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ELUCIDATING THE MOLECULAR RESPONSE OF MICROBIAL NITROGEN FIXATION IN ESTUARINE SEDIMENTS TO HYPOXIA

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ELUCIDATING THE MOLECULAR RESPONSE OF
MICROBIAL NITROGEN FIXATION IN ESTUARINE
SEDIMENTS TO HYPOXIA

BY

ANDRAYA LOUISE EHRLICH

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OF
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ABSTRACT

Thermally-induced stratification and anthropogenic inputs of nutrients are major contributors to the onset of seasonal hypoxia in coastal estuaries. Hypoxia, defined by the U.S. Environmental Protection Agency as less than or equal to 2.3 mg L⁻¹ dissolved oxygen (DO), has been recorded globally in over 400 systems. Hypoxia in estuarine systems is predicted to become more prevalent with climate change exacerbating eutrophication-driven hypoxic conditions. This study investigated impacts of hypoxic stress on microbes that drive nitrogen (N) fixation in estuarine sediments from Narragansett Bay, RI. Previous studies have indicated organic matter loading and depleted oxygen conditions may be driving factors of benthic N fixation in Narragansett Bay and demonstrated that diazotrophs may provide an additional and unanticipated source of N input to benthic ecosystems. Previous studies also indicated that anaerobes related to sulfate reducers (e.g. *Desulfovibrio* spp.) and the iron reducers, *Geobacteraceae*, have the potential to fix N in Narragansett Bay, as indicated by the detection of active expression of the gene (*nifH*) encoding a subunit of the protein complex that catalyzes N fixation, the nitrogenase iron protein. Interestingly, severely hypoxic conditions in Narragansett Bay appear to lead to an increase in *nifH* expression from these microbes. To elucidate the relationship between hypoxic stress, organic matter loading, and microbial N fixation, we performed a two-part study, coupling biogeochemical profiling of sediment cores (oxygen and total sulfide concentrations, pH, and carbon and N content analysis) with N fixation rate measurements and microbial analysis targeting *nifH* (quantitative PCR and RT-PCR). First, we collected sediment cores, capturing a natural DO gradient at a severely

hypoxic site near a wastewater treatment plant (Greenwich Cove) and a site at the lower end of the nutrient gradient of Narragansett Bay that occasionally experiences hypoxia (Mid Bay). To understand the impact of spreading and intensifying oxygen depletion and elevated organic matter on the benthic community of diazotrophs, an incubation experiment was performed with Mid Bay sediment samples at the U.S. Environmental Protection Agency, Atlantic Ecology Division laboratory (Narragansett, RI). Mid Bay sediment cores were incubated under four treatments: normoxic, hypoxic, normoxic + organic matter, and hypoxic + organic matter. For the Narragansett Bay field study and incubation experiment, higher diazotrophic activity by sulfate reducers was observed, as measured by both total sulfide concentration and N fixation rates, under depleted DO. N fixation rates and total sulfide concentration were higher under the organic matter addition than the non-organic matter counterparts. Consistent with these data uncultivated sulfate reducers were the dominant group expressing *nifH* at the Greenwich Cove site and in the organic matter treated cores. Hypoxia, but not organic matter addition, stimulated *nifH* expression by uncultivated *Geobacteraceae* in the incubation experiment. N fixation rates and total sulfide concentration were higher under the organic matter addition than the non-organic matter counterparts during the incubation. This study indicates that two dominant groups of diazotrophs in Narragansett Bay sediments, uncultivated *Geobacteraceae* and sulfate reducers, may respond to different environmental drivers that may result from exacerbated hypoxia.

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PREFACE

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INTRODUCTION

Exacerbating Hypoxic Conditions in Estuarine and Coastal Systems

Oxygen depletion, or hypoxia, of estuarine and coastal waters stress over 400 systems globally (Diaz and Rosenberg, 2008), and is increasing in prevalence (Diaz, 2001). Hypoxia may form when high respiration rates, which result from organic matter decomposition, are coupled with water column stratification (Diaz and Rosenberg, 2008). In some systems, such as in the oxygen minimum zones off Chile (Pizarro *et al.*, 2002), upwelled nutrients stimulate large photosynthetic blooms. The detritus from the bloom will sink and decompose, resulting in the formation of oxygen minimum zones below upwelling regions (Rabalais *et al.*, 2010). While hypoxia may form naturally in these nutrient upwelling regions, human activities are exacerbating oxygen depletion in traditionally hypoxic areas and resulting in new areas being impacted worldwide (Diaz and Rosenberg, 2008). For instance, periodic hypoxia has been observed for several decades in Narragansett Bay (Codiga *et al.*, 2009; Melrose *et al.*, 2007; Deacutis *et al.*, 2006; Bergondo *et al.*, 2005), where hypoxia is driven by the nutrient enrichment, or eutrophication (Nixon, 1995). These hypoxic conditions are accelerated by anthropogenic inputs of N from fertilization, wastewater treatment plants, and other activities (NSTC 2000; Bricker *et al.*, 2000), which may increase hypoxic duration and intensity (Diaz and Rosenberg, 2008). Yet, increases in temperature and precipitation related to climate change may further exacerbate hypoxia, resulting in greater intensity, duration, and a higher prevalence (Middelburg and Levin, 2009).

A combination of physical, chemical, and biological factors, though their contribution may vary by site, may cause dissolved oxygen (DO) concentrations to fall below the hypoxic threshold (2.3 mg L^{-1} DO (U.S. EPA, 2000)) and persist (Rabalais *et al.*, 2010). In Narragansett Bay, nutrients from natural and anthropogenic sources are carried into the bay with an influx of river freshwater (Codiga *et al.*, 2009), followed by an increase in primary productivity (Melrose *et al.*, 2007). As the resulting organic matter is microbially consumed through aerobic respiration on the sediment surface, the benthic community is depleted of DO (Melrose *et al.*, 2007). When this microbial respiration occurs at a faster rate than oxygen replenishment, hypoxia is established (Rabalais *et al.*, 2010). Thermohaline stratification, strengthened by intervals of reduced wind and tidal mixing prevents oxygen replenishment in the benthos, thus allowing hypoxia to persist (Melrose *et al.*, 2007). Hypoxia is most prevalent at peak water temperature and stratification (Diaz and Rosenberg, 2008). While many factors, such as chlorophyll, tidal range, wind direction, and density stratification, are important drivers of hypoxia, river flow is the most important variable for Narragansett Bay as a driver for stratification, nutrient input, and flushing rate (Codiga *et al.*, 2009).

Due to the influence of anthropogenic activities, hypoxia appears to progress in many systems from episodic or periodic, persisting from days to weeks, to severe, seasonal hypoxia (Diaz and Rosenberg, 2008). Additional pressure from climate change may intensify the severity, length, and frequency of hypoxia (Middelburg and Levin, 2009). As described by Rabalais *et al.* (2010), stratification, strengthened by

warming surface water temperature, is sufficient on its own to exacerbate hypoxia and contribute to the formation in new areas.

Despite predictions of increased hypoxia in the future, the impacts of hypoxia on ecosystems are already alarming. Hypoxia has direct impacts on the mortality of many aquatic larvae and adult organisms, particularly sessile organisms that cannot escape a low oxygen zone. Low DO levels also indirectly impact ecosystems by reducing growth rates, compressing habitats, and altering predator-prey interactions (Codiga *et al.*, 2009). Hypoxia also shifts biogeochemical cycles, which are microbial driven and regulate the availability of N species and nutrient cycles (Burgin *et al.*, 2011). Under aerobic conditions, coupled nitrification-denitrification occurs in the sediments and removes inputs of ammonium, a nutrient that promotes eutrophication. Nitrification is an oxygen-requiring process that converts ammonium to nitrate, which is often converted to the inert N₂ gas through denitrification in coastal sediments. This coupled process removes reactive forms of N from the estuary, acting as an N sink (Gruber, 2008). However, the lack of oxygen disrupts the link between these processes. As oxygen is depleted in the water column, oxygen penetration into the sediment is reduced, thus extending anoxic (no oxygen) regions and broadening the range of anaerobic microbes, including sulfate reducers (e.g. *Desulfovibrio* spp.) and the iron reducers, *Geobacteraceae*, which use electron acceptors other than oxygen for their growth (Seitzinger *et al.*, 1984). The accumulation of hydrogen sulfide, as a byproduct of anaerobic sulfate and sulfur reduction performed by sulfate and sulfur reducing bacteria in the sediment, exerts more pressure on the aquatic organisms already stressed by hypoxia. Burrowing polychaetes and other infauna may leave their

burrows to escape sulfide poisoning (Jørgensen, 1980). Other N reactions, such as nitrification and anaerobic ammonium oxidation, are inhibited by sulfide (Thamdrup and Dalsgaard, 2002).

N fixation by sulfate reducers and *Geobacteraceae* has been detected in a variety of environments (Bazylinski *et al.*, 2000; Welsh *et al.*, 1996), including benthic estuarine sediments frequently stressed by hypoxia (Brown and Jenkins, 2014; Brown, 2013; Fulweiler *et al.*, 2013; Bertics *et al.*, 2012; Burns *et al.*, 2002). We hypothesize that, under certain environmental conditions, N fixation may provide N to primary producers and establish a positive feedback loop, fueling hypoxia. A stronger shift in the N cycle paradigm may result from increasing interactions between climate change and anthropogenic N inputs, which may further exacerbate hypoxia.

Thesis Motivation and Outline

Sulfate reducers have previously been found to drive N fixation in sediments colonized by *Zostera* spp., where N fixation rates were measured using the acetylene reduction assay (McGlathery *et al.*, 1998, Welsh *et al.*, 1996). This assay indirectly measures the activity of nitrogenase, the enzyme complex that catalyzes N fixation (Capone, 1993). The dominant diazotrophs were confirmed to be sulfate reducers with the addition of molybdate, a specific inhibitor of sulfate reduction (McGlathery *et al.*, 1998; Welsh *et al.*, 1996). A recent study measuring net N₂ flux rates indicated that the non-vegetative estuarine sediments of Narragansett Bay were an N source (net N fixation), and this phenomena was suggested to be the result of oligotrophication (Fulweiler *et al.*, 2007). This N₂ flux reversal from net denitrification to net N fixation dispelled the paradigm that N removal by denitrification dominated N cycling in

estuarine sediments (Fulweiler *et al.*, 2007) with negligible N fixation (Howarth *et al.*, 1988). To identify the microbes responsible for the observed N fixation, sediment samples were collected concurrently and analyzed for the *nifH* gene (Fulweiler *et al.*, 2013). This highly conserved gene encodes for one of the enzymatic subunits (dinitrogenase reductase) of the N fixation enzyme (nitrogenase), which is used to convert N₂ into ammonium (Canfield *et al.*, 2010). *nifH* expression was detected in Narragansett Bay sediments, thus indicating benthic microbes were expressing the metabolism to fix N. The active diazotrophs were phylogenetically identified as sulfate reducers and *Geobacteraceae* (Brown and Jenkins, 2014; Brown, 2013, Fulweiler *et al.*, 2013). Brown and Jenkins (2014) found a correlation between severely hypoxic conditions and an increase in *nifH* expression, indicating hypoxic conditions may be an important driver of microbial N fixation by sulfate reducers and *Geobacteraceae* in estuarine sediments.

In contrast to the oligotrophication hypothesis, whereby decreased nutrient inputs stimulate N fixation in sediments (Fulweiler *et al.*, 2013), other studies focused on the response of estuarine sediments to nutrient loading and suggested that N fixation may become more active under elevated nutrient loading. In fact, nutrient loading was detected to stimulate N fixation by sulfate reducers and *Geobacteraceae*. For instance, addition of exogenous carbon (glucose) and N (ammonium) increased N fixation rates in vegetative sediment cores from Limfjord, Denmark (McGlathery *et al.*, 1998). Glucose addition also stimulated N fixation in estuarine sediment cores from Tay Estuary, Scotland (Herbert, 1975). Acetate was found to increase the growth of *Geobacteraceae* and expression of a core nitrogenase subunit gene (*nifD*) in

chemostat cultures and crude oil-contaminated subsurface sediments from Minnesota (Holmes *et al.*, 2004). Rates of N fixation and sulfate reduction were integrated for the hypoxic sediments of Eckernförde Bay, Germany and found to negatively correlate with bottom water DO concentrations, but positively correlate with bottom water temperature and phytoplankton blooms (Bertics *et al.*, 2012). Increased organic matter and depleted oxygen conditions were demonstrated to stimulate sulfate reduction in sediments below mussel beds in a seasonally anoxic Danish fjord (Jørgensen, 1980).

These contrasting observations provided motivation for the current study, which coupled biogeochemical and molecular analysis to elucidate the influence of exacerbated hypoxia and increased organic matter loading on the two dominant groups of diazotrophs in Narragansett Bay. We captured a DO gradient and targeted the active diazotrophs that dominate the benthic sediments at two sites, located at the high and low ends of the hypoxic and nutrient gradients of Narragansett Bay. An incubation experiment was also performed to investigate the influence of prolonged hypoxia and the addition of organic matter on sediment from the low risk hypoxic site. Globally, hypoxic conditions are expected to increase in intensity, frequency, and duration with continued N inputs from anthropogenic sources and added pressure from climate change (Middelburg and Levin, 2009). As the oxygen-depleted regions expand, the impact on the N budget of the ecosystem is unclear and is the target of this study.

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Manuscript 1

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Biogeochemical and molecular response of microbial nitrogen fixation in
hypoxic estuarine sediments

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Abstract:

Hypoxia is one of the most concerning impacts on coastal ecosystems resulting from eutrophication. The continued influx of anthropogenic nitrogen (N), along with pressures from climate change, is expected to increase the intensity, duration, and frequency of hypoxia in coastal systems worldwide. Recently, microbial N fixation in benthic estuarine sediments has been identified as a potentially important source of N for the ecosystem, but it is unclear how exacerbated hypoxia conditions influence N cycling dynamics. To elucidate the influence of exacerbated hypoxia and elevated organic matter (OM) loading on N fixation in estuarine sediments, two experiments were conducted with sediment cores from Narragansett Bay, RI, an estuary that experiences periodic hypoxia. The first study analyzed biogeochemical profiles and N fixation activity in field cores, collected at different sites and times under a range of DO concentrations. Manipulation experiments were also conducted with cores collected from the occasionally hypoxic Mid Bay site and incubated for three weeks with varied oxygen availability and OM loading. Biogeochemical profiling of the field and incubated cores (including oxygen and total sulfide concentrations, carbon and N content analysis) was coupled with N fixation rate measurements and molecular analysis, targeting the expression of the nitrogenase subunit (*nifH*) gene. The highest N fixation rates and total sulfide concentrations were measured under low DO in the field and with OM addition in the incubation experiment. Natural and laboratory-induced hypoxia stimulated *nifH* expression by uncultivated *Geobacteraceae* spp. and sulfate reducers. Uncultivated sulfate reducers were the dominant group expressing *nifH* at the site with the lowest DO and highest N fixation rates. The microbial group

also dominated *nifH* expression in OM treatments, where N fixation rates increased in response to the OM additions. In contrast, OM addition repressed *nifH* expression by uncultivated *Geobacteraceae*. Our data show that microbes driving N fixation in estuarine sediments respond differently to DO depletion combined with elevated OM levels, and predicting hypoxia-stimulated inputs of N depend on the type of dominant diazotrophs at a given site.

Introduction:

Hypoxia, or low dissolved oxygen, is one of the most concerning pressures on estuarine and coastal waters resulting from eutrophication (Diaz and Rosenberg, 2008). Anthropogenic inputs from fertilization, wastewater treatment plants, and other activities exacerbate eutrophication-driven hypoxic conditions (NSTC, 2000; Bricker *et al.*, 2000), which already stress over 400 systems globally, including Narragansett Bay, RI (Diaz and Rosenberg, 2008). As anthropogenic activities continue to generate a nitrogen (N) surplus, there is also the potential for added pressure from climate change, which may increase hypoxic intensity, duration, and frequency of events (Middelburg and Levin, 2009).

Hypoxic conditions, defined as less than or equal to 2.3 mg L⁻¹ dissolved oxygen (DO) (U.S. EPA, 2000), occur at peak water temperature and stratification (Diaz and Rosenberg, 2008). Hypoxia results from a series of interactions, beginning with an influx of river freshwater and nutrients from natural and anthropogenic sources (Codiga *et al.*, 2009), followed by an increase in primary productivity (Melrose *et al.*, 2007). Subsequent microbial-driven, aerobic decomposition at the sediment surface depletes the benthic community of DO, and thermohaline

stratification, strengthened by intervals of reduced wind and tidal mixing, prevents oxygen replenishment (Melrose *et al.*, 2007). The duration and intensity of hypoxia may be exacerbated with a greater volume of OM delivered to the sediments, resulting from eutrophication (Diaz and Rosenberg, 2008).

Under hypoxic stress, many aquatic organisms face altered growth and mortality rates and predator-prey interactions (Codiga *et al.*, 2009) and biogeochemical cycles are shifted (Burgin *et al.*, 2011). Although sediments of coastal margins represent only a small portion of the total area of marine sediments (<9%), they perform the majority (83%) of the biogeochemical cycling in the benthos (Jørgensen, 1983). Microbes drive major biogeochemical cycles, including N, and their activity regulates estuarine nutrient availability (Burgin *et al.*, 2011), thus providing a critical ecosystem service to the estuary.

Hypoxic conditions induce a shift in the N cycling of the estuarine sediments. The availability of electron donors and acceptors (e.g. oxygen, nitrate, sulfate) influence this microbial-driven cycle (Burgin *et al.*, 2011). Under oxygen-rich, aerobic conditions, coupled nitrification-denitrification act as an N sink by removing reactive forms of N from the environment and exporting it as the inert N₂ gas. However, oxygen depletion inhibits the aerobic conversion of ammonia to nitrate through nitrification, thus disrupting this coupled reaction. Instead, the sediment's anoxic regions presumably expand the niche for anaerobic microbes, such as sulfate reducers (e.g. *Desulfovibrio* spp.) and the iron reducers, *Geobacteraceae*, which use alternative electron acceptors (other than oxygen) for energy generation (Seitzinger *et al.*, 1984).

The metabolism of sulfate reducers and *Geobacteraceae* performs two

functions to further shift the N cycle. These anaerobic microbes produce sulfide, which inhibits nitrification (Thamdrup and Dalsgaard, 2002; Joye and Hollibaugh, 1995). Studies targeting the expression of the nitrogenase iron protein gene (*nifH*) have indicated that these anaerobes have the potential to fix N and add it back to the ecosystem (Brown and Jenkins, 2014; Brown, 2013; Fulweiler *et al.*, 2013). This highly conserved gene encodes for one of the enzymatic subunits, dinitrogenase reductase, of the N fixation enzyme, nitrogenase (Canfield *et al.*, 2010), which is used to convert inert N₂ gas into biologically available ammonium. If *nifH* is detected in RNA samples, microbes are expressing *nifH*, and thus, may be fixing N.

In 2006, N₂ flux measurements indicated that Narragansett Bay sediments were seasonally an N source (net N fixation); dispelling the denitrification-dominated paradigm (Fulweiler *et al.*, 2007) and the theory that N fixation occurred only in the water column (Gruber, 2008). *nifH* expression was also detected during the 2006 season (Fulweiler *et al.*, 2013), which was severely hypoxic (Codiga *et al.*, 2009). Interestingly, Brown (2013) found a correlation between severely hypoxic conditions and an increase in *nifH* expression, indicating hypoxic conditions may be an important driver of microbial N fixation by sulfate reducers and *Geobacteraceae* in estuarine sediments. We hypothesize that the resulting N could potentially stimulate primary producers, and establish a positive feedback loop; exacerbating hypoxia.

Since eutrophication increases the volume of organic matter deposited on the sediments, hypoxia tends to persist and spread in area. In many systems, hypoxic conditions degrade over time, progressing from episodic or periodic hypoxia to seasonally persistent (Diaz and Rosenberg, 2008). The impact of more frequent,

severe hypoxia and elevated anthropogenic loading on the N fixation activity of sulfate and sulfur reducers is unclear. To elucidate this relationship, this study focused on the diazotrophs of the Narragansett Bay sediments using a combination of field sampling and environmental core incubations in a DO table at the U.S. Environmental Protection Agency, Atlantic Ecology Division (Narragansett, RI). In both cores from the field and the DO table incubation, Unisense microprofiles of downcore oxygen and total sulfide concentrations were coupled with molecular biology analysis to target changes in diazotrophic activity under hypoxia and elevated organic matter loading. Field samples were collected under a range of DO concentrations from Greenwich Cove, a high-risk hypoxic site (Melrose *et al.*, 2007; Deacutis *et al.*, 2006) located near a wastewater treatment plant (Calabretta and Oviatt, 2008). Sediment was collected from the Mid Bay site for the field study and incubation experiment. The Mid Bay site is located at the lower end of the nutrient gradient of the bay (Calabretta and Oviatt, 2008) and occasionally experiences hypoxia (Codiga *et al.*, 2009; Melrose *et al.*, 2007; Deacutis *et al.*, 2006).

Results:

Field Measurements and DO Table Experimental Conditions

Sediment cores were collected from two sites in Narragansett Bay: Greenwich Cove (GC: July 19, July 30, August 12) and Mid Bay (MB: July 15, August 6, September 25). A hypoxic gradient was studied at GC over the 2013 season, with bottom water DO conditions at anoxic, hypoxic, and normoxic levels, with only small variation in bottom water temperature (Table 1). All MB field cores were collected under normoxic conditions, with bottom water temperatures ranging from 18.0 to

20.1°C (Table 1). For each time point, three cores were collected for immediate microprofiling, followed by sectioning down to the 2.0 cm depth, sampling for N fixation rate measurements and molecular analysis. Fifty-one additional cores were collected from MB on September 25 under normoxic (6.34 mg L^{-1}) bottom water DO conditions at 18.0°C (Table 2) and incubated for three weeks in a continuous flow system (termed "DO table") at the EPA. The EPA DO system allows field cores to be incubated at a constant temperature under multiple DO concentrations and OM levels simultaneously. The system continuously draws in water from the bay and passes it through temperature and DO controls, constantly providing fresh seawater to the cores. Unlike field studies, the DO table allowed us to limit the number of variables and observe the influence of prolonged hypoxia and the addition of OM in the form of spray dried phytoplankton to the MB sediment cores. The cores were divided among four treatments: normoxic, hypoxic, normoxic + organic matter (OM), and hypoxic + OM. Throughout the experiment, the DO table system remained stable and maintained the cores at 7.0 mg L^{-1} (normoxic) or 0.5 mg L^{-1} DO (hypoxic) at 20.0°C (Table 2, Supplemental Figure 1).

Biological and Biogeochemical Characteristics of Sediment Cores

Fine, white filaments of *Beggiatoa* spp. were observed growing on the surface of the GC cores on August 12, as well as, in the normoxic + OM and hypoxic + OM treatments in the DO table experiment. In the DO table experiment, black sediment (iron sulfide) was observed in the hypoxic cores and OM cores. The bands of black sediment were thicker and closer to the surface of the OM treatment cores.

The July 30 GC sediment cores had higher weight percent carbon (C) and N than all of the MB cores collected over the season (Table 3). The C:N ratio of the July sediment cores from MB and GC were similar at depths lower than 0.5 cm (Table 3). However, the C:N ratio of the 0-0.5 cm depth was higher for the MB site than the GC site for the July sediment samples (Table 3). As the season progressed, a decrease in the C:N ratio of the MB field cores was observed at all depths (Table 3).

Prior to incubation, the MB sediment cores had a C:N ratio of 9.7 (± 0.11 SEM) at 0-0.5 cm and 10.1 (± 0.23) at 0.5-2.0 cm (Table 3). The spray dried phytoplankton used for the organic matter (OM) treatments in the incubation experiment had a C:N ratio of 26.40 (± 1.28 SEM), more than double the ratio measured in the sediment cores (Table 3).

As reflected in the oxygen profiles (Figure 2), the August 6 MB field cores had the highest percent saturation oxygen (57.3%) of all field cores at the sediment-water interface. The lowest oxygen saturation levels at the sediment water interface measured in the GC field cores were collected on July 19 and July 30 (0% and 28.5% respectively, Figure 2). Oxygen was depleted by the depth of 0.5 mm in the July 19 GC and September 25 MB cores and by 1 mm in the remainder of the field cores (Figure 2).

The total sulfide concentrations in the GC cores ($0-873 \mu\text{mol L}^{-1}$) were higher than the concentrations measured for the MB cores ($0-16.6 \mu\text{mol L}^{-1}$) (Figure 3). The July 30 GC cores had the highest concentration overall, with an increase in concentration at approximately 5-6 mm in depth (Figure 3). Although the total sulfide

concentration increased with depth in all of the GC cores, a spike in concentration occurred in the July 19 GC cores near the sediment surface, at 0.75 mm (Figure 3).

Oxygen was completely depleted by 5 mm in the September 25 MB field cores and after 5 days of incubation in the DO table cores. Oxygen penetration was deeper in the DO table cores treated with OM where it was detected at $5.27\% \pm 3.86\%$ SEM saturation (normoxic + OM) and $3.79\% \pm 1.45\%$ saturation (hypoxic + OM) at 1 mm in contrast to the field cores and other treatments, where it was $0.96\% \pm 0.42\%$ (field), $1.76\% \pm 0.01\%$ (normoxic), and $0.74\% \pm 0.73\%$ (hypoxic) by 1 mm (Figure 4). Oxygen penetration at the surface was higher after 5 days of incubation for the normoxic and normoxic + OM cores, which had higher percent oxygen saturation ($17.1\% \pm 1.31\%$; $33.5\% \pm 8.95\%$ respectively) than both hypoxic counterparts and field cores ($9.27\% \pm 2.38\%$ (field); $10.1\% \pm 1.46\%$ (hypoxic); $8.68\% \pm 2.08\%$ (hypoxic + OM)) (Figure 4). The average pH of the cores from September 25, the hypoxic treatment, and the OM treatments ranged from 7.17 to 7.64 with the normoxic table cores showing an elevated pH of 9.08 (Table 4). As indicated in Figure 5, the total sulfide concentration of the hypoxic cores only slightly increased with depth (maximum $4.95 \mu\text{mol L}^{-1}$ at 19.5 mm), a small increase relative to the OM treatments, where relatively large sulfide peaks were observed. The sulfide peak ($421 \mu\text{mol L}^{-1}$) occurred at the sediment-surface interface in the hypoxic + OM treatment and at a depth of 3.5 mm in the normoxic + OM cores ($430 \mu\text{mol L}^{-1}$) (Figure 5). The downcore sulfide concentrations of the normoxic cores were low and not included in Figure 5, as the sulfide probe loses sensitivity at pH concentrations above 9 (Jeroschewski *et al.*, 1996).

Nitrogen Fixation Rates in Field and Incubated Cores

Overall, the GC site had higher N fixation rates than the MB site (Figure 6A, Spinette *et al.*, in prep). Rates of N fixation were highest at GC on July 19 for all depths (top depth: 15.60 nmol/g.d; bottom depth: 12.50 nmol/g.d) and decreased over the season, with the lowest level of activity on August 12 (Figure 6A). A seasonal decrease was also observed in the bottom depth samples (>0.5 cm) from the MB field cores (Figure 6A). The MB samples from the top 0-0.5 cm depth had similar levels of activity on July 15 and September 25, but lower activity on August 6 (top depth: 6.04 nmol/g.d; bottom depth: 4.87 nmol/g.d) (Figure 6A).

After three weeks of incubation, the top 0-0.5 cm of sediment from the normoxic and hypoxic cores had similar, but relatively low N fixation rates (1.87 and 1.60 nmol/g.d respectively, Figure 6B) compared to the rates measured for the field cores used to set up the experiment on September 25 (rate data courtesy: R. Spinette and A. Jones, URI). Despite this reduction, higher N fixation rates were measured in both the normoxic + OM and hypoxic + OM treatments. The normoxic + OM samples had the highest N fixation rates, which was comparable to rates measured in the field prior to the start of the incubation (Figure 6B).

Identifying the Active Diazotrophs and Targeting the Abundance and Activity of Important Groups

To identify the dominant groups of diazotrophs in Narragansett Bay sediments, expressed *nifH* sequences from field samples were cloned, sequenced, and phylogenetically compared (Figure 7). Phylogenetic analysis indicates that *nifH* expression was restricted to two phylogenetic groups: Clusters I and III (Chien *et al.*,

1996). These sequences from the top 2 cm of sediment grouped with known sulfate reducers and the iron reducers, *Geobacteraceae* (Figure 7). As indicated in the phylogenetic tree (Figure 7), the expressed *nifH* sequences extracted from the MB field cores grouped with two clusters of diazotrophs: NB3 (uncultivated *Geobacteraceae* with the closest cultivated relative *Pelobacter carbinolicus*) and NB7 (uncultivated sulfate reducers with *Desulfovibrio vulgaris* and *D. salexigens* as the most closely related species). The GC sequences primarily grouped with two regions of the cluster I: NB7 and NB5 (Figure 7). *Desulfatibacillum alkenivorans* was the most likely closely related cultivated species to NB5 (Figure 7).

Primer and probe sets, previously described by Brown and Jenkins (2014) and Brown (2013), were used to target groups NB3 and NB7. However, we identified new sequences from GC that grouped in regions of the tree that were outside the qPCR-targeted groups (e.g. NB5) (Figure 7). Since the majority of the sequences for sulfate reducers from the GC site grouped in NB5, we designed primers to specifically target NB5, excluding NB7. Despite trying a range of concentrations, annealing temperatures, and two qPCR chemistries (SYBR and probe-based qPCR), attempts were unsuccessful to design qPCR primers and probes to target NB5. Two fluorescent peaks, corresponding to primer-dimers and the amplicon, were detected in the dissociation curve plots for SYBR-based qPCR. When probe-based qPCR was used to specifically target the amplicon, the samples and lower concentration standards could not be detected. Thus, the abundance and expression of NB5 in the extracted sampled could not be quantified.

The abundance of uncultivated group NB7 sulfate reducers was followed in all cores using qPCR. This group had a similar cellular abundance across time points and sediment depths in the MB field samples, ranging from 6.43×10^7 to 1.01×10^8 gene copies per gram of sediment (Figure 8A). For the 0-0.5 cm depth of the July 30 GC cores, the uncultivated NB7 sulfate reducer abundance was comparable to the MB field cores (Figure 8A). Lower abundance was measured in the deeper depths (>0.5 cm) of the July 30 GC cores, as well as in the July 19 and August 12 GC cores (Figure 8A).

While the abundance of uncultivated NB7 sulfate reducers indicated the number of bacteria with the genetic potential to fix N, *nifH* expression of NB7 was measured to compare the NB7 activity in all cores. The MB July 15 cores had the highest *nifH* expression by the uncultivated sulfate reducers at the 1.0 cm depth (Figure 8B). Lower expression was detected in the GC cores (Figure 8B). While no expression was detected at 0-0.25 cm depth on August 6, the MB 2.0 cm depth on September 25 had the lowest detectable *nifH* expression by uncultivated sulfate reducers (Figure 8B).

Since *nifH* expression by uncultivated group NB3 *Geobacteraceae* was not detected in GC samples with cloning and sequencing, only the MB field cores were analyzed for the abundance and expression of NB3. The NB3 abundance in MB field cores ranged from 1.19×10^7 to 1.46×10^8 gene copies per gram of sediment (Figure 8A). The cores from August 6 at all depths had the highest abundance (8.57×10^7 to 1.46×10^8 gene copies per gram of sediment) (Figure 8A). The July 15 MB samples had the overall lowest abundance of uncultivated *Geobacteraceae* (Figure 8A). The

highest *nifH* expression was detected for the group uncultivated *Geobacteraceae* in the bottom depth (0.5-2.0 cm) of the September 25 MB cores (Figure 8B).

The group NB7 uncultivated sulfate reducers were more than group NB3 uncultivated *Geobacteraceae* in the top 0.5 cm of field cores collected on September 25 used to initiate the DO and OM manipulation experiments (Figure 9). After three weeks of incubation, the uncultivated sulfate reducing group NB7 had similar levels of abundance to the field samples and the uncultivated group NB3 *Geobacteraceae* had increased abundance (Figure 9). Overall, in the incubation experiment, both NB7 and NB3 reached similar abundances, with the exception of the OM treatment in normoxic conditions, which stimulated the growth of uncultivated sulfate reducer NB7 (Figure 9). The uncultivated NB7 sulfate reducers had a similar level of *nifH* expression in the top 0-0.5 cm of the incubation cores and September 25 field samples (Figure 10). Although *nifH* expression by the uncultivated group NB3 *Geobacteraceae* was not detected in the OM treatments, the expression increased in the normoxic and hypoxic cores compared to the field (Figure 10). Furthermore, the hypoxic cores had a higher level of *nifH* expression than the normoxic cores (Figure 10).

Discussion:

Periodic hypoxic conditions have been recorded in Narragansett Bay for the past few decades (Codiga *et al.*, 2009; Melrose *et al.*, 2007; Deacutis *et al.*, 2006; Bergondo *et al.*, 2005) and are strongly influenced by river flow and temperature (Codiga *et al.*, 2009). Conditions promoting hypoxia are predicted to increase under climate change, further exacerbating eutrophication-driven hypoxic conditions (Zhang *et al.*, 2010; Middelburg and Levin, 2009). Low oxygen environments create an

expanded niche for anaerobic microorganisms that use terminal electron acceptors other than oxygen for energy metabolism (Seitzinger *et al.*, 1984). Recent studies suggest that low oxygen may promote the growth of these organisms (Brown and Jenkins *et al.*, 2014; Brown, 2013).

Recently, N fixation was detected in estuarine sediments (Fulweiler *et al.*, 2007) and was estimated to have a larger role in N cycling than previously believed (Bertics *et al.*, 2010). The microbes responsible for the majority of benthic N fixation are anaerobes that can flourish in low oxygen systems. In particular, N fixation was linked to sulfate reducers and the iron reducers, *Geobacteraceae*, in the sediments of Narragansett Bay (Brown and Jenkins, 2014; Fulweiler *et al.*, 2013), Catalina Harbor (CA) (Bertics *et al.*, 2010), and Eckernförde Bay (Baltic Sea) (Bertics *et al.*, 2012). Furthermore, low DO and temperature may be key in promoting N fixation in Narragansett Bay because they stimulate the expression of an essential gene in N fixation, *nifH* (Brown and Jenkins, 2014; Brown, 2013). In Narragansett Bay, the highest *nifH* expression was detected at sites that frequently experience hypoxia and at the top of the north-south nutrient gradient (Brown and Jenkins, 2014).

Since areas of oxygen depletion are predicted to increase in the future climate, we wanted to determine the response of N fixing microbes to a natural DO gradient. In addition, we used experimental manipulations with coupled biogeochemical and molecular analyses to test the influence of prolonged hypoxia and elevated OM loading on N fixation.

Oxygen Penetration Into Sediments is Influenced by Bottom Water DO Levels

The lowest oxygen percent saturations in field sediments were observed at the GC site (Figure 2), which frequently experiences severe, periodic hypoxia (Codiga *et al.*, 2009). Interestingly, the oxygen penetration at GC paralleled the observed range of bottom water DO concentrations (Table 1) with the highest percent saturation at the GC sediment surface under normoxic conditions on August 12 and lowest during anoxic conditions on July 19 (Figure 2, Table 1). The rapid oxygen depletion (Figure 2) suggests high oxygen consumption in sediment from the GC site. Gundersen and Jørgensen (1990) also observed shallow oxygen penetration depths (2.2-2.5 mm) in Danish coastal sediments of Aarhus Bay, the result of high microbial oxygen consumption rates (Gundersen and Jørgensen, 1990; Howarth *et al.*, 2011; Middelburg and Levin, 2009).

Oxygen penetration was deeper in MB sediments which experience occasional episodic hypoxia (Codiga *et al.*, 2009; Melrose *et al.*, 2007; Deacutis *et al.*, 2006). Oxygen depletion depths ranged from 0.5 to 1.0 mm (Figure 2). The highest oxygen saturation at MB was measured in the August 6 cores. Although the MB bottom water on September 25 was more oxygenated than earlier in the season (Table 1), the September 25 cores had the lowest percent saturation oxygen at the sediment surface with oxygen depletion by 0.5 mm depth (Figure 2). This suggests another factor, other than oxygen, was influencing the oxygen microprofiles. As suggested by Morse and Rowe (1999), differences in sediment oxygen demand may be due to an increase in temperature. In MB sediments, the reduced oxygen penetration may be the result of

lower benthic activity due to lower water temperature. The lowest oxygen penetration (Figure 2) was observed in the coldest water temperature (Table 2).

Sulfate Reducers and *Geobacteraceae* may be Influenced by Dissolved Oxygen, C and N Content

As the oxygen was depleted in the sediment cores (Figure 2), the total sulfide concentration increased with depth in the anoxic regions of the GC sediment (Figure 3). Interestingly, the sulfide concentration increased for all GC time points at the 4-5 mm depth, when the oxygen was completely depleted (Figures 3, 2). A spike in sulfide concentration was also observed at 0.75 mm in one of the GC cores collected under anoxic conditions on July 19 (Table 1), but the concentration was variable at this depth among the replicate cores (Figure 3). McCarthy *et al.* (2008) also recorded microprofiles of hypoxic cores from Corpus Christi Bay (TX) and detected low sediment oxygen demand and high hydrogen sulfide concentration in the surface sediments. Bertics *et al.* (2012) and Jørgensen (1977) detected seasonal variation in sulfate reduction rates in hypoxic sediments of Baltic Sea (Bertics *et al.*, 2012) and Limfjorden, Denmark (Jørgensen, 1977). Highest rates were observed in the summer (Bertics *et al.*, 2012; Jørgensen, 1977), when the highest rates ascended to the top few centimeters of sediment (Jørgensen, 1977). This change was most visible during hypoxia, when reducing conditions were close to the sediment surface and additional OM was available for anaerobic decomposers (Jørgensen, 1977). Bertics *et al.* (2012), who integrated rates of N fixation and sulfate reduction, found that the rates negatively correlated with bottom water DO concentrations, but positively correlated with bottom water temperature and phytoplankton blooms.

Interestingly, in the current study, the highest sulfide concentrations were measured under hypoxia (July 30), not anoxia (July 19) (Figure 3, Table 1). The differences in sulfide concentration may reflect the DO conditions prior to the sediment collection. Using a YSI buoy in the Narragansett Bay Fixed-Site Monitoring Network to estimate DO concentration at GC over the entire season (data courtesy: H. Stoffel, URI GSO), the July 19 anoxic cores were collected at the beginning of the hypoxic season, whereas the July 30 hypoxic cores were collected after a potentially long exposure to hypoxia (Table 1, Figure 11A). The microbial activity may have already been ramped up, resulting in a higher sulfide concentration on July 30.

When comparing sulfide concentrations across sites, however, additional factors may drive differences in sulfide concentration in the bay. GC consistently had higher total sulfide concentrations, even under normoxic conditions (Figure 3, Table 1). In contrast, MB site had low sulfide concentrations (Figure 3) despite oxygen depletion in the sediment by a depth of 1 mm (Figure 2). In addition, the majority of expressed *nifH* sequences extracted from GC (Figure 7) and from the other regions of the upper bay (Brown and Jenkins, 2014) grouped with sulfate reducers. The 2014 study measured the diversity of expressed *nifH* sequences along the estuarine gradient of Narragansett Bay and detected higher diversity of active diazotrophs in the upper region of the bay than the MB site (Brown and Jenkins, 2014). Furthermore, *nifH* expression by NB10, NB5, and other groups of sulfate reducers was limited to the upper bay (Brown and Jenkins, 2014).

Since sulfate reducers are more active in the upper bay, this suggests the environment of the upper bay is promoting sulfate reducer *nifH* expression and sulfide

production. Another factor, other than oxygen, may be influencing sulfide concentration. Westrich and Berner (1984), for instance, analyzed surface and 1-meter deep cores from Long Island Sound sediments; determining decomposition kinetics and measuring microbial sulfate reduction rates using the ^{35}S radiotracer technique. Sulfate reduction was directly proportional to the amount of planktonic C added (Westrich and Berner, 1984). When comparing the GC and MB sediment in the current study, the July 30 GC cores had higher C and N content than the MB samples collected over the entire season (Table 3). This difference in C and N content is not surprising, as the study sites are at opposite ends of the nutrient gradient of Narragansett Bay (Calabretta and Oviatt, 2008). GC is closer to point sources of anthropogenic sources of N and OM, as it is adjacent to the East Greenwich Wastewater Treatment Plant. MB, at the lower end of the gradient, is farther from the point sources (Calabretta and Oviatt, 2008). A similar C:N trend that followed the Narragansett Bay nutrient gradient was reported by Brown (2013). These results suggest that, in addition to DO concentration, OM may influence the activity of diazotrophs. Sulfate reduction may be C limited at the MB site. The July GC sediment samples had higher total sulfide concentration (Figure 3) and higher weight percent C and N than MB samples collected over the entire field season (Table 3).

Hypoxia Promotes N Fixation Rates, but Variable *nifH* Expression

As described by Spinette *et al.* (in prep), N fixation rates in the sediment field cores were also influenced by bottom water DO conditions. The highest N fixation rates were measured under anoxic conditions (July 19), followed by hypoxic conditions (July 30) at GC (Table 1, Figure 6A). Lower rates were recorded under

normoxic conditions at MB site and on August 12 at GC (Table 1, Figure 6A). The N fixation rates negatively correlated with depleted DO bottom water (Spinette *et al.*, in prep). Although the quantification of N fixation rates is important, it does not provide the phylogenetic identity of the microbes performing N fixation (Burns *et al.*, 2002). Thus, expressed *nifH* sequences were phylogenetically compared and qRT-PCR was used to target and quantify the *nifH* expression by the dominant diazotrophs. While uncultivated *Geobacteraceae* and uncultivated sulfate reducers were previously found to dominate *nifH* expression in other regions in Greenwich Bay (Brown and Jenkins, 2014; Brown, 2013), the expressed *nifH* sequences extracted from the GC samples were dominated by uncultivated sulfate reducers, primarily grouping with the groups NB5 and NB7 (Figure 7). As described by Morse and Rowe (1999), hypoxic conditions result in sulfate reducing bacteria dominating the heterotrophic activity. These data suggest that sulfate reducers may be the dominant anaerobic N fixers in sediments frequently exposed to hypoxic conditions, such as GC (Melrose *et al.*, 2007, Deacutis *et al.*, 2006). Conversely, both *Geobacteraceae* and sulfate reducers were detected in expressed *nifH* sequences from MB cores, primarily grouping with NB3 and NB7 (Figure 7).

Quantitative PCR primer and probe sets were selected to follow microbial groups that dominated *nifH* expression at both sites: uncultivated *Geobacteraceae* at MB and uncultivated sulfate reducers at MB and GC. When targeting group NB7 uncultivated sulfate reducers, higher levels of gene abundance were measured in the MB samples compared to GC (10^7 - 10^8 and 10^6 - 10^7 gene copies per gram of sediment, respectively) (Figure 8A), thus supporting the phylogenetic comparison of expressed

nifH sequences (Figure 7). Unlike MB sulfate reducers, which primarily grouped with NB7, GC sulfate reducers grouped with NB5 and NB7. Group NB7 was also detected by Brown (2013) in Narragansett Bay sediment at sites in close proximity to MB and GC (near MB and Greenwich Bay, respectively). These sites had a similar abundance of NB7: 10^8 (Greenwich Bay) and 10^7 - 10^8 (near MB) gene copies per gram of sediment (Brown, 2013). However, higher *nifH* expression by NB7 was detected in Greenwich Bay (10^5 - 10^7 (Greenwich Bay); 10^5 - 10^6 (near MB) transcript copies per gram of sediment) (Brown, 2013). In the current study, *nifH* expression by NB7 was lower and more variable, ranging from 9.03×10^1 to 1.93×10^3 at GC and 1.66×10^1 to 2.52×10^4 at MB transcript copies per gram of sediment (Figure 8B).

The expression by group NB7 uncultivated sulfate reducers may have been lower than in Brown (2013) as a reflection of the observed shift in diversity of sulfate reducers to include group NB5 that was not detected by our qPCR primers (Figure 7). Our GC site was at a different location than where gene expression was measured by Brown *et al.* (2013). In addition, the observed variability may reflect the heterogeneous environment of the estuarine sediments. As described by Jørgensen (1977), natural variation may exist in the cores due to the heterogeneous distribution of microbial activity, which create microscale patchiness (Paerl and Pinckney, 1996). Macrofaunal burrows, for instance, provide ideal conditions for N fixation, thus create “hot spots” of elevated rates of N fixation (Bertics *et al.*, 2010).

Although the hypoxic July 30 cores had a higher abundance of uncultivated sulfate reducers above the 1.0 cm depth (Figure 8B) and had the highest sulfide concentration (Figure 3), no trends in expression followed the hypoxic gradient.

Despite differences in oxygen penetration between time points, the *nifH* expression by the targeted groups did not appear to be influenced by DO level. However, we were unable to collect samples for *nifH* analysis on the same spatial scale as oxygen depletion measurements. The cores could only be sectioned down to 0.25 cm (large cores) or 0.5 cm (small cores) depth horizons, whereas the microprofiles were measured on a finer scale with intervals of 0.25 mm and 1.0 mm.

Core Incubation: Influence of Dissolved Oxygen and Organic Matter on Biogeochemical Profiles

After five days of incubation, the influence of DO conditions on the sediment microprofiles was more apparent in the OM treatments, which received OM with a C:N ratio 2.4 times greater than the MB seasonal average (Table 3). Although oxygen was rapidly depleted in all cores by approximately 0.5 mm (field cores) or 1 mm (experimental cores) (Figure 4), the normoxic and normoxic + OM treatments had higher percent oxygen saturation at the sediment surface, compared to their hypoxic counterparts and the field cores (Figure 4). Despite the observed differences in oxygen penetration between treatments, low concentrations of total sulfide were measured in the normoxic and hypoxic treatments (Figure 5), suggesting that five days of incubation in hypoxic water was too early to observe changes in sulfide in these cores. However, higher total sulfide concentrations were observed in the microprofiles of the OM treatments (Figure 5), showing that OM addition rapidly stimulates sulfate reduction. The OM cores (Figure 5) and the GC field cores (Figure 3) had a similar sulfide concentration range above 5 mm, but the sulfide peaks differed in magnitude and depth. The sulfide concentration increased at the sediment surface of the hypoxic

+ OM cores (Figure 5), where oxygen saturation was less than 10% (Figure 4). In the normoxic + OM cores, which had deeper oxygen penetration than the hypoxic + OM cores (Figure 4), the sulfide concentration peaked deeper in the cores (Figure 5), where the oxygen concentration was reduced (Figure 4). For both treatments, the sulfide concentration increased near the top of the core (above 5 mm), but decreased at depths greater than 5 mm (Figure 5). Conversely, the GC sulfide peaks were greater in magnitude and primarily occurred at deeper depths (> 5 mm) (Figure 3).

In this study, the highest sulfide concentrations were detected in the GC field cores and DO table OM treatment cores (Figures 3 and 5), which also had the highest C and N contents (Table 3). The C and N content of the GC cores, though less than the spray dried phytoplankton added to the OM cores, was higher than the MB sediment used for the incubation experiment (Table 3). This suggests that OM stimulated the activity of sulfate reducers. Though the type, timing, and amount of OM may influence microbial activity (Babbin and Ward, 2013; Bauer *et al.*, 2013; Herbert, 1999), increased OM was demonstrated to stimulate sulfate reduction in sediments below mussel beds in a seasonally anoxic Danish fjord (Jørgensen, 1980). N fixation rates were also found to increase with additions of glucose (vegetative sediment cores from Limfjord, Denmark – McGlathery *et al.*, 1998; estuarine sediment cores from Tay Estuary, Scotland – Herbert, 1975) and ammonium (McGlathery *et al.*, 1998).

Conversely, higher levels of OM in the GC and OM treatment cores may have also created anoxic microniches, which may have stimulated sulfate reducers. Higher rates of decomposition increase sediment oxygen consumption, resulting in decreases in benthic oxygen availability (Sturdivant, *et al.*, 2012; Zhang *et al.*, 2010). Under

reduced oxygen conditions, sulfate reduction, among other reducing reactions, occurs at shallower depths and hydrogen sulfide proliferates (Jørgensen, 1980). The steep oxygen gradient and the availability of essential metabolites in the microniche provide a favorable habitat for N fixers (Paerl and Pinckney, 1996).

Core Incubation: Diazotrophic Community After Prolonged Hypoxia

After three weeks of incubation, the normoxic and hypoxic cores had similar rates of N fixation, but had lower rates than the field (Figure 6B) (rate data courtesy R. Spinette and A. Jones, URI). Since the field and incubated cores were collected at the same time, this suggests a decrease in N fixation rates occurred during the incubation. As described by McGlathery *et al.* (1998), Herbert (1999), and Herbert (1975), who measured N fixation rates, N fixation may be limited by a lack of oxidizable C substrates. The normoxic and hypoxic cores may have been OM limited, as their OM counterparts had the highest N fixation rates of the incubation experiment (Figure 6B). Combined N was originally thought to repress N fixation (Postgate, 1982), but recent evidence suggests N fixation (Knapp, 2012) and active diazotrophs in NB sediments (Brown, 2013) may be less sensitive to dissolved inorganic N (Knapp, 2012) and combined N levels (Brown, 2013) than previously thought. Instead, the combined N may not repress nitrogenase, or microbes at the sediment surface may simply rapidly assimilate it.

Higher N fixation rates were measured for the OM cores than the non-OM cores (Figure 6B). The normoxic + OM samples had the highest average rate, which was comparable to the field (Figure 6B). These measurements suggest that, during the duration of the incubation, the N fixing activity in the OM treatments was maintained.

Since we were unable to sample at earlier time points, it is currently not known if rates exceed those observed in the field samples.

At first glance, the high sulfide concentration and N fixation rate measurements in the normoxic + OM treatment seemed paradoxical for normoxic DO conditions. However, the observed total sulfide concentration peak (Figure 5) occurred under reduced oxygen levels, below the sediment-water interface, at a depth of 3-4 mm (Figure 4). Decomposers, which fed on the OM at the sediment surface and received oxygen from the circulating water, may have caused the oxygen levels to be rapidly depleted at the sediment surface thus allowing the anaerobic sulfate reducers and *Geobacteraceae* to produce higher levels of sulfide near the sediment surface than was observed in the non-OM normoxic treatment (Figure 5). Although the abundance of both uncultivated *Geobacteraceae* and uncultivated sulfate reducers was measured in OM treatments (Figure 8A), only *nifH* expression by uncultivated sulfate reducers was detected (Figure 9B). Thus, OM addition stimulates *nifH* expression by uncultivated sulfate reducers, but appears to repress expression by uncultivated *Geobacteraceae*.

Revsbech and Jørgensen (1986) suggest sediments with a high organic turnover may cause the oxygen-hydrogen sulfide interface to move up towards the sediment-water interface, allowing the white filamentous mats of *Beggiatoa* spp. to grow on sediment surface. Despite the circulation of overlying water at normoxic conditions, Revsbech and Jørgensen (1986) attributed the microoxic or anoxic conditions at the sediment surface to the diffusive boundary layer and high oxygen consumption by *Beggiatoa* spp., thus corroborating our observations of *Beggiatoa*

spp., N fixation rates, and high total sulfide concentration near the sediment surface of the normoxic + OM cores. Although water DO concentrations were lower in the hypoxic + OM treatment, the high organic turnover also explained the high total sulfide peak, high N fixation rate, and growth of *Beggiatoa* spp. near the sediment surface. Both OM treatments had higher total sulfide levels and N fixation rates than their non-OM counterparts suggesting an influence of OM. As proposed by McCarthy *et al.* (2010), hydrogen sulfide may determine N fates near the sediment surface. Their study site with the lowest oxygen penetration and the highest hydrogen sulfide concentration in Corpus Christi Bay (Texas) showed N fixation regardless of season (McCarthy *et al.*, 2010).

Previous work measuring net N₂ fluxes in field cores and mesocosm incubations with Narragansett Bay sediments suggest that denitrification is stimulated by OM loading, whereas N fixation is promoted in oligotrophic conditions (Fulweiler *et al.*, 2007; Fulweiler *et al.*, 2013; Fulweiler and Heiss, 2014). Spray dried phytoplankton (C:N 10.5) was added to MB sediment in varying amounts in a long-term mesocosm experiment, testing the impact of quantity and temporal addition of OM on benthic N₂ fluxes. The highest net denitrification rates were measured soon after OM addition. The lowest net rates and increased *nifH* expression were detected in sediments starved of OM (Fulweiler *et al.*, 2013). However, it is important to recognize that N₂ flux measurements only determine the magnitude of N₂ flux out of or into the sediment and are not direct measurements of N₂ rates. Therefore, high rates of denitrification with OM addition could be masking elevated N₂ fixation. Supporting

this notion, expression of *nifH* and the functional gene for denitrification (*nirS*) were detected concurrently (Fulweiler *et al.*, 2013).

As indicated by phylogenetic relationship of expressed *nifH* sequences, uncultivated *Geobacteraceae* and uncultivated sulfate reducers continued to dominate MB sediment after three weeks of incubation (Figure 7). Both groups were abundant in all treatments (Figure 9). Although the abundance of uncultivated *Geobacteraceae* was similar across treatments and remained at levels observed in the field prior to the start of the incubation (Figure 9), the hypoxic conditions stimulated *nifH* expression by uncultivated *Geobacteraceae* (Figure 10). This group may be more active under depleted oxygen conditions in low OM sediments.

Although hypoxia stimulated the uncultivated *Geobacteraceae*, the OM treatment completely repressed *nifH* expression. In the OM treatments, *nifH* expression was not detected for NB3 in the normoxic + OM and hypoxic + OM treatments (Figure 10) and this repression may be the result of N addition in the OM treatments. These observations are consistent with the hypothesis that oligotrophication may drive N fixation in MB sediment (Fulweiler *et al.*, 2003). N fixation in uncultivated *Geobacteraceae* in particular may be highly sensitive to ammonium concentrations. The *Geobacteraceae* group was the focus of a study on chemostat cultures and crude oil-contaminated subsurface sediments from Minnesota, which found that expression of another nitrogenase gene (*nifD*) decreased two days after adding ammonium (Holmes *et al.*, 2004). In our study, *nifH* expression by uncultivated *Geobacteraceae* may have been inhibited by ammonium present in the OM treatment.

Different drivers may influence the abundance and expression of uncultivated *Geobacteraceae*. The abundance of the uncultivated *Geobacteraceae* increased in all treatments during the incubation (Figure 9), which may be an indication of the influence of temperature. The bottom water temperature was 18.0°C on the day of core collection (Table 1), whereas the cores were incubated in 20°C water in the DO table (Table 2). Temperature was also thought to drive *nifH* expression in Narragansett Bay sediment cores in a study by Brown and Jenkins (2014), which targeted *nifH* expression by uncultivated sulfate reducers. Expression was the highest when the water temperature was the warmest, during the summer months (Brown and Jenkins, 2014).

Unlike uncultivated *Geobacteraceae*, for which *nifH* expression was not detected in the OM treatment, expression by uncultivated sulfate reducers was stimulated with the addition of OM (Figure 9). Interestingly, uncultivated sulfate reducers dominated GC (Figure 7) and other regions of the upper bay (Brown and Jenkins, 2014; Brown, 2013). Expression by uncultivated sulfate reducers was previously detected to dominate Greenwich Bay and other regions in the upper bay (Brown, 2013), located at the top of the hypoxic and nutrient gradient (Codiga *et al.*, 2009; Melrose *et al.*, 2007). The GC site also had higher total sulfide concentrations compared to the MB site (Figure 3). In the DO table experiment, the OM treatments had higher total sulfide concentrations (Figure 5) and N fixation rates (Figure 6B) than their non-OM counterparts, suggesting diazotrophic activity of sulfate reducers was also stimulated by the OM addition. The sulfate reducers were the likely diazotrophs contributing to the elevated rates. As with the GC field samples, other groups of

sulfate reducers were detected, but could not be targeted. Yet, these groups likely contributed to the overall N fixation rate.

Although the influence of low DO and elevated OM were detected in measurements of total sulfide concentration and N fixation rates, the molecular methods targeting *nifH* expression were essential to elucidating drivers on N fixation activity of two dominant groups of diazotrophs in Narragansett Bay. When studying the entire community, higher N fixation rates and sulfide concentrations were detected under hypoxia and elevated OM loading. However, N fixation rates were a bulk measurement of all the active diazotrophs in the sediment (Burns *et al.*, 2002). Therefore, we used molecular tools to elucidate controls on individual groups in the community. In this study, only molecular analysis indicated that N fixation activity of uncultivated *Geobacteraceae* and sulfate reducers increased in response to hypoxia, but these microbial groups responded differently to the OM addition. Thus, the molecular data helps partition the "black box" of a net biogeochemical signal. Determining the environmental controls and niche partitioning of distinct diazotrophs is critical for understanding and managing N cycle dynamics under exacerbated hypoxia.

Conclusion:

Although hypoxia and organic matter loading may be tightly coupled (Middelburg and Levin, 2009), we performed two experiments to elucidate the influence of hypoxia and OM on the activity of heterotrophic diazotrophs and found two distinct microbial groups functioning differently under these controls. Uncultivated sulfate reducers and uncultivated *Geobacteraceae*, known N fixers

(Brown and Jenkins, 2014; Brown, 2013; Fulweiler *et al.*, 2013; Bertics *et al.*, 2012; Burns *et al.*, 2002; Bazylinski *et al.*, 2000; Welsh *et al.*, 1996; Herbert, 1975), were detected in the field and in the incubation samples, though at different levels. Higher total sulfide concentrations and N fixation rates were detected under hypoxic stress at GC than in MB field cores and in the OM addition treatments of the DO table incubation. This suggests that oxygen depletion stimulates N fixation. However, using molecular tools to target *nifH*, a shift in the community of active diazotrophs was also detected. The dominant diazotrophs appeared to respond to different environmental drivers: one group to low oxygen and the other to low oxygen and OM. Gene expression by uncultivated *Geobacteraceae* dominated the MB site, but was not a major diazotrophic group at the GC site. In the DO table experiment, *nifH* expression by uncultivated *Geobacteraceae* was responsive to low DO, but it was repressed under the OM addition. Conversely, uncultivated sulfate reducers were also detected at MB, but the group dominated *nifH* expression at GC, a site with higher C and N content and high risk for severe hypoxia (Melrose *et al.*, 2007, Deacutis *et al.*, 2006). In addition, *nifH* expression by uncultivated sulfate reducers appeared to be stimulated with the addition of OM in the DO incubation experiment. When cores were exposed to higher OM levels, the dominant species expressing *nifH* shifted towards uncultivated sulfate reducers in OM treatment samples. This suggests that the OM may have stimulated the uncultivated sulfate reducers and/or its decomposition may have lowered the DO conditions, which in turn, stimulated benthic N fixation.

Hypoxia is a growing threat to estuarine and coastal systems worldwide, accelerated by anthropogenic N loading (Diaz and Rosenberg, 2008). Hypoxic

conditions are expected to intensify in frequency and severity with added pressure from climate change (Zhang *et al.*, 2010). However, the impact on benthic N fixation is unclear, as it was only recently found to significantly contribute to the N budget (Fulweiler *et al.*, 2007). The activity and vertical distribution of the sulfate reducers and *Geobacteraceae* that dominate the diazotrophic community of Narragansett Bay may be stimulated by exacerbated DO conditions and elevated OM loading. These anaerobes may provide an additional source of biologically available N, thus establishing a positive feedback loop, exacerbating hypoxia. The sulfide also produced by these microbes may poison benthic organisms and other microbes driving the N cycle, further impacting N cycle dynamics, thus altering the overall N budget of the ecosystem.

Materials and Methods:

Site Characteristics

Narragansett Bay is a medium-sized (370 km²), urban estuary and coastal embayment located in the northeastern US (Nixon *et al.*, 1995) with an average depth at mean tide of 8.31 m (Pilson, 1985). This temperate estuary is relatively well mixed (Melrose, *et al.*, 2007) and has a 4766.2 km² watershed (Boothroyd and August, 2008). Anthropogenic nutrients in Narragansett Bay primarily originate from sewage (Melrose *et al.*, 2007). One of the study sites, Greenwich Cove (41° 39.504' N 71° 26.631' W), is located across from the East Greenwich Wastewater Treatment Plant (Calabretta and Oviatt, 2008) and is part of the larger Greenwich Bay. Greenwich Bay is a shallow (2.6 m mean depth) subembayment (Deacutis *et al.*, 2006) with a poor flushing rate (Abdelrhman, 2004) and weak currents (Spaulding and Swanson, 2008).

Consequently, it is at a high risk for severely hypoxic conditions (Melrose *et al.*, 2007, Deacutis *et al.*, 2006). The Mid Bay site (MB, 41° 35.558'N 71° 22.218'W) is at the low end of the nutrient and anthropogenic influence gradient of Narragansett Bay (Calabretta and Oviatt, 2008). It experiences hypoxia less frequently and severely (Codiga *et al.*, 2009; Melrose *et al.*, 2007; Deacutis *et al.*, 2006).

Field Methods

Environmental sediment cores were collected from the Mid Bay site and Greenwich Cove (GC, 41° 39.504' N 71° 26.631'W) in Narragansett Bay, RI on six time points (MB: July 15, August 6, and September 25, 2013; GC: July 19, July 30, and August 12, 2013). Three cores were collected and sub-sampled at each time point. In addition, fifty-one MB sediment cores were collected from MB on September 25 for an incubation experiment conducted at the U.S. Environmental Protection Agency (EPA, Atlantic Ecology Division, Narragansett, RI).

Divers collected large cores (30.5 cm height, 10 cm inner diameter, 22 cm sediment height) on August 6. The Van Veen grab, with the assistance of the EPA, was used to collect cores for all other time points. These cores (7 cm height, 6.6 cm inner diameter) were inserted into the sediment, while maintaining the sediment depth profile, and filled to a height of approximately 5.5 cm. The boat was moved a few feet throughout the collection period to ensure the collection of “fresh” mud. At each time point, a 60 mL syringe was used to gently top off each core with bottom water, collected with a Niskin bottle. The cores were then capped and stored on ice, protected from light.

Prior to every collection, an YSI (Model #6920, YSI Incorporated, Yellow Springs, CO, USA) was used to record the salinity, temperature, and DO level at the sampling site, generating a depth profile for the water column. A second YSI, attached to a nearby buoy, was deployed for the entire season as part of the Narragansett Bay Fixed-Site Water Quality Monitoring Network, providing salinity, pH, and DO data for the entire season (Heather Stoffel, University of Rhode Island, Graduate School of Oceanography).

Mid Bay Dissolved Oxygen Table Incubation

After collecting the September 25 cores, they were randomly placed into plastic aquaria within the DO table at the EPA. The cores were fully submerged in seawater that was continuously circulated by the DO system, which controlled the water temperature (heated to 20°C) and DO concentration, among other factors. After pulling in water from the bay, a vacuum system established two DO concentrations: 7.0 mg L⁻¹ (normoxic treatment) and 0.5 mg L⁻¹ (hypoxic treatment). Since N₂ gas may also be removed by the vacuum system, excess N₂ gas was gently bubbled into the incoming water as starting material for the N fixation reaction.

The cores were randomly divided into four treatments: normoxic, hypoxic, normoxic + organic matter, and hypoxic + organic matter, with 12 cores per treatment. 0.543 g of organic matter (Spray Dried Marine Phytoplankton, E.S.V. Aquarium Supplies, Hicksville, NY, USA) was sprinkled on top of each core within the organic matter treatments. Four plexiglass aquaria (25 cm x 46 cm x 15 cm) separated the cores based on treatment. Plastic lids covered the aquaria to inhibit the exchange of

oxygen between the water and air. Aphotic conditions, characteristic of the estuarine floor, were maintained by the use of opaque, black plastic sheets secured with rocks.

Two YSIs were placed in a hypoxic and a normoxic aquarium, which contained no cores. The YSIs recorded the conductivity, DO, and temperature every 15 minutes for the duration of the experiment. To account for the long body of the YSI, the plastic lid did not completely cover the YSI aquaria, thus introducing some oxygen to the YSI aquaria. Consequently, the recorded DO levels were higher than the aquaria containing cores. YSI recordings were used to determine the stability of the system and to provide a continuous log of the temperature and approximate DO levels.

During the first week of incubation, the incoming water was filtered and Winkler titrations were conducted every 2 to 3 days to monitor the DO levels and calibrate a DO probe (Hack Model HQ10, Loveland, CO, USA). The DO level and temperature was also monitored daily with the DO probe. However, one week into the incubation, the federal government was shutdown for 16 days. As visiting scientists, our access to the EPA facility was denied. Fortunately, a few essential EPA employees were able to check on the incubation experiment and change the N₂ gas tank as needed, allowing the experiment to continue. During this period, the YSIs continued to record the experimental conditions. After the shutdown ended, the DO concentrations were measured using the DO probe and Winkler titrations. Originally, the experiment was planned for two weeks with sediment subsampling at the incubation midpoint and the conclusion of the experiment. However, as a consequence of the shutdown, the incubation was extended to three weeks and there was no midpoint collection.

Unisense profiles were recorded upon collection from the field and after five days of incubation, before the government shutdown. Sediment samples for nitrogenase activity and molecular analysis were collected from the field and on the last day of incubation. The field cores were also sectioned for carbon:nitrogen content analysis samples.

Carbon:Nitrogen Content

The sediment collected for carbon:nitrogen analysis was placed in aluminum tins and dried overnight, at 60°C. After removing shells, the samples were homogenized and ground with a mortar and pestle. The mortar and pestle were cleaned with distilled water and ethanol between samples. Samples were stored in Wheaton 2 mL glass vials (Millville, NJ USA) until analysis. A microbalance was used to place approximately 10-15 mg of sediment was placed into an ultra-clean tin capsule. After crimping the capsule closed, it was placed into a nickel sleeve. The CHN Lab at the University of Rhode Island, Graduate School of Oceanography analyzed the carbon to nitrogen content of the sediment samples using acetanilide standards and an Elemental Analyzer Model CE 440 (Exeter Analytical Inc., North Chelmsford, MA, USA) with PC Compatible CE-490 Interface Unit.

Unisense

Sediment depth microprofiles of DO, hydrogen sulfide, and in some cases, pH, were measured using a Unisense microprofiling system (Unisense A/S, Denmark: Microsensor Multimeter, Micromanipulator MM33-2, Laboratory Stand LS18) prior to core sectioning. When 7 cm cores were collected, two (DO incubation experiment) or three (field) cores were measured, with one profile set per core. When 30 cm cores

were sampled, one core per treatment was used for the Unisense profiling. Due to the large surface area of these cores, three profiles per core were measured. The cores were moved randomly so the triplicate profiles measured different areas of the core.

The pH and hydrogen sulfide profiles were used to calculate total sulfide concentrations, following the manufacturer's instructions. The measurements were then averaged, generating the sediment oxygen penetration profile and the hydrogen sulfide concentration and pH depth profiles. The pH profile could not be collected on some sampling days, in which case pH values from the hypoxic incubation treatment or MB September 25 cores were used to calculate total sulfide. The oxygen profiles were used to determine the depth of the water-sediment interface.

Prior to use, the oxygen and hydrogen sulfide probes went through a pre-polarization period for a minimum of two hours. Probe calibration and measurements followed instructions provided by the manufacturer (Unisense OX-50 (oxygen), H2S-100 (hydrogen sulfide), and pH-100 (pH)). Measurements were collected in 250 to 1000 μm increments to depths of 10,000 to 30,000 μm using a micromanipulator. July 15 measurements were collected, but not analyzed due to equipment failure.

Sediment Core Subsampling

For every collection, three sediment cores were extruded and subsampled. The field cores were divided into two sections (top: 0-0.5 cm, bottom: 0.5-2.0 cm) on July 19 (GC), August 12 (GC), and September 25 (MB) and three sections (top: 0-0.5 cm, middle: 0.5-1.0 cm, bottom: 1.0-2.0 cm) on July 15 (MB) and July 30 (GC). The August 6 MB cores were sectioned into three sections: 0-0.25 cm, 0.25-0.5 cm, and 0.5-1.0 cm. Only the top 0-0.5 cm was collected from the DO table experimental

cores, which were sectioned on the day of core collection and after three weeks of incubation.

Sediment samples were collected from each depth in duplicate or triplicate and immediately flash frozen in liquid nitrogen to be later extracted for RNA (0.5 mL or 1.0 mL of sediment) and DNA (0.25 mL or 1.0 mL of sediment). 5 mL of sediment from each section of the July 30 GC and the MB field cores was saved in an aluminum tin for C:N analysis. 4 mL of sediment from the 0-0.5 cm depth (top sample) and, for the field cores, 4 mL of sediment from the lower depths (>0.5 cm, bottom sample) were collected in duplicate or triplicate and placed into test tubes for the acetylene reduction assay. As previously described (Capone 1993), the headspace of the samples was flushed with nitrogen gas and then spiked with acetylene gas. The production of ethylene from acetylene was measured by a Shimadzu GC8 gas chromatograph (Shimadzu Corporation, Kyoto, Japan).

Molecular Analysis

The PowerSoil DNA Kit (MO Bio Carlsbad, CA, USA) was used to extract total DNA from 0.25 g of sediment, following the protocol from the manufacturer. Samples containing 1.0 g of sediment were aliquoted into four tubes of 0.25 g (DNA) or two tubes of 0.5 g (RNA) of sediment and pooled into one tube at final elution step. After quantifying the total DNA concentration with the Qubit dsDNA BR Assay Kit (Invitrogen/Life Technologies, Grand Island, NY, USA), the samples were diluted to $1 \text{ ng } \mu\text{L}^{-1}$ for quantitative real-time PCR.

Total RNA was extracted from 0.5 g sediment samples with the PowerSoil RNA Isolation Kit (MO Bio Carlsbad, CA, USA). A modified protocol was used, as

the manufacturer protocol is designed for 2.0 g of sediment. After the precipitation of RNA, the RNA pellet was dried and resuspended in 100 μL nuclease-free water. The Ambion TURBO DNA-*free* Kit (Ambion/Life Technologies, Grand Island, NY, USA) was used for DNA removal. After the addition of 10 μL of 10X TURBO DNase Buffer and 2 μL of TURBO DNase, the samples were incubated at 37°C for 30 minutes. A 5-minute incubation followed the addition of 10 μL DNase Inactivation Regent to deactivate and remove DNase. The Qiagen RNeasy Mini Kit (Valencia, CA, USA) was used for RNA purification. The manufacturer protocol was coupled with the addition of Qiagen RNase Free DNase Set to the spin column. After the elution of the RNA, the RNA concentration was measured with the Qubit RNA HS or BR Kits and diluted to 4 ng μL^{-1} . The SuperScript III First-Strand Synthesis System (Invitrogen/Life Technologies) was used to synthesize first-strand cDNA to prepare the samples for PCR and quantitative real-time PCR. The manufacturer's protocol was used, but with minor revisions. For every sample, two tubes of 8 μL of the diluted RNA were mixed with 1 μL of 2 μM outer reverse *nifH* and *nirS* primers and 1 μL of dNTPs. The first tube received the SuperScript III reverse transcriptase. The second tube served as the control to verify that there was not any DNA contamination in the sample. After the addition of the reverse transcriptase, the samples were incubated at 50°C for 50 minutes and 85°C for 5 minutes. The reverse transcriptase samples then received 0.5 μL of RNase.

Nested PCR was used to isolate and amplify the *nifH* gene. As previously described by Brown and Jenkins (2014), degenerate outer primers (*nifH4* and *nifH3*) and inner primers (*nifH1* and *nifH2*) were used with Bioline Bio-X-Act Short Mix

(Taunton, MA, USA). The two rounds of PCR were performed using the following program: a 2 minute initial 94°C denaturation step, 30 cycles of a 94°C denaturation step for 30 seconds, a 50°C annealing step for 30 seconds, and a 72°C extension step for 1 minute, followed by a final 7 minute extension step at 72°C.

Following PCR amplification, the samples were run on a 1.0% agarose (wt/vol) TAE gel. If bands were present in the lanes containing no reverse transcriptase samples, the RNA samples received another DNase treatment using the Ambion TURBO DNA-*free* Kit and the cDNA synthesis and PCR steps were repeated. Otherwise, bands of approximately 350 bp in length were cut and extracted from the gel using Qiagen QIAquick Gel Extraction Kit. The concentration of the eluted sample was measured with the Thermo Scientific NanoDrop 2000c. The pGEM-T Vector System (Promega, Madison, WI, USA) was used to clone the purified PCR products. The clones were then transformed into JM 109 *E. coli* competent cells (Zymo Research, Irvine, CA, USA). Colonies with the insert were selected using blue-white screening and used to inoculate 2 mL LB broth, 2 µL carbenicillin. After a 16 hour incubation at 37°C, the Qiagen QIAprep Spin Miniprep Kit was used for plasmid purification. The concentration of the purified product was measured on the nanodrop. Each sample was diluted to 400 ng per reaction. 20 µL of the diluted sample sequenced at the RI Genomic Sequence Center at the University of Rhode Island on the Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The sequence results were imported into Geneious R6 (Biomatters, New Zealand). The *nifH* sequences were converted to their reversed complement as needed,

trimmed to only include the region between the inner *nifH* primers, and translated into NifH amino acid sequences. BLAST searches of these sequences were used to identify closely related, cultivated species. The translated environmental sequences and the translated cultivated species were then aligned with MUSCLE, the multiple sequence alignment tool (Edgar, 2004) within Geneious. This alignment was used to build a *nifH* maximum likelihood tree of expressed *nifH* sequences using PhyML in Geneious (1000 bootstrap replicates).

Using the Stratagene Mx3005 qPCR system (Agilent Technologies Santa Clara, CA, USA) and LightCycler 480 Probes Master (Roche, Switzerland), quantitative real-time PCR (qPCR) was performed to amplify and detect the *nifH* gene in DNA and cDNA samples. As described by Brown and Jenkins (2014), degenerate qPCR primers and dual-labeled TaqMan probes were used to target two *nifH* groups, NB3 (uncultivated *Geobacteraceae*) and NB7 (uncultivated sulfate reducers), whose most closely related cultivated species were *Pelobacter carbinolicus* and *Desulfovibrio vulgaris* respectively. For every qPCR run, a standard curve was generated from a plasmid dilution series and used to determine the absolute *nifH* gene copy or transcript copy number in the sediment samples. The dilution series, performed in triplicate, contained a sequenced *nifH* clone from the targeted group. The 10-fold dilution series ranged from 1 ng to 1 ag of linearized plasmid. Each reaction (20 μL total) contained 10 μL of the Roche LightCycler 480 Probes Master, 5.7 μL of water, 2 μL of the primer/probe mix, 0.3 μL of the Stratagene Brilliant II qPCR ROX reference dye, and 2 μL of sample (1 ng μL^{-1} DNA or 1.5 ng μL^{-1} cDNA). The primer/probe mixes were at concentrations of 0.4 μM (NB3) and 0.2 μM (uncultivated

sulfate reducers). The qPCR thermocycling conditions were: 1 cycle at 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, and 60°C (uncultivated *Geobacteraceae*) or 55°C (uncultivated sulfate reducers) for 1 minute. Since three cores were collected for each time point and treatment, two to three replicates were used for each depth and time point. A few cDNA samples were not analyzed due to DNA contamination in the no RT controls. DNA and cDNA samples were performed in duplicate and triplicate respectively.

Attempts were made to design a primer and probe set to specially target NB5 and exclude NB7. We tried two qPCR chemistries (LightCycler 480 SYBR Green I Master and LightCycler 480 Probes Master) and attempted to optimize the reaction with a range of primer and probe concentrations and annealing temperatures. Two fluorescent peaks, corresponding to primer-dimers and the amplicon, were detected in the dissociation curve plots when using SYBR-based qPCR. After designing a probe to selectively amplify the amplicon, the lower standard concentrations and the extracted samples were not detected with probe-based qPCR. The process was further complicated by the highly conserved, GC rich characteristic of the *nifH* region. Consequently, only a few primer/probe combinations could be designed to specifically target the NB5 group.

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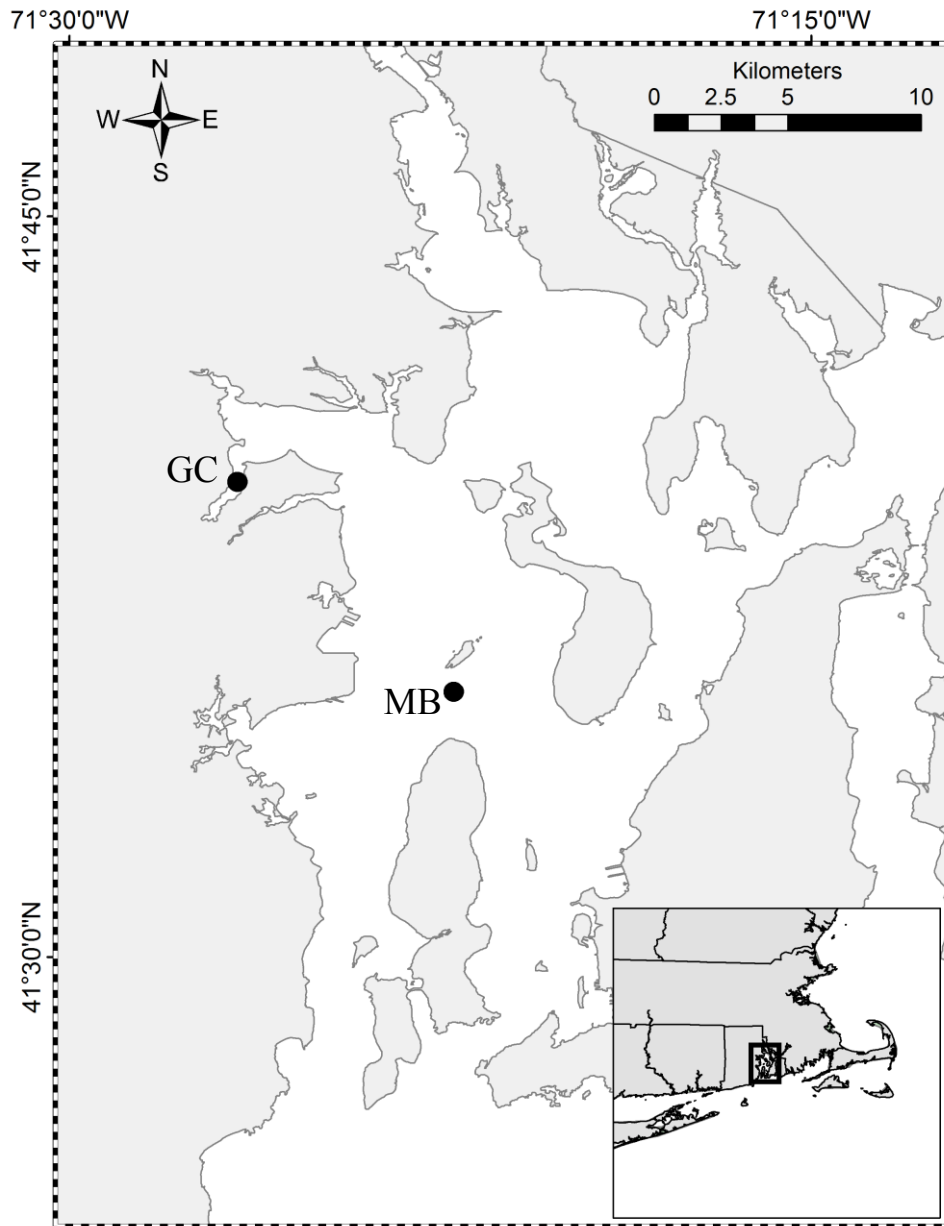


Figure 1: Map of Narragansett Bay, RI. Black circles denote sampling sites: Greenwich Cove (GC) and Mid Bay (MB). (Courtesy C. McManus.)

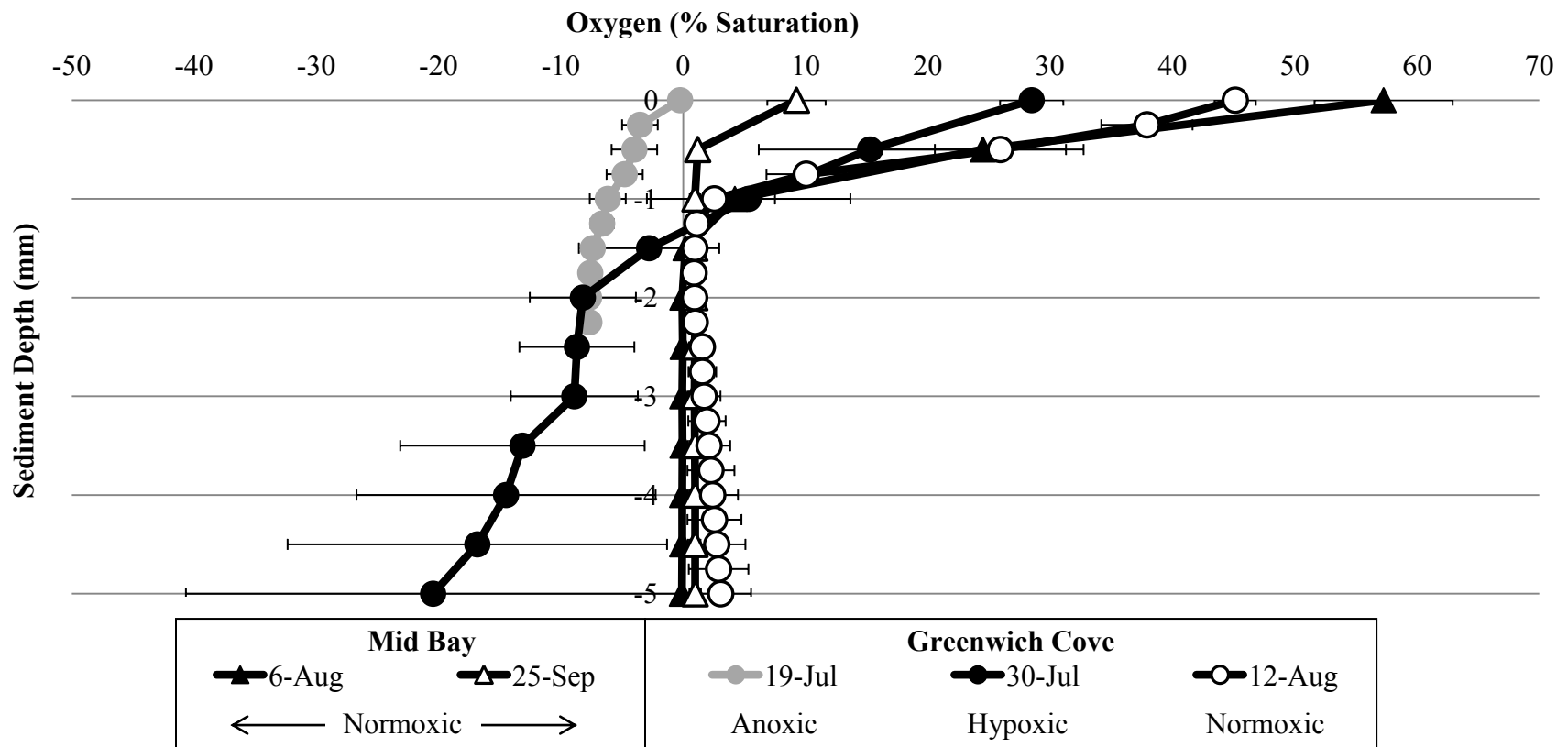


Figure 2: Average downcore oxygen profiles for field samples. Measurements were collected down to 10 or 30 mm for most time points, but no changes were observed beyond 5 mm. Error bars represent standard error of mean.

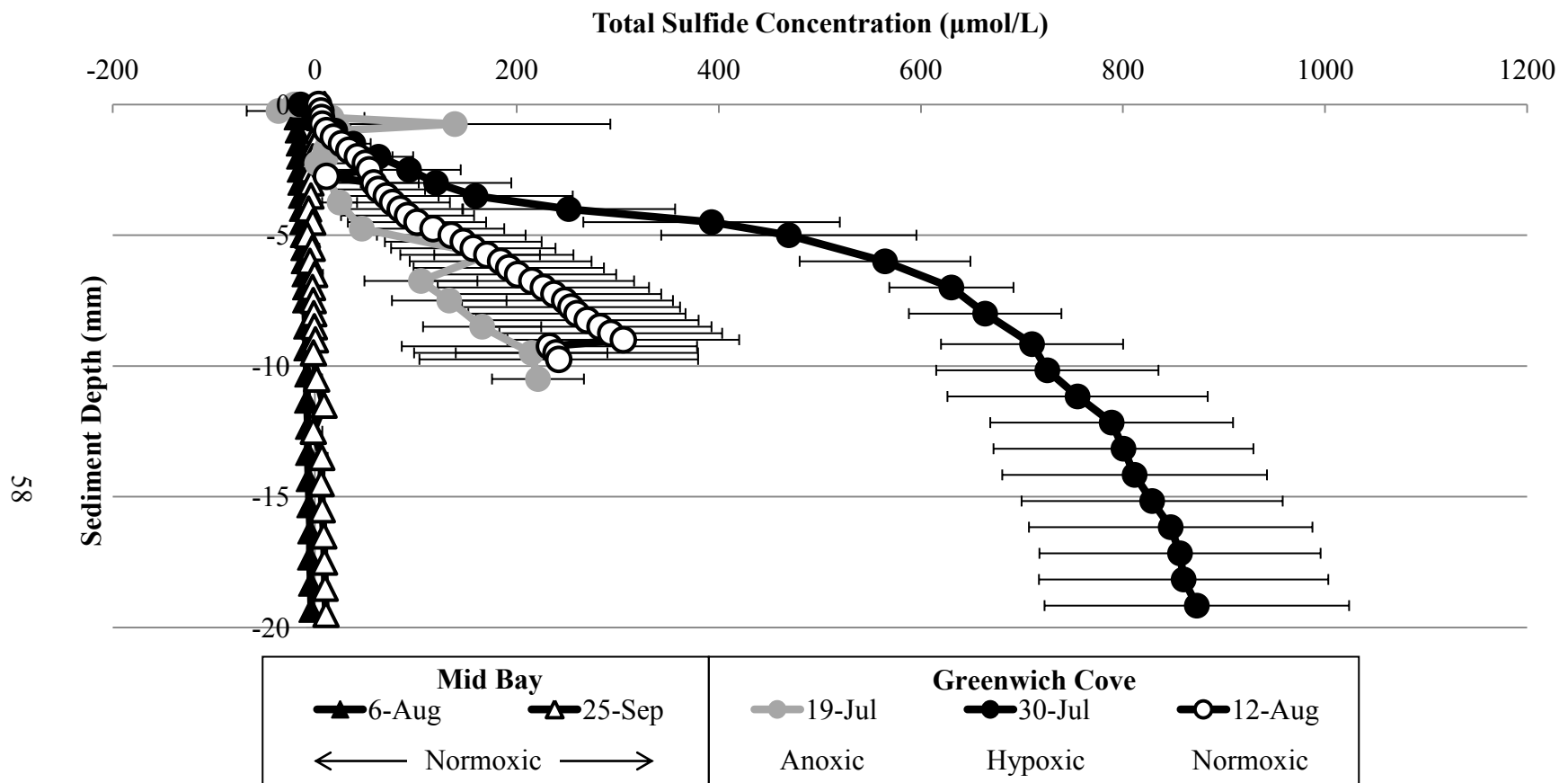


Figure 3: Average downcore total sulfide profiles for field samples. Measurements were collected down to 30 mm for July 30, August 6, and September 25, but no changes were observed beyond 20 mm. Error bars represent standard error of mean.

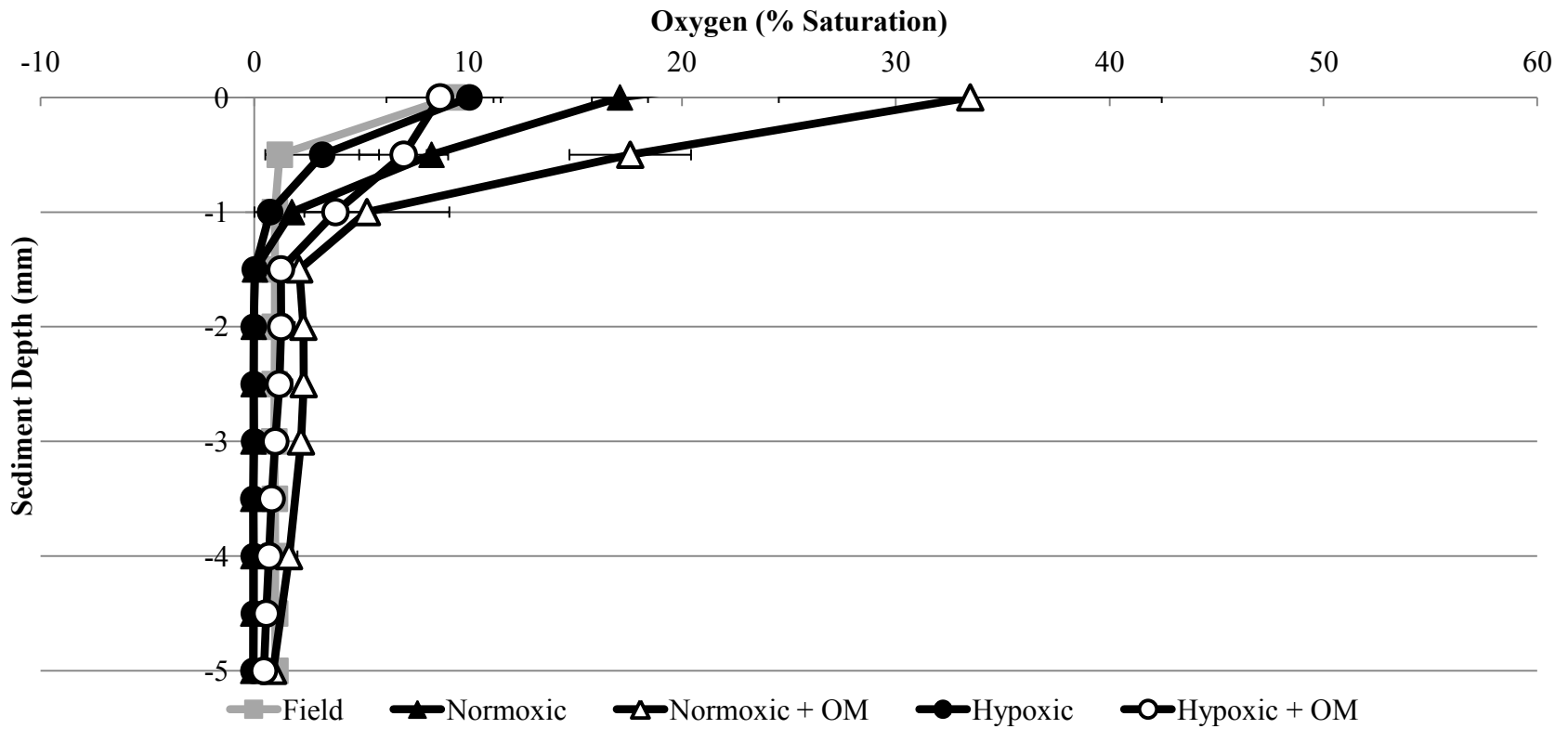


Figure 4: Average downcore oxygen profiles for September 25 (field) and DO table incubation cores. Measurements were collected down to 30 mm, but no changes were observed beyond 5 mm. Error bars represent standard error of mean.

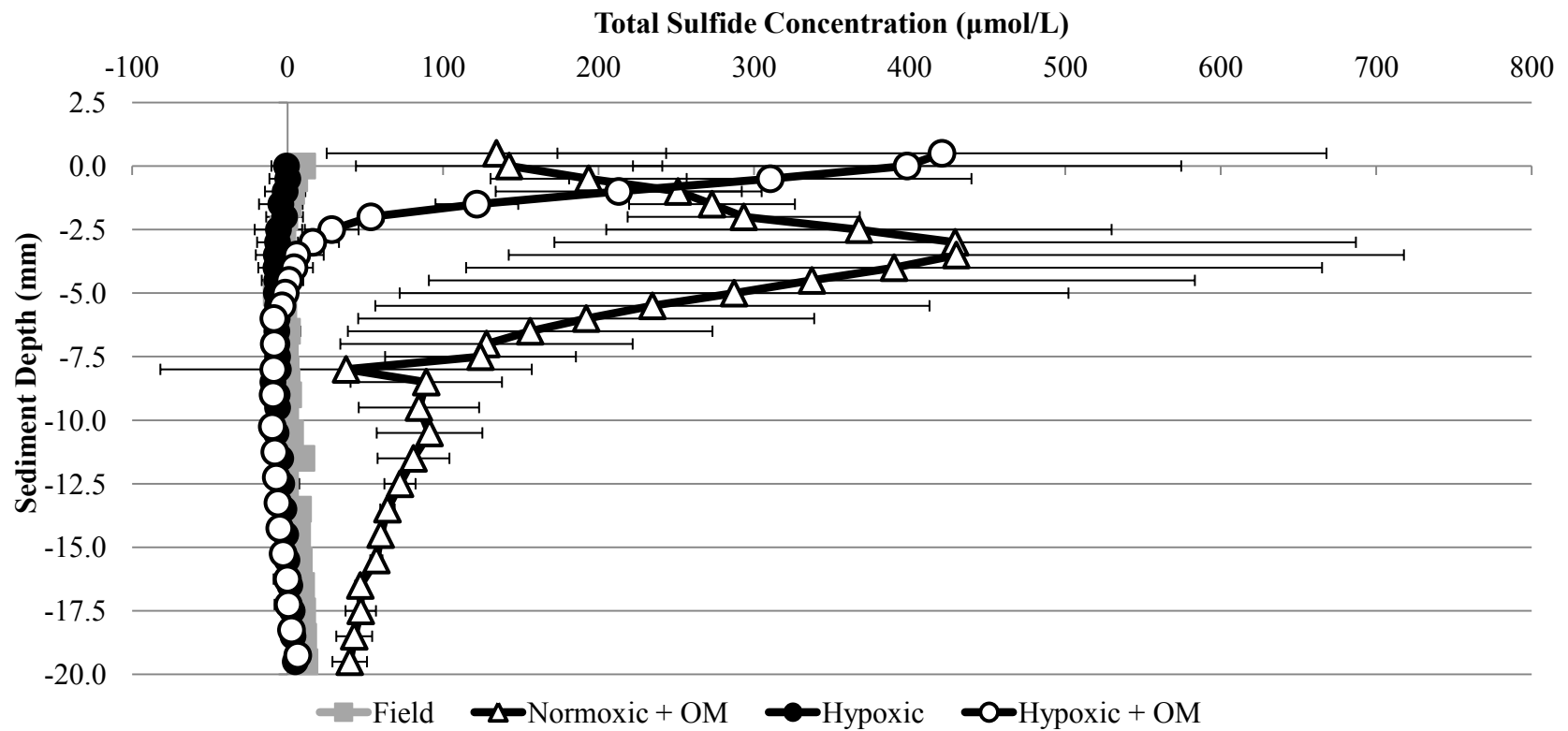


Figure 5: Average total sulfide profiles for September 25 (field) and DO table incubation cores after five days of incubation. Measurements were collected down to 30 mm, but no changes were observed beyond 20 mm. Error bars represent standard error of mean. The normoxic profile was omitted due to low sulfide concentrations and high pH (Table 4).

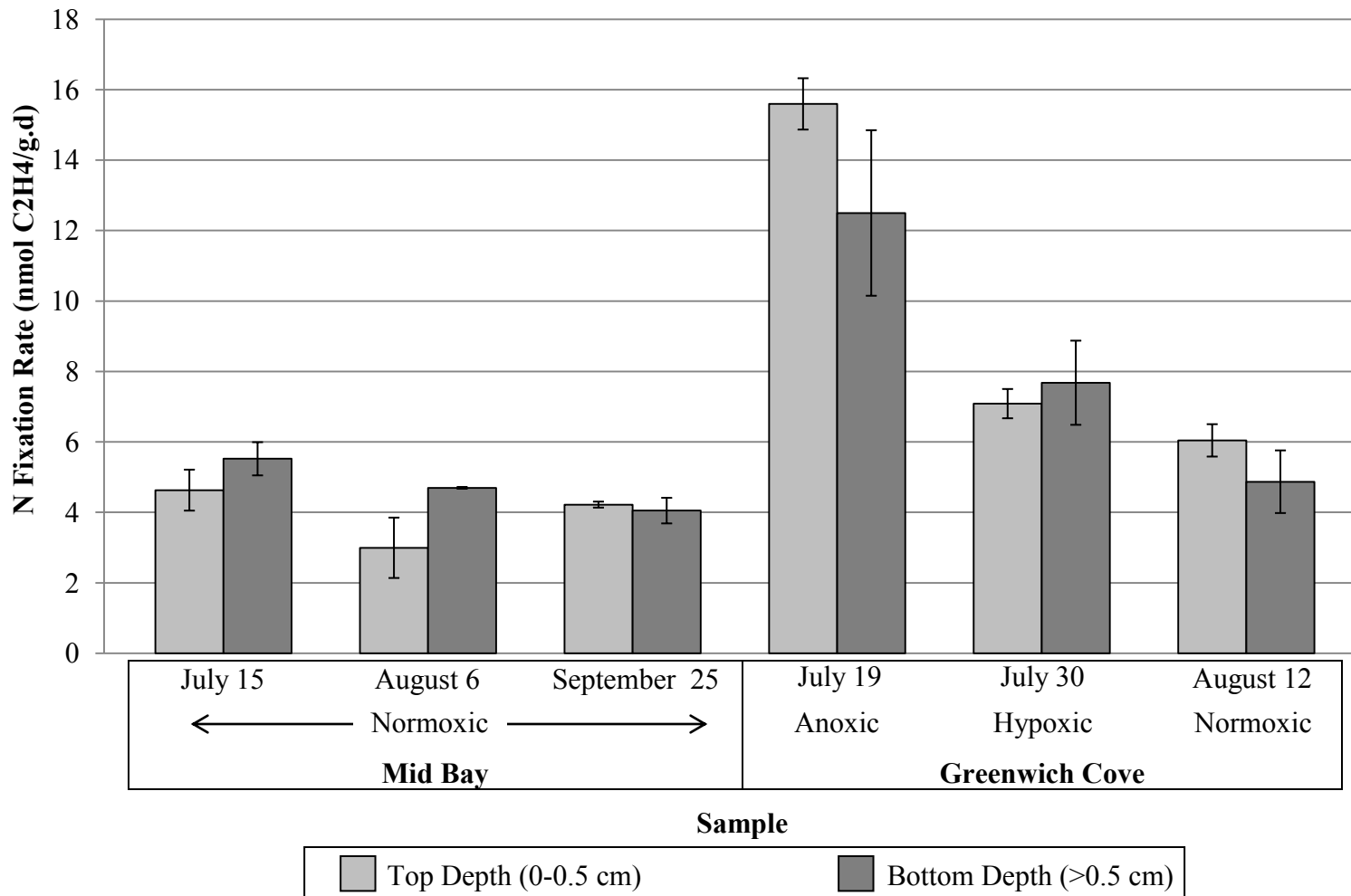


Figure 6A: Nitrogen fixation rates in field. Rates measured by acetylene reduction assay (Spinette *et al.*, in prep). Error bars represent standard error of mean.

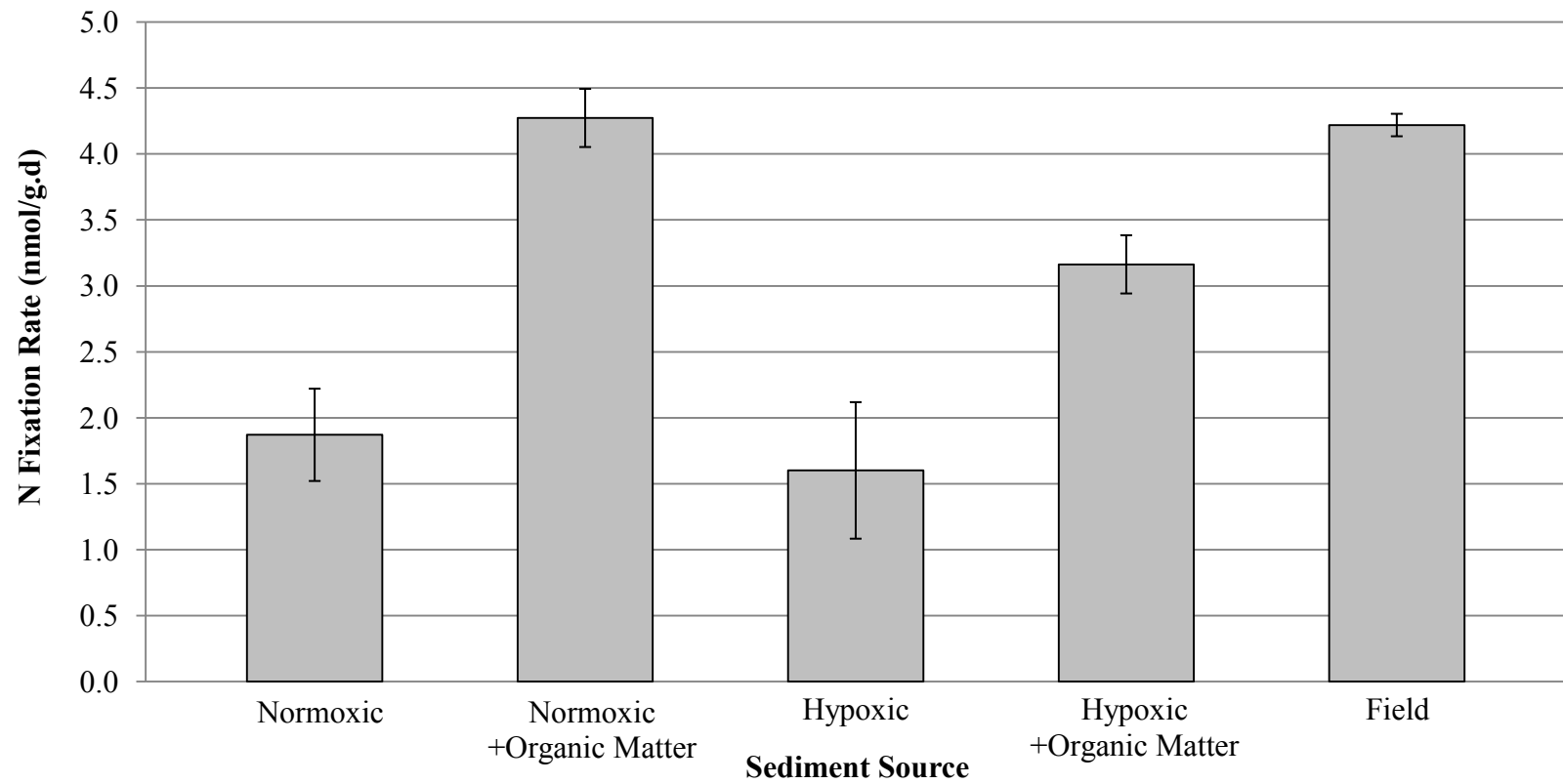


Figure 6B: Nitrogen fixation rates in DO table incubation. The field measurement represents the rate for the field cores that were used to set up the incubation, when the cores were collected from the field on September 25 (rate data courtesy: R. Spinette and A. Jones, URI). Error bars represent standard error of mean.

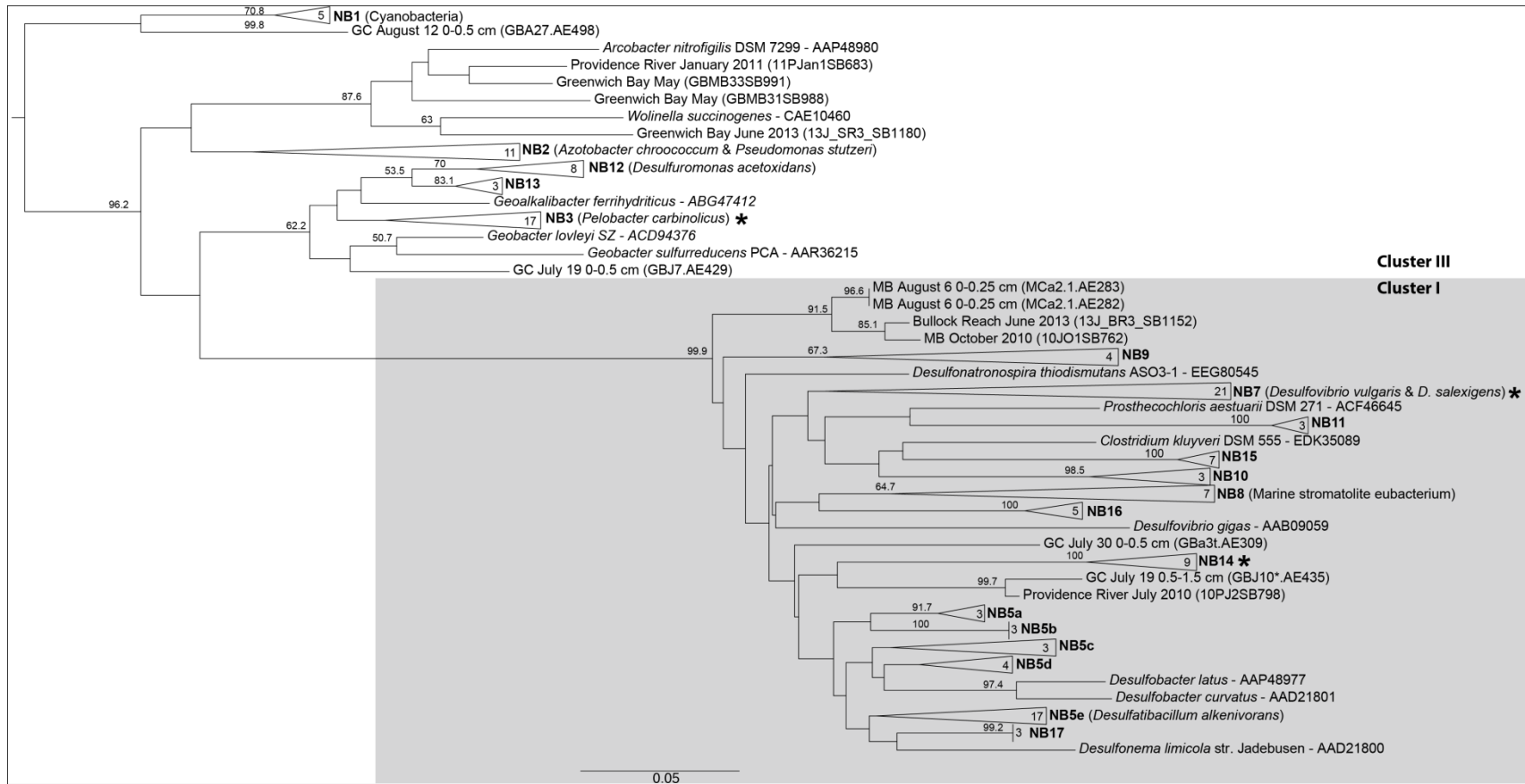


Figure 7: Maximum likelihood protein tree of expressed *nifH* sequences extracted from sediment samples and closely related cultivated species. Cluster I and III designations were assigned according to Chien and Zinder (1996). Groups NB1-11 (Fulweiler *et al.*, 2013; Brown and Jenkins, 2014) and NB12-14 (Brown, 2013) were previously reported. The total number of sequences contained in each group are indicated by the number within the grouping. The values at listed at the node represent the bootstrap values (1000 replicates). Only bootstrap values above 50% were listed. Groups targeted for quantitative PCR are denoted with an asterisk. Only some sequences within NB14 are targeted by NB7 primer set.

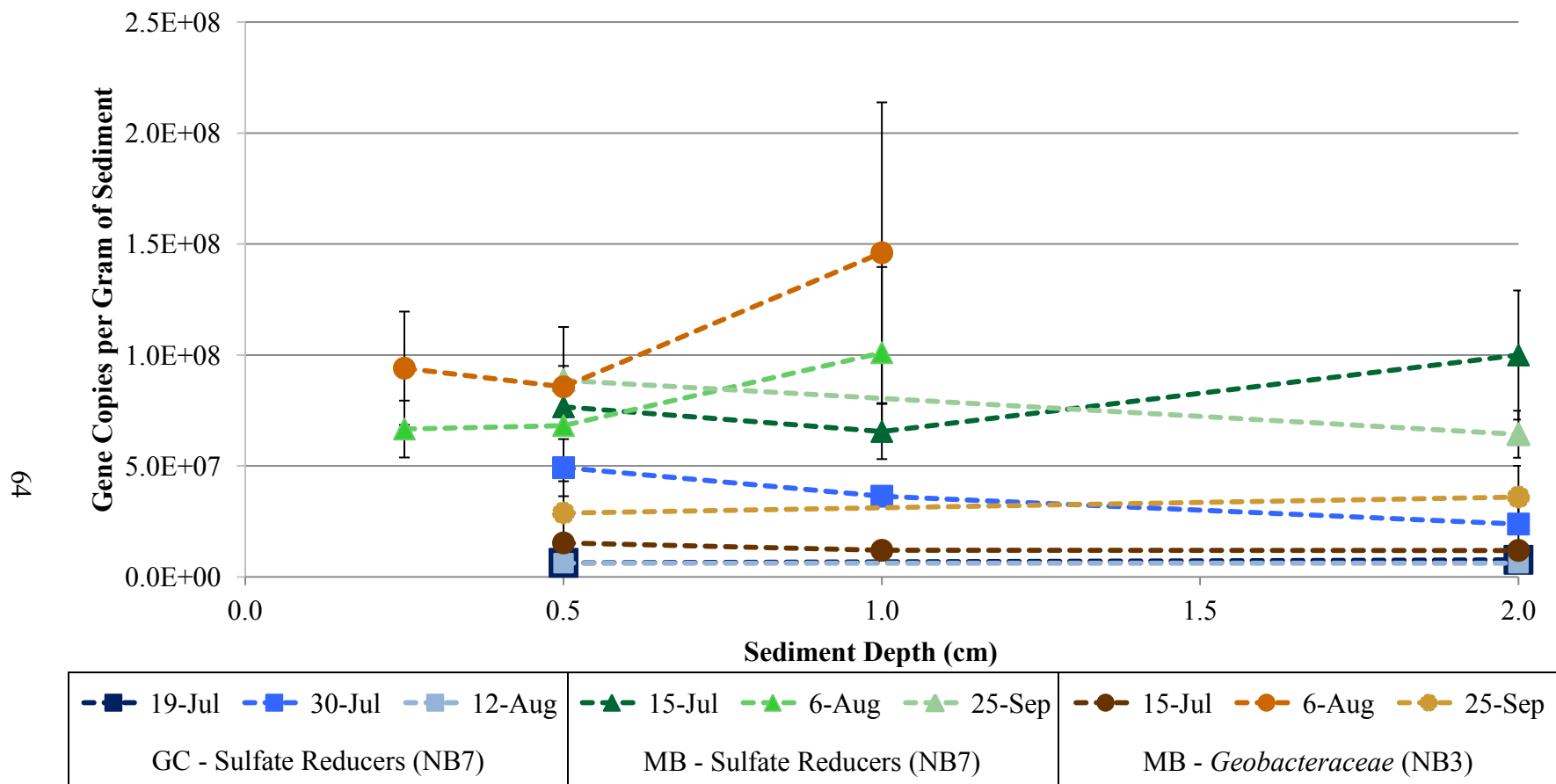


Figure 8A: Abundance of groups NB3 and NB7 extracted from field samples (average). Sampling increments varied, but ranged between 0.25 – 1.5 cm. Data for each sediment section was graphed using the lowest depth (i.e. 2 cm was used for the 1.0-2.0 cm section). Error bars represent standard error of mean.

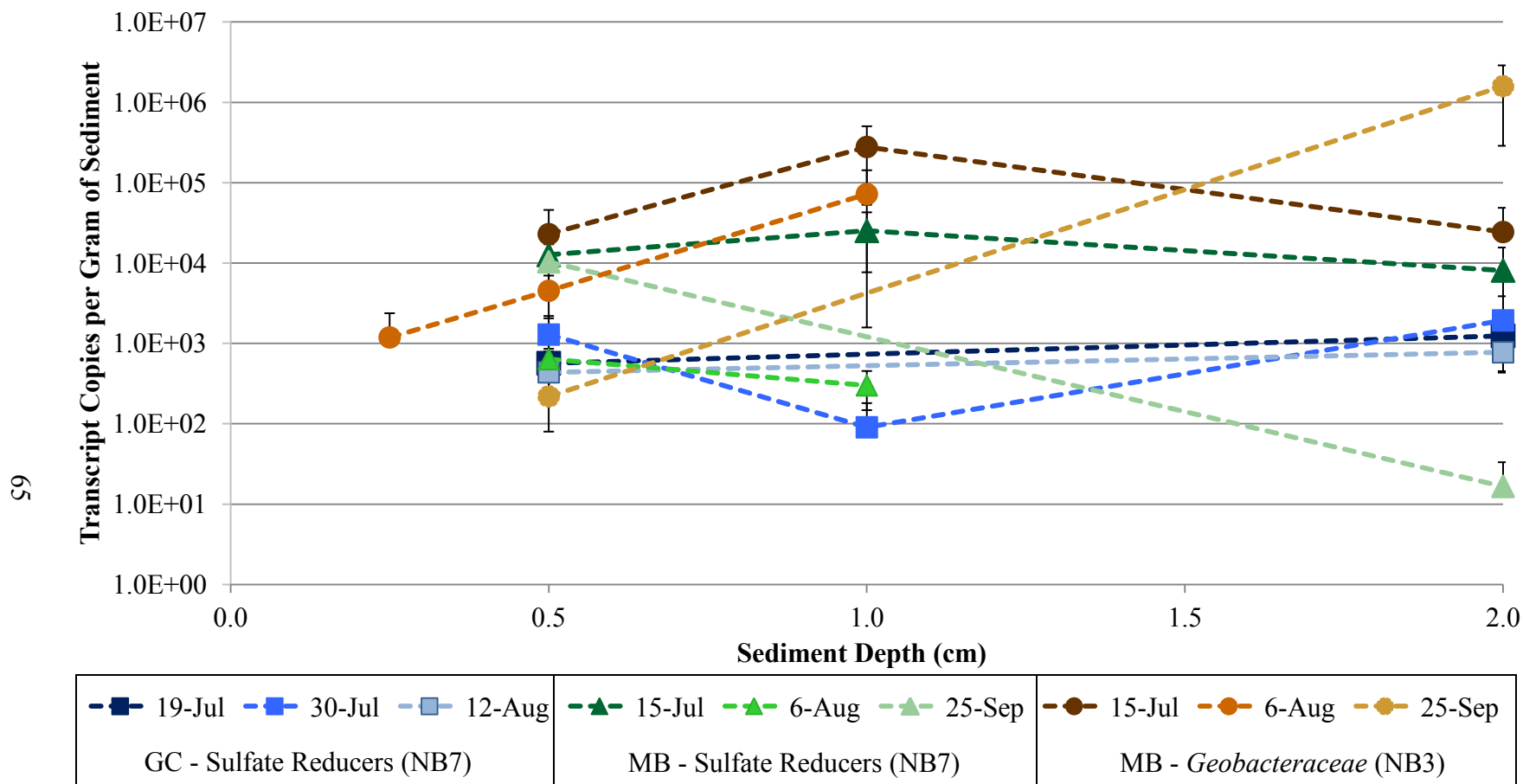


Figure 8B: Expression of groups NB3 and NB7 extracted from field samples (average). Sampling increments varied, but ranged between 0.25 – 1.5 cm. Data for each sediment section was graphed on a logarithmic scale, using the lowest depth (i.e. 2 cm was used for the 1.0-2.0 cm section). Error bars represent standard error of mean. No *nifH* expression by group NB7 was detected at the MB 0.25 cm depth on August 6.

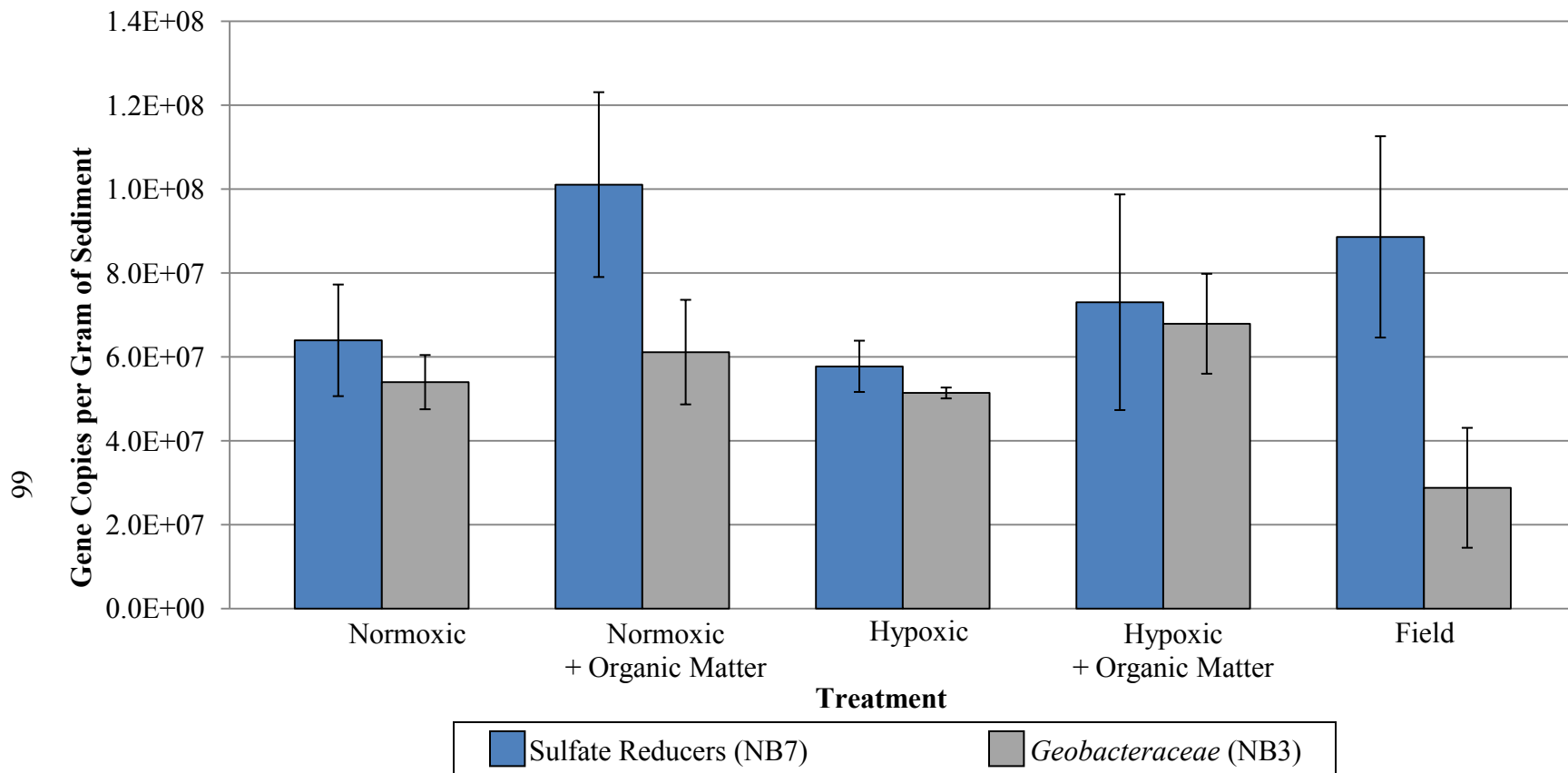


Figure 9: Abundance of groups NB3 and NB7 extracted from the 0-0.5 cm depth of the DO table incubation cores (average). Error bars represent standard error of mean. The field measurement represents the September 25 field cores, which were used to set up the incubation.

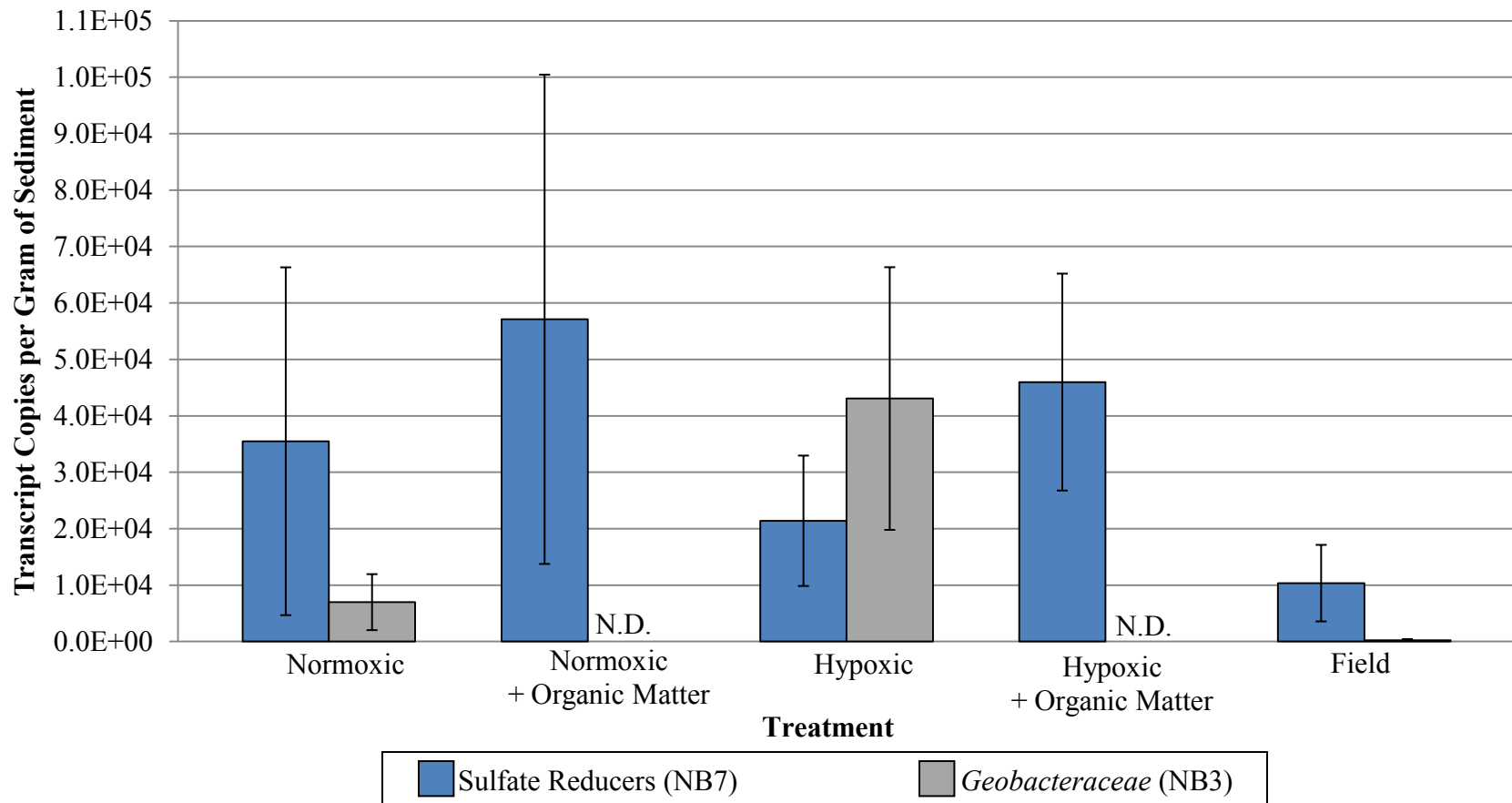


Figure 10: *nifH* expression of groups NB3 and NB7 extracted from the 0-0.5 cm depth of the DO table incubation cores (average). Error bars represent standard error of mean. The field cores were collected on September 25 and used to set up the incubation experiment.

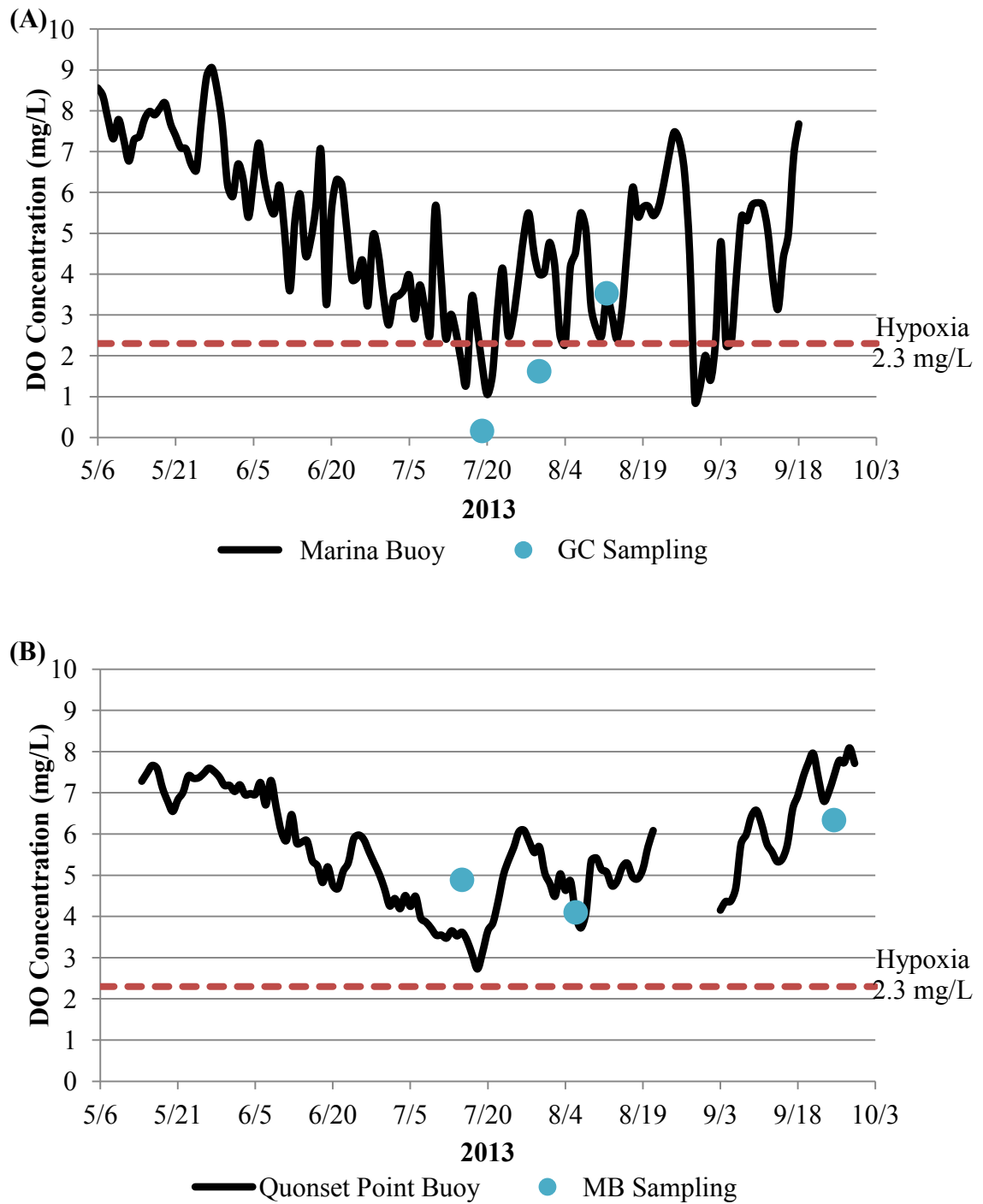


Figure 11: DO concentrations over the 2013 season. Buoys monitored bottom water conditions at sites in close proximity to the study sites. (A) The field cores were collected at Greenwich Cove under anoxic (July 19), hypoxic (July 30), and normoxic (August 12) conditions. (B) All Mid Bay field cores were collected under normoxic conditions. The buoy data was used to estimate the DO conditions at the sampling sites for the entire 2013 season and were part of Narragansett Bay Fixed-Site Monitoring Network (buoy data courtesy: H. Stoffel, URI GSO).

Table 1: Site coordinates and bottom water conditions at time of sediment core collection. Three cores were collected on each time point.

Site	Latitude	Longitude	Date Sampled (2013)	Temperature (°C)	Dissolved Oxygen (mg L⁻¹)	Salinity (ppt)
MB	41° 35.558' N	71° 22.218'W	July 15	19.3	4.90	28.46
			August 6	20.1	4.10	30.07
			September 25	18.0	6.34	30.01
GC	41° 39.504'N	71° 26.631'W	July 19	23.3	0.16	27.17
			July 30	23.6	1.60	26.94
			August 12	23.5	3.53	25.87

Table 2: DO table conditions. DO and water temperature were measured before and after the government shutdown with a DO probe. Two YSIs recorded measurements every 15 minutes for the duration of the incubation experiment. The DO levels of the YSI aquaria were slightly higher than in the aquaria containing sediment cores. Oxygen was introduced into the YSI aquaria as a result of YSI placement.

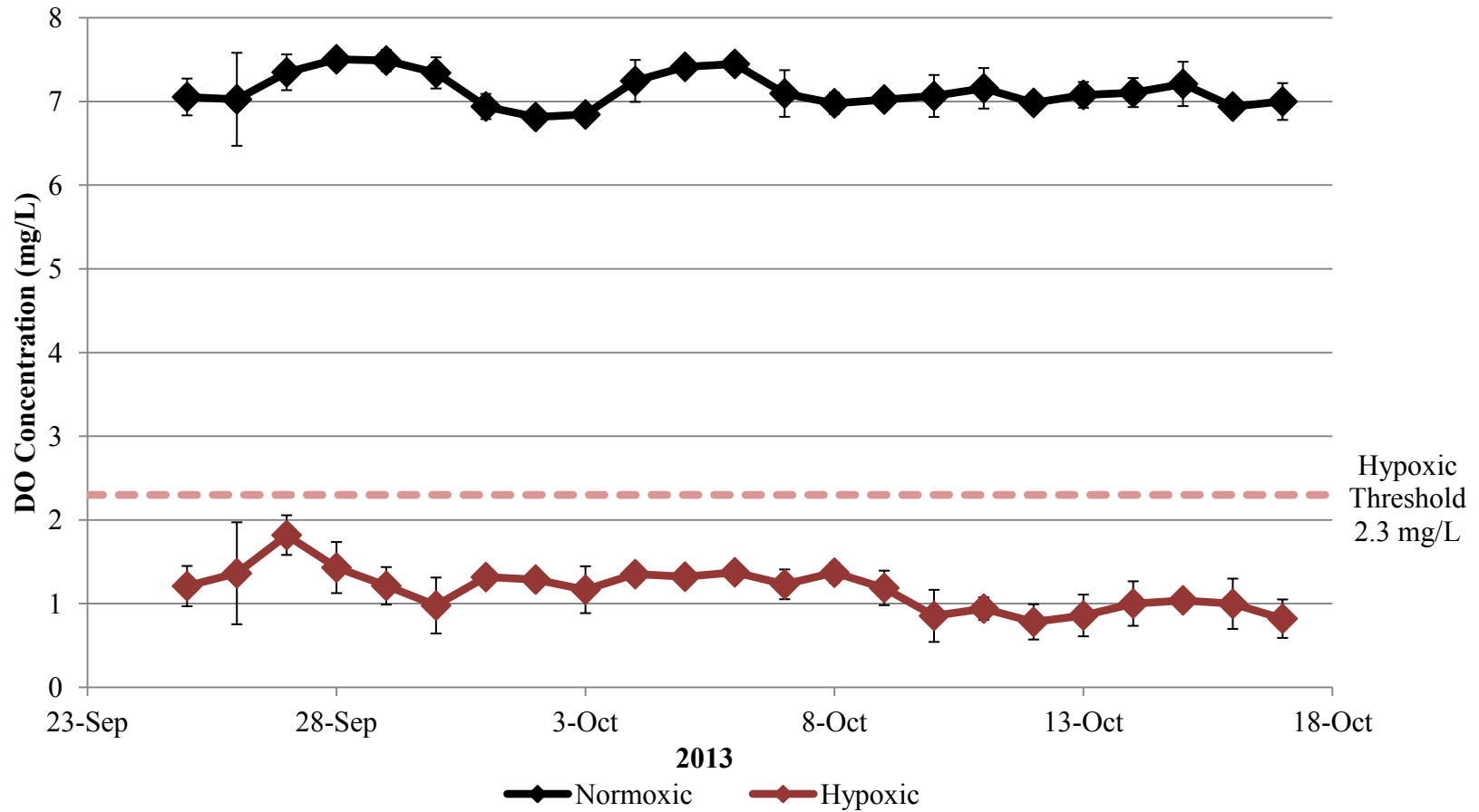
Treatment	YSI Measurements (Average; n=2109 per treatment)			DO Probe Measurements			
	Temperature (°C)	Dissolved Oxygen (mg L ⁻¹)	Conductivity (mS/cm)	Pre-Shutdown (Average; n=4)		Post-Shutdown (October 17; n=1)	
				Temperature (°C)	Dissolved Oxygen (mg L ⁻¹)	Temperature (°C)	Dissolved Oxygen (mg L ⁻¹)
Normoxic	19.9 (±0.82)	7.14 (±0.29)	47.3 (±1.09)	19.7 ±0.2)	6.42 (±0.22)	20.9	6.17
Normoxic + OM	N/A			20.0 ±0.4)	6.40 (±0.30)	20.8	5.89
Hypoxic	21.3 (±0.82)	1.18 (±0.34)	47.8 (±0.34)	21.3 (±0.4)	0.60 (±0.05)	22.2	0.23
Hypoxic + OM	N/A			21.3 (±0.3)	0.63 (±0.10)	22.3	0.43

Table 3: Downcore carbon and nitrogen content of field cores to depth of 2.0 cm. Sediment samples were collected from duplicate or triplicate cores. The carbon and nitrogen content of the organic matter used for the DO table incubation treatment is also included. Values are averaged with standard error of the mean in parentheses.

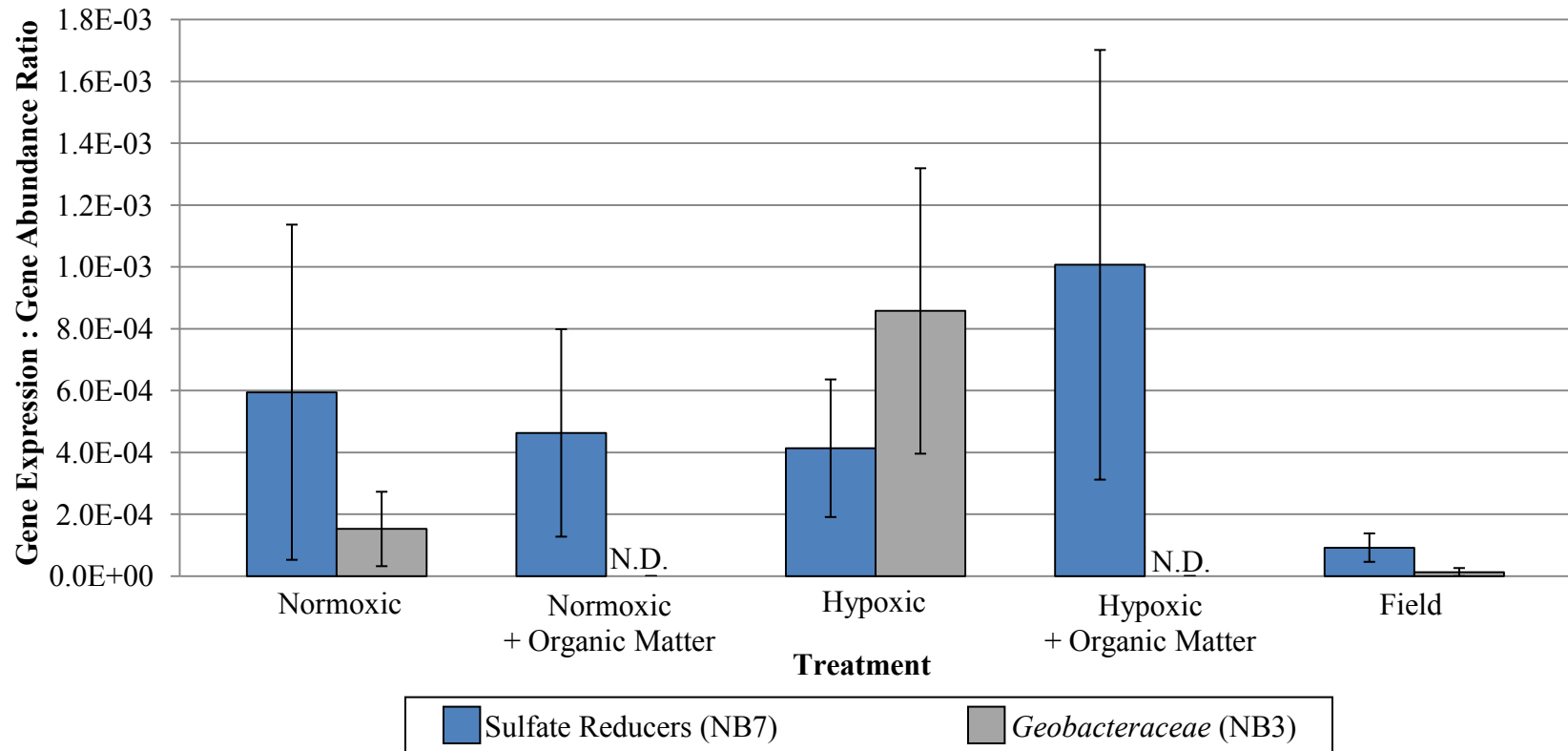
Site/ Sample Type	Date Sampled (2013)	Depth (cm)	Weight Percent N (%)	Weight Percent C (%)	C:N Ratio
MB Sediment	July 15	0-0.5	0.21 (± 0.01)	2.10 (± 0.05)	11.9 (± 0.38)
		0.5-1.0	0.19 (± 0.01)	1.94 (± 0.17)	11.6 (± 0.57)
		1.0-2.0	0.20 (± 4.9 x10 ⁻³)	2.01 (± 0.01)	11.7 (± 0.31)
	August 6	0-0.25	0.19 (± 4.1 x10 ⁻⁵)	1.83 (± 2.6 x10 ⁻⁴)	11.0 (± 0.11)
		0.25-0.5	0.19 (± 1.1 x10 ⁻⁴)	1.84 (± 9.5 x10 ⁻⁴)	11.1 (± 0.06)
		0.5-1.0	0.19 (± 4.3 x10 ⁻⁵)	1.77 (± 6.4 x10 ⁻⁴)	10.8 (± 0.21)
		1.0-1.5	0.18 (± 1.7 x10 ⁻⁴)	1.70 (± 1.5 x10 ⁻³)	11.2 (± 0.12)
		1.5-2.0	0.18 (± 2.6 x10 ⁻⁵)	1.75 (± 1.7x10 ⁻⁵)	11.3 (± 0.21)
	September 25	0-0.5	0.24 (± 3.3 x10 ⁻⁴)	1.99 (± 0.03)	9.7 (± 0.11)
		0.5-2.0	0.22 (± 0.01)	1.89 (± 0.01)	10.1 (± 0.23)
	GC Sediment	July 30	0-0.5	0.66 (± 0.01)	6.25 (± 0.09)
0.5-1.0			0.65 (± 0.02)	6.49 (± 0.03)	11.6 (± 0.41)
1.0-2.0			0.63 (± 0.01)	6.32 (± 0.06)	11.6 (± 0.21)
Spray Dried Phytoplankton (organic matter treatment for DO table incubation)	N/A	N/A	2.50 (± 0.13)	56.4 (± 0.21)	26.4 (± 1.28)

Table 4: Average pH over sediment depth profile of cores (0-2.0 cm). Measurements were recorded in duplicate (incubation cores) or triplicate (field cores). Standard deviation is listed in parentheses.

Sediment Source	Date/Treatment	Average pH
MB	September 25	7.39 (± 0.08)
MB DO Table: 5 days incubation	Normoxic	9.08 (± 0.05)
	Normoxic + Organic Matter	7.17 (± 0.22)
	Hypoxic	7.64 (± 0.13)
	Hypoxic + Organic Matter	7.51 (± 0.21)



Supplemental Figure 1: Daily average of DO concentrations during DO table incubation. Error bars represent standard deviation. The YSIs recorded the DO concentrations every 15 minutes for the entire duration of the incubation (n=2109 per treatment). Oxygen was introduced into the YSI aquaria due to YSI placement, causing the levels to be slightly higher than in the aquaria containing sediment cores. However, these measurements reflect the stability of the system throughout the incubation.



Supplemental Figure 2: Gene expression to abundance ratio of groups NB3 and NB7 for the DO table incubation cores. The samples were extracted in triplicate from the 0-0.5 cm depth and averaged. Error bars represent standard error of mean. The field cores were collected on September 25 and used to set the incubation experiment.

Supplementary Table 1: List of expressed *nifH* sequences. The list includes sequences from this study, uncultivated species (Brown and Jenkins, 2014; Brown, 2013), and closely related cultivated species (with GenBank accession numbers) contained within groups NB1-NB3, NB5, and NB7-NB17 (Figure 7).

Group Name	Cultivated Species	Expressed <i>nifH</i> sequences from this study			Uncultivated Species
		GC	MB	DO Table	
NB1	<i>Cyanobacterium</i> KM9-AAP42776	GBJ4.AE432			<i>KF285296</i> ; <i>KF285290</i> ; GBJ31SB817
NB2	<i>Azotobacter chroococcum</i> – ACF22078; <i>Pseudomonas stutzeri</i> A1501 – ABP7902	GBA31*.AE444; GBA22.AE420; GBA22.AE448			<i>KF285304</i> ; <i>KF285313</i> ; <i>KF285314</i> ; <i>KF285319</i> ; <i>KF285342</i> ; <i>KF285386</i>
NB3	<i>Pelobacter carbinolicus</i> DSM 2380-ABA89338		MJBa1m.AE371; MJBa2b.AE372; MJBa3m.AE391; Mca1.3.AE 246-249, 330-333; MSBa2T.AE397	MHZa6t.AE460; MNZa5tAE479; MNZa6t.AE455	<i>KF285284</i>
NB5a		GBa1t.AE298; GBa1t.AE328; GBA31*.AE451			
NB5b				MNPb5t.AE 499 -501	
NB5c					<i>KF285306</i> ; <i>KF285320</i> ; <i>KF285321</i>
NB5d		GBA28*.AE438; GBa1t.AE300; GBa1t.AE325		MNPb5t.AE502	

Supplementary Table 1 (continued)

Group Name	Cultivated Species	Expressed <i>nifH</i> sequences from this study			Uncultivated Species
		GC	MB	DO Table	
NB5e	<i>Desulfatibacillum alkenivorans</i> AK-01-ACL03220	GBJ1*.AE422; GBJ1*.AE424; GBJ4*.AE430; GBJ4*.AE431; GBa2m.AE307; GBa2m.AE329; GBa3t.AE310; GBA28*.AE439; GBA28*.AE440; GBA28*.AE442	MSBa2B.AE398; MSBb1B.AE402		<i>KF285308</i> ; GBJ11SB941; GBJ15SB636; GBJ41SB644
NB7	<i>Desulfovibrio salexigens</i> DSM 2638-ACS78514; <i>Desulfovibrio vulgaris</i> DP4-ABM30101	GBA22.AE419	MJBa3m.AE375; MCA2.1.AE287; MCA2.1.AE288; MCA2.1.AE318; MSBa1T.AE394	MNZa5t.AE480	<i>KF285307</i> ; <i>KF285316</i> ; <i>KF285356</i> ; <i>KF285359</i> ; <i>KF285384</i> ; <i>KF285385</i> ; <i>KF285387</i> ; <i>KF285392</i> ; <i>KF285372</i> - <i>KF285375</i>
NB8	Marine stromatolite eubacteria HB(0697) A100-AAG23908; HB(0898) Z02-AAG23903		MJBa3m.AE392	MNZa6t.AE452; MNZa6t.AE453	<i>KF285309</i> ; <i>KF285322</i>
NB9					<i>KF285297</i> ; <i>KF285312</i> ; GBJ41SB642; 10JOSB766
NB10		GBA28*.AE441			<i>KF285303</i> ; <i>KF285388</i>

Supplementary Table 1 (continued)

Group Name	Cultivated Species	Expressed <i>nifH</i> sequences from this study			Uncultivated Species
		GC	MB	DO Table	
NB11					<i>KF285329 - KF285331</i>
NB12	Desulfuromonas acetoxidans DSM 684 – EAT 15955		MJBa1m.AE389; MJBa3t.AE374; MJBa3t.AE373; MJBa3t.AE390	MHZa4t.AE457; MHZa4t.AE458; MHZa6t.AE459	
NB13					13J_BR1_SB1143; 13J_BR1_SB1168; 10PO1SB681
NB14		GBJ16.AE 416 - 418			13A_BR1_SB1167; 13J_BR3_SB1150; 13J_SR3_SB1177; GBJ13SB631; GBJ41SB641; GBJ41SB643
NB15			MSBa1T.AE393; MSBa1T.AE 376-378		13J_BR3_SB1151; 13J_BR3_SB1149; 13A_BR3_SB1169
NB16					GBJB31SB935; GBJB31SB936; GBJB31SB 993 - 995
NB17		GBJ10*.AE434; GBJ10*.AE436; GBJ10*.AE437			

CONCLUSION

Benthic nitrogen (N) fixation, an energy expensive, anaerobic process (Canfield *et al.*, 2010), was only recently measured to provide a significant contribution of N to the overall N budget of the ecosystem under certain environmental conditions (Fulweiler *et al.*, 2007). Sulfate reducers and *Geobacteraceae* have been found to be the dominant diazotrophs in a variety of estuarine systems including sediments of Narragansett Bay (Brown and Jenkins, 2014; Fulweiler *et al.*, 2013), Catalina Harbor (CA) (Bertics *et al.*, 2010), and Eckernförde Bay (Baltic Sea) (Bertics *et al.*, 2012). These systems suffer from seasonal oxygen depletion, or hypoxia. The N fixation activity of these microbes may be influenced by depleted oxygen conditions (Brown, 2013) and nutrient additions (McGlathery *et al.*, 1998; Herbert, 1975). Furthermore, production of hydrogen sulfide was detected to increase and ascend towards the sediment surface under oxygen depletion (Jørgensen, 1980). Yet, it is unclear how the N fixation activity of these microbes will respond to exacerbated hypoxic conditions from continued anthropogenic N loading and the rising threat of climate change (Middelburg and Levin, 2009).

This thesis aimed to couple biogeochemical profiling of sediment cores with analysis of N fixation rates and gene expression to identify the potentially active diazotrophs and follow their activity under hypoxic stress and varying organic matter loading. The activity of N fixers was analyzed over a range of dissolved oxygen (DO) conditions at two locations in Narragansett Bay. Higher total sulfide concentration and N fixation rates were detected under hypoxic conditions. Greenwich Cove (GC), which frequently experiences severe hypoxic conditions (Melrose *et al.*, 2007,

Deacutis *et al.*, 2006), had higher total sulfide concentrations and N fixation rates under normoxic conditions than the Mid Bay (MB) site, which experiences hypoxia infrequently (Codiga *et al.*, 2009; Melrose *et al.*, 2007; Deacutis *et al.*, 2006). While *Geobacteraceae* and sulfate reducers were the dominant diazotrophs at the MB site, sulfate reducers dominated at GC, which also had a higher carbon and N content. Despite these observations, the abundance and *nifH* expression of the dominant groups of diazotrophs did not follow the oxygen gradient captured in the field, as we were only able to quantitatively detect and follow one dominant subset of sulfate reducers using primers and probes in this study. Using bulk amplification methods, we could detect *nifH* gene expression by other sulfate reducers, but we could not develop primer sets to specifically follow these groups. Their active gene expression suggests these microbes were likely contributing to the N fixation rate. This suggests diversity surveys of the entire community of active N fixers, similar to the high throughput sequencing of *nifH* DNA and RNA used by Farnelid *et al.* (2013), are needed to better understand the microbial contribution to the overall N budget of hypoxic-stressed systems.

Since oxygen depleted areas are predicted to increase under continued anthropogenic inputs and pressures from climate change, an incubation experiment was conducted at the U.S. Environmental Protection Agency to further investigate the link between depleted DO and organic matter loading. The study collected sediment cores from the MB site to understand how the diazotrophic community might respond to exacerbated hypoxia. *nifH* expression by uncultivated *Geobacteraceae* was stimulated under prolonged hypoxic conditions. However, the highest total sulfide

concentration and N fixation rates were detected in the OM treatments. Furthermore, only *nifH* expression by uncultivated sulfate reducers was detected in the OM treatments. This suggests the activity of uncultivated sulfate reducers may have been stimulated by the OM addition or the depletion of DO resulting from OM decomposition.

This two-part study suggests different environmental drivers control N fixation activity of the dominant diazotrophs of Narragansett Bay. When capturing a DO gradient in the field, hypoxia stimulated N fixation rates. When MB sediment was incubated in the DO tables, the two dominant groups of diazotrophs were influenced by different factors. N fixation by uncultivated *Geobacteraceae* may be driven by low DO, but was inhibited by high OM loading. The combined N provided by the OM addition may have repressed the uncultivated *Geobacteraceae*. Carbon may also stimulate the uncultivated *Geobacteraceae* in the Narragansett Bay sediments, but additional incubation experiments are needed to investigate this relationship. A 2004 study, which focused on chemostat cultures and crude oil-contaminated subsurface sediments from Minnesota, found that acetate increased the growth of *Geobacteraceae* and expression of a core nitrogenase subunit gene (*nifD*), but *nifD* expression decreased two days after adding ammonium (Holmes *et al.*, 2004). Conversely, OM or the resulting DO depletion may stimulate N fixation by uncultivated sulfate reducers. This microbial group also dominated the frequently hypoxic stressed GC site, which was measured to have a higher carbon and N content than the MB site. Therefore, the N fixation activity by these dominant microbial groups has the potential to be

influenced by exacerbated hypoxia, resulting from increased pressure from climate change and anthropogenic inputs of N.

This study is important to increase our understanding of the N budget for coastal management. Recent regulations have been implemented to reduce eutrophication in Narragansett Bay. Despite efforts to reduce N loading (10-15% of total annual) from sewage effluent in Narragansett Bay, immediate reductions in total N, hypoxia, and primary productivity were not observed (Codiga *et al.*, 2009; Krumholz, 2012; Smith, 2011). However, as described by Rabalais *et al.* (2010) eutrophication occurs over a long time scale and characteristics of the ecosystem change, responding to these conditions (i.e. the development of hypoxia). Larger systems may not immediately, if ever, return to its initial conditions (Rabalais *et al.*, 2010; Duarte *et al.*, 2009). Thus, it is even more imperative to determine how N fixation responds to future environmental stressors to improve coastal management.

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