification dominated) to a net source (nitrogen fixation dominated). According to this relationship, the switch should occur if no organic matter were to reach the benthos for approximately 320 d. It seems unlikely that no deposition would reach the sediments for almost a year. Thus, these results suggest that other key factors are important in phytoplankton phenology and subsequent bloom deposition. Changes in the timing of phytoplankton blooms can alter organic matter deposition to the benthos in at least 2 ways. First, organic matter deposition to the benthos is considerably reduced from blooms during warmer water temperatures (e.g. Rudnick & Oviatt 1986, Keller et al. 1999). Second, the C:N ratio of phytoplankton bloom deposition is known to change with temperature. Phytoplankton blooms during cold months deposit organic matter with a low C:N while organic matter deposited during warm months has a higher C:N and thus lower nutritional value (Smetacek 1984, Keller & Riebesell 1989). In Narragansett Bay, both the total amount and timing of production has shifted such that the quality and quantity of organic matter deposited to the benthos has changed. It is this combination that we hypothesize is driving the previously observed nitrogen fixation rates. Perhaps we did not observe net nitrogen fixation in this most recent experiment because we gave all treatments the same amount of organic matter and only focused on days since deposition, not changes in the quality of the organic matter. The quality may be particularly important in terms of nitrogen fixation because we know that nitrogen fixers can use a wide variety of carbon sources (Hansen 1993, Rabus et al. 1993, Annachhatre & Suktrakoolvait 2001, Eckford et al. 2002). Because we only measured positive net N_2 fluxes we cannot say how nitrogen fixation responded to these deposition events. For that, we must turn to our molecular results.

Microbial genetics

It was no surprise that we observed the presence of *nifH* in these sediments as the genetic potential to fix nitrogen is widespread in prokaryotic taxa (Zehr et al. 2003) and in estuarine water columns (Jenkins et al. 2004) and sediments (Burns et al. 2002, Moisander et al. 2007). The expressed *nifH* sequences observed in this study were only found within 2 phylogenetic groups (*nifH* Clusters I and III) that contain sulfur and sulfate reducing bacteria. Included in this group are *Desulfovibrio* spp. *and Desulfobacter* spp., which have been shown to fix nitrogen in culture (Sisler & ZoBell 1951, Widdel 1987). Research in seagrass beds (Welsh et al. 1996) and salt marshes (Gandy & Yoch 1988) has also shown sulfate reducers to be primarily responsible for observed rates of nitrogen fixation. Thus, our phylogenetic data indicating sulfate reducers as the dominant nitrogen fixers are consistent with other findings. In addition, the expressed *nifH* sequences we observed phylogenetically group with DNA sequences recently reported from coastal California sediments: Bertics & Ziebis (2010) measured nitrogen fixation indirectly using the acetylene reduction assay and found that nitrogen fixation significantly decreased when sulfate reduction was inhibited. They proposed that the nitrogen fixation rate data and the phylogenetic placement of the sequences provided evidence that sulfate reducers may largely be responsible for their observed nitrogen fixation. While our phylogenetic data are consistent with their findings, we also observed concurrent *nirS* expression. The overlapping expression of these genes in both space and time indicate that both nitrogen fixation and denitrification co-occur within relatively small spatial scales. However, our directly measured sediment N_2 fluxes highlight that denitrification was still the dominant process during this experiment.

The expression of *nirS* was measureable in fewer samples than *nifH* and more difficult to detect. This may be due to the short half-life of *nirS* mRNA (Hartig & Zumft 1999) or low transcript copy levels in the environment (Nogales et al. 2002, Bulow et al. 2008). The majority of expressed *nirS* sequences in Narragansett Bay were found in groups NB1, NB2, and NB3. These are most closely related to *Azoarcus tolulyticus*, a bacterium notable for its ability to both denitrify and fix nitrogen (Zhou et al. 1995). Group NB1 also contains *nirS* sequence CB3-S-16 (DQ675925), one of only 3 archetypes in which mRNA expression was detected in Chesapeake Bay (Bulow et al. 2008), indicating that these microbes may be an important

group of denitrifiers in estuarine ecosystems. Other groups of expressed *nirS* sequences (NB4 and NB5) are related to the metabolically diverse bacteria *Paracoccus denitrificans*, *Roseobacter denitrificans*, and *Ruegeria pomeroyi*. *P. dentrificans* can grow aerobically or as a chemolithoautotroph, using hydrogen and sulfur compounds as possible energy sources. *R. denitrificans* has genes for inorganic sulfur oxidation, dimethyl sulfoxide reduction, and dimethyl sulfoniopropionate methyltransferase, suggesting this, and related microbes from our samples, might have the potential to metabolize sulfur compounds (Swingley et al. 2007).

A conceptual model

The relationship of expressed genes from nitrogen fixing and denitrifying bacteria in Narragansett Bay sediments with those from organisms known to respire sulfur compounds suggest the nitrogen and sulfur (S) cycles may be mediated by the same organisms. Further, because the timing and quantity of organic matter deposition appears, at least in part, to control the net sediment N_2 flux, the carbon cycle is intimately linked to both N and S cycling. From the results of this study and numerous others we present the following conceptual model which puts forth our hypothesis explaining the co-occurrence of denitrification and nitrogen fixation in heterotrophic marine sediments (Fig. 5).

Canonical denitrification follows 2 possible pathways in heterotrophic systems: direct or coupled nitrification-denitrification. In direct denitrification, water column nitrate diffuses into anaerobic sediments and is reduced to N_2 gas. In coupled nitrification-denitrification, nitrifying bacteria convert ammonium to nitrate in the aerobic surface sediment layer. This nitrate is then available via diffusion for reduction to N_2 by denitrifying bacteria in deeper anoxic sediments. In most coastal ocean sediments (including these), coupled nitrification-denitrification

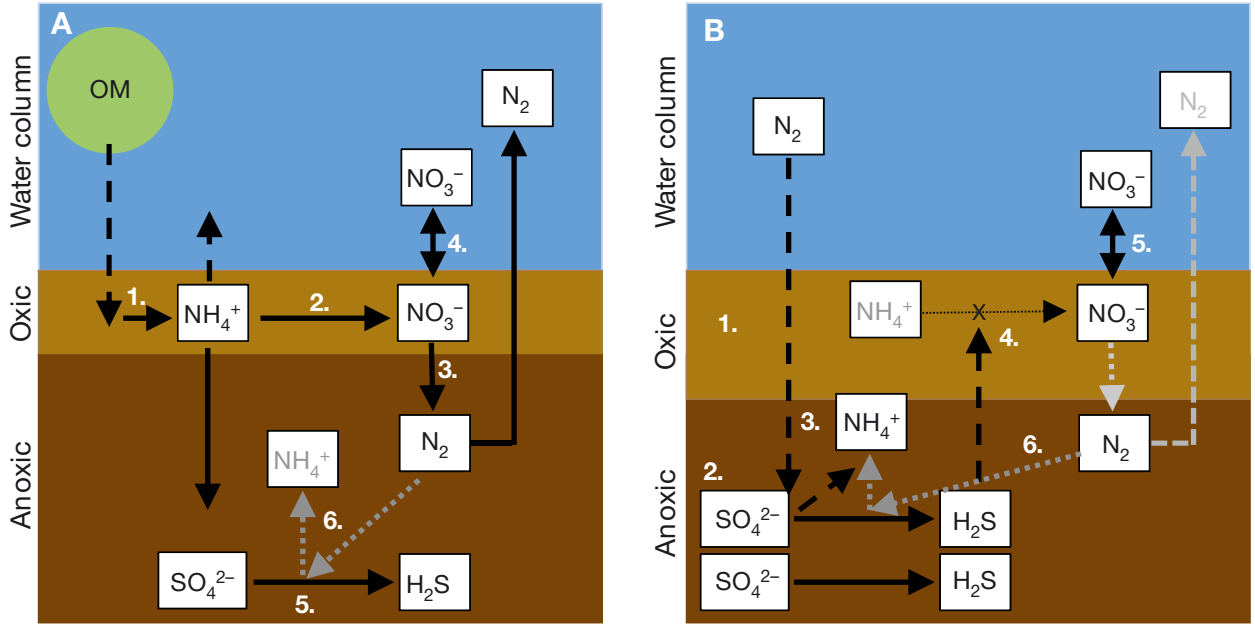

Fig. 5. Conceptual model explaining the co-occurrence of nitrogen fixation and denitrification in heterotrophic marine sediments. (A) N cycle under typical organic matter loading: (1) Aerobic bacterial decomposition degrades organic matter releasing ammonium and depleting oxygen, (2) ammonium is nitrified to nitrate, (3) nitrate is then denitrified to N_2 gas via coupled nitrification-denitrification, and released into the overlying water, or (4) via direct denitrification, (5) sulfate reduction occurs in deeper anoxic sediment and may be accompanied by (6) low rates of N-fixation. (B) N cycle under organic matter starvation: (1) With little organic matter to degrade, ammonium concentrations are low, aerobic decomposition rates are repressed, and the oxic sediment layer thickness increases, (2) because reduced ammonium is low and the available carbon is recalcitrant, sulfate reducing bacteria expand in the sediment column and (3) begin to fix N at higher rates, (4) increased sulfate reduction produces toxic hydrogen sulfide which inhibits nitrification and shuts down the pathway for coupled nitrification-denitrification, (5) direct denitrification continues to occur as long as nitrate is available, (6) denitrifiers capable of metabolizing toxic sulfur compounds may begin to grow, perhaps in a relationship with the sulfate reducing bacteria. Black lines show dominance of process (solid: high rates; dashed: medium rates; dotted: low or repressed rates); grey dotted lines show proposed processes

is the dominant denitrification pathway (Seitzinger et al. 1984, Nowicki 1994). This is particularly important because rapid and substantial inhibition of nitrification at hydrogen sulfide concentrations commonly occurs in estuarine sediments (Joye & Hollibaugh 1995) and direct denitrification is inhibited at even lower hydrogen sulfide concentrations (Joye 2002). Joye & Hollibaugh (1995) proposed that this inhibition was the explanation for the routine observation of low sediment nitrification and denitrification accompanied by high rates of ammonium regeneration in summer N cycling studies (Kemp et al. 1990).

It is well established that estuarine sediments exhibit high rates of sulfate reduction because they are rich in organic matter, exposed to high concentrations of sulfate in seawater, and are anaerobic beneath the top few centimeters (Canfield 1989). Despite recent decreases in water column productivity, changes in bloom phenology, and the subsequent decline in the deposition of fresh organic matter to the benthos, Narragansett Bay sediments are still rich in organic matter. But the quality of organic matter available for microbial decomposition in these sediments has changed from a mean C:N ratio of 7.6 in the 1970s to a mean of 10.5 (range 9.5 to 13.9) (Nixon et al. 2009). Thus, a microbial community able to use this recalcitrant carbon could potentially flourish. Sulfate reducers are notoriously flexible in their ability to use a wide variety of carbon sources (Hansen 1993, Rabus et al. 1993, Annachhatre & Suktrakoolvait 2001).

Building on these studies, we propose that while both nitrogen fixation and denitrification co-occur in marine sediments, they are not necessarily coupled. Instead, nitrogen fixation mediated by sulfate reducers represses coupled nitrification-denitrification because of the toxic by-product of sulfate reduction, hydrogen sulfide (H_2S). Thus, we hypothesize that sulfate reduction provides energy for the fixation of nitrogen while simultaneously producing a toxic by-product that inhibits competition from other microbes for reduced nitrogen. While nitrogen fixation is an energetically intensive process, much of the energy costs go to protecting nitrogenase from oxygen (Großkopf et al. 2012) and thus in anaerobic sediments the energetic costs must be significantly reduced.

Sediment microbial activity is heterogenic both spatially and temporally. All of the sediment N cycling processes described above vary depending on the *in situ* environmental conditions. Many factors, including inputs of organic matter, will help determine the thickness of the aerobic sediment zone (Brune et al. 2000). As environmental conditions change, so too will the location of the oxic-anoxic sediment boundary layer. While some nitrifying bacteria are motile, those that are not will be exposed to a variety of conditions within the sediment column. In the marine sediments tested here, the lack of fresh organic matter addition to the benthos over time decreased microbial respiration and, in turn, would have increased the depth of the aerobic boundary layer. In 120 d starvation experiments using cores from mid Narragansett Bay and maintained in the dark at a constant 15°C, Kelly & Nixon (1984) found that oxygen uptake declined at about 0.6 % d^{-1} while NH_4^+ releases declined at twice this rate, thus increasing the C:N of the remaining organic matter. In the mesocosm experiment described here, as the sediment became starved of fresh organic matter inputs, the competition for labile carbon and available N would have increased. Under these conditions, sulfate reducing bacteria would have the advantage as they have the ability to metabolize refractory carbon (using the N they fix) while concurrently repressing competition for available ammonium by inhibiting nitrifying bacteria. The increase in *nifH* expression, from being undetectable in one replicate and observed highly only at 3.0 cm depth in the other on Day 8, to being medium or high throughout the sediment column on Day 214, supports this hypothesis as does our phylogenetic analysis. Furthermore, over this same period we observed a decrease in net N_2 sediment fluxes which indicates that denitrification rates were decreasing or, perhaps more likely given our genetic data, that nitrogen fixation rates were increasing.

Despite the decrease in net denitrification over the course of this experiment, it was still the overall dominant process. The observed N_2 production could have been from coupled nitrification-denitrification taking place within sediment microzones, from direct denitrification, or from anaerobic oxidation of ammonium (anammox). To date, no studies have reported rates of anammox production in Narragansett Bay. However, it is unlikely that anammox is important in this estuary, as anammox is thought to only account for a small portion of the total N_2 production in shallow systems. For example, anammox only accounted for 6 % (±2 %) of the total N_2 production in systems 16 m or shallower (Dalsgaard et al. 2005). Autotrophic denitrification could also have occurred where reduced sulfur compounds were used as the electron donor and nitrate was respired (Batchelor & Lawrence 1978). In fact, this seems likely as *nirS* sequences were closely related to bacteria that have

the genetic machinery to metabolize sulfur compounds. These bacteria may function similarly to *Roseobacter denitrificans*, and use reduced sulfur compounds as electron donors and respire nitrate. It is also possible that there is a syntrophic relationship between these denitrifiers and the sulfate reducing bacteria like that seen between sulfate reducers and methanogens (Hoehler et al. 1994).

Conclusions

Decreases in water column primary production in many coastal systems and throughout the global ocean have recently been reported. In some cases these observations are linked to warming water temperatures and changes in light (Nixon et al. 2009, Boyce et al. 2010); in other cases, these decreases are directly linked to management intervention and a reduction in nutrient loading (Carstensen et al. 2006, Greening & Janicki 2006). These ecosystems will continue to have sediments high in recalcitrant carbon despite the reduction in fresh organic matter. We predict that nitrogen fixation mediated by anaerobic bacteria that respire sulfur compounds will be found in these systems and may be a significant component of the nitrogen budget. The question remains whether nitrogen fixation was a dominant process before anthropogenic changes in climate and nutrient loading altered productivity, or whether these systems are bearing the legacy of human impact in this new and unpredicted way.

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