



# Diazotroph activity in surface Narragansett Bay sediments in summer is stimulated by hypoxia and organic matter delivery

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**ABSTRACT:** Bacteria that carry out many processes of the nitrogen cycle inhabit estuarine sediments. Denitrification is known to be a dominant process causing estuarine sediments to behave as net nitrogen sinks. However, measurements of nitrogen fluxes in the sediments of Narragansett Bay, Rhode Island, USA, have at times revealed high rates of net nitrogen (N<sub>2</sub>) fixation. Whereas changes in primary production, in magnitude and phenology, within Narragansett Bay have been identified as possible causes for these changes in nitrogen cycling within the benthos, a factor that has not been examined thus far is seasonal hypoxia. Since anaerobic diazotrophs figure so prominently within the sediments of Narragansett Bay, we hypothesized that dissolved oxygen concentrations in the bottom waters affect their activity. In order to explore this relationship, we measured the activity of diazotrophs in the surface sediments of 3 study areas during the summers of 2013 and 2014 using the acetylene reduction assay. We explored the effects of several water quality parameters on nitrogenase activity including, among others, dissolved oxygen and chlorophyll concentrations. Our measurements of nitrogenase activity were generally low, ranging between 2 and 5 nmol ethylene g<sup>-1</sup> d<sup>-1</sup> but spiked to 16 nmol ethylene g<sup>-1</sup> d<sup>-1</sup> at an area experiencing severe hypoxia in July 2013. Our data suggest that diazotrophy in estuarine sediments is enhanced when the benthos experiences very low dissolved oxygen in conjunction with recent influxes of autochthonous organic matter. Experiments with sediment core incubations conducted in the laboratory support our hypothesis that low dissolved oxygen and organic matter additions promote N<sub>2</sub> fixation.

**KEY WORDS:** N<sub>2</sub> fixation · Diazotrophy · Hypoxia · Estuary · Sediments

## 1. INTRODUCTION

Estuaries, residing at the interface of freshwater systems and oceans, are attractive habitats for human populations. Human activities impact estuarine ecosystems in a variety of ways, including anthropogenic nutrient loading from agriculture, sewage treatment, and fossil fuel emissions. A consequence of anthro-

pogenic nutrient delivery to estuaries is increased hypoxic conditions, something that has been documented as growing in severity in estuaries worldwide (Bricker et al. 2008, Diaz & Rosenberg 2008, Kemp et al. 2009, Rabalais et al. 2010, Howarth et al. 2011). Narragansett Bay, a temperate estuarine system located at 41.59° N, 71.38° W in Rhode Island, USA, is not exempt from these impacts, and the shallow

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upper bay proximal to the largest population densities experiences seasonal hypoxia during summer months (Deacutis 2008, Saarman et al. 2008, Codiga et al. 2009). Hypoxic conditions occur at depth when dissolved oxygen is depleted faster (by bacterial respiration) than it can be replenished. These conditions are typically established when water temperatures are high and surface waters are stratified leading to high rates of biological activity and elevated primary production in the surface water. The elevated surface production is heightened due to temporal inputs of nutrient-laden freshwater and ultimately feeds organic matter to the benthos. Greatest hypoxic expression is found in the upper third of the estuary, where the major rivers enter and the major wastewater effluents discharge. These conditions generate a clear gradient of chlorophyll, surface salinity, and hypoxia in Narragansett Bay (Oviatt et al. 2017).

Estuaries, particularly those that are anthropogenically impacted, have high primary production (Cloern et al. 2014). The resultant organic matter is rapidly deposited to the bottom sediments due to shallow water depths (Nixon et al. 1995). This sedimentary organic matter delivery fuels a range of biogeochemical processes that both remove and return nutrients to the water column, notably nitrogen (N) (Herbert 1999). In an aerobic estuary, oxygenated bottom water overlays anoxic sediment creating a redox gradient. Within the sediment, the oxidation of organic matter produces dissolved inorganic N (DIN) as ammonium ( $\text{NH}_4^+$ ) and consumes oxygen ( $\text{O}_2$ ). Once  $\text{O}_2$  is exhausted in the sediments, nitrate ( $\text{NO}_3^-$ ) is consumed and  $\text{NH}_4^+$  continues to be released to sedimentary pore fluids (Burdige 2006). In aerobic zones within shallow sediment, nitrifying bacteria oxidize  $\text{NH}_4^+$  to nitrite then  $\text{NO}_3^-$ . Denitrifying bacteria living in adjacent anoxic zones respire  $\text{NO}_3^-$  and remove N from the system as dinitrogen gas. This nitrification–denitrification pairing decreases the return flux of N to the water column and serves as a net N removal process for the estuary. When hypoxia is established and bottom water dissolved oxygen (DO) vanishes, nitrification is inhibited and denitrification is reduced or suppressed (Joye & Hollibaugh 1995, Cornwell et al. 1999, Howarth et al. 2011). Thus, hypoxia can exert strong control on benthic N cycling and can reduce net N removed via denitrification. For Narragansett Bay, denitrification has been estimated to be 13–26% of the N entering the bay (Nixon et al. 1995).

In addition to seasonal hypoxia altering N cycling in estuaries due to a decline in estuarine denitrification, shifts in other N cycling processes have been documented. There are increasing reports of N in-

puts to estuarine sediments from sedimentary N fixation (Bertics et al. 2013, Fan et al. 2015). Initially reported for Narragansett Bay sediments in 2006 (Fulweiler et al. 2007), inputs of N from N fixation measured by  $\text{N}_2:\text{Ar}$  flux methods greatly exceeded prior measurements of benthic N fixation from Narragansett Bay and similar systems (Howarth et al. 1988, Herbert 1999). Our previous studies, targeting the microbes actively expressing the genetic machinery for N fixation in Narragansett Bay sediments in 2006 and subsequent years, revealed that the major contributors to this process were 2 groups of anaerobes belonging to *Desulfovibrionaceae* and *Geobacteraceae* (Fulweiler et al. 2013, Brown & Jenkins 2014). Anaerobic bacteria have also been identified as important N fixers in other estuarine systems (Bertics et al. 2013, Bentzon-Tilia et al. 2015, Fan et al. 2015, Newell et al. 2016, Thajudeen et al. 2017).

Given the combined observations that anaerobic bacteria are the active diazotrophs in Narragansett Bay sediments and that the highest sedimentary N fixation rates reported to date were for a year in which Narragansett Bay was marked by severe hypoxia (Codiga et al. 2009), we hypothesized that hypoxic events may create an expanded niche for N fixation mediated by anaerobic bacteria. We tested this hypothesis using a multifaceted approach. We conducted time-series measurements of N fixation using the acetylene reduction assay (ARA) at multiple sites in Narragansett Bay during 2 sequential summer seasons (2013 and 2014). We used statistical modeling to examine the potential influences of water column characteristics including magnitude and duration of chlorophyll fluorescence and dissolved oxygen on N fixation rates. In addition, we conducted small-scale incubation experiments with Narragansett Bay sediments to test the role of organic matter deposition, and duration of oxic and hypoxic periods on modulating rates of N fixation. These experiments address the environmental controls that may stimulate N fixation in estuarine sediments and help us predict when conditions are ripe for diazotrophs to introduce additional N into Narragansett Bay sediments.

## 2. MATERIALS AND METHODS

### 2.1. Sample collection and preparation

Sediments cores were collected at 5 sites in Narragansett Bay (see Fig. 1). Average bottom depths were as follows: Greenwich Cove (GC): 3.0 m; Sally Rock (SR): 4.4 m; Bullock Reach (BR): 8.1 m; Quonset Point

(QP): 7.3 m; and Mount View (MV): 8.1 m. Cores were collected in duplicate or triplicate in 2013 and triplicate in 2014 as indicated in Table S1 in the Supplement at [www.int-res.com/articles/suppl/m614\\_p035\\_supp.pdf](http://www.int-res.com/articles/suppl/m614_p035_supp.pdf). Large sediment cores (10 cm diameter, 30.5 cm depth) were collected by SCUBA divers on 31 May 2013, and 5 and 6 August 2013. Sediment collected on all other days in 2013 and 2014 were collected with a Van Veen grab sampler from a vessel. Sediment in the grab sampler was subsampled with smaller core tubes (7 cm diameter, 7 cm depth). For cores of either size, the captured sediment was immediately overlain with bottom water collected on site with a Niskin bottle. In addition, at the time of collection, *in situ* measurements of DO, chlorophyll (2014 only), temperature, and salinity were made using a YSI 6920 V2 sonde. Upon collection, the cores and their matching waters were immediately transported on ice in a cooler to the laboratory where they were processed.

In 2013, sediments from 2 layers were sampled as indicated in Table S1: a top layer from 0–0.5 cm below the surface, and a bottom layer from 0.5–2 cm. In 2014, the top 3 cm of the core was sampled since the top and bottom layers gave similar nitrogenase activity (NA) results in 2013. Sediment from the depth horizons was sampled by pushing sediment from the bottom of the core sleeve and slicing off the top at the appropriate depth. Each layer was gently mixed to homogenize the samples over the core width. Some of the mixed sediment from layers sampled was used for the measurement of NA, and some was used for determination of pore water  $\text{NH}_4^+$  concentrations. For the NA measurement, 4 ml of sediment slurry from a given layer was collected using a 5 ml syringe with the tip cut off and placed in an anaerobic culture tube (Chemglass CLS 4209); 10 ml of bottom water from the corresponding site was then added, and the headspace of the tube was flushed with pure  $\text{N}_2$  gas before capping with septa and aluminum crimp tops. For pore water  $\text{NH}_4^+$  determination, mixed sediment was placed in 50 ml conical tubes and centrifuged at 4000 rpm for 5 min. For further clarification, pore water was collected, placed in a second 15 ml conical tube and centrifuged at 4000 rpm for 5 min. Supernatants from this second spin were used for pore water  $\text{NH}_4^+$  determination.

## 2.2. Small-scale incubations

To complement our field observations, a limited set of laboratory incubations were conducted. On 2 sam-

pling cruises, 3 sediment cores (7 cm diameter, 7 cm depth) were collected from each of 2 sites (GC and QP), and stored at 4°C until use. Six cores collected on 21 August 2014 were used for an incubation experiment started on 30 September 2014; and 6 cores collected on 26 September 2014 were used on 5 November 2014. Both incubation experiments were conducted as follows. To one core from each site, 0.25 g of agar (Sigma A1296) was added. To another core, 0.25 g of phytoplankton (ESV spray-dried marine phytoplankton) was added. To the remaining core, nothing was added. Organic matter was added to the appropriate cores by homogeneously spreading the powdered material over the core and gently working the material into the top 1–2 mm of surface sediment using a spatula. The cores were then incubated for 7 d in the dark at room temperature (22°C) under approximately 3 cm of 0.2  $\mu\text{m}$  filtered Narragansett Bay water and open-air condition. The cores remained in this 'oxic' state for a total of 7 d. At specific times throughout the oxic period (Days 1, 3, 5, and 7), 2 ml plugs of sediment were collected from the cores using a 5 ml syringe with a cut-off tip (reaching sediment to a depth of 3 cm) and placed in Hungate tubes (Chemglass CLS 4208). A total of 3 ml of the overlying water was then added to each tube before they were flushed with pure  $\text{N}_2$  gas and capped. Some of these tubes were analyzed for NA right away using the methods described below; others were incubated at room temperature with the sediment subjected to the 'anoxic' conditions created within the Hungate tubes for time periods of 1 to 3 d before NA was measured. These oxic–anoxic incubations were designed to replicate patterns that occur in Narragansett Bay where oxic conditions in the benthos are followed by hypoxic conditions. The spray-dried phytoplankton and agar were added to simulate the influx of primary production biomass to the benthos.

## 2.3. Pore water $\text{NH}_4^+$

Pore water  $\text{NH}_4^+$  was determined according to Koroleff (1983), except for a proportional reduction of the volumes of the necessary reagents so that a smaller sample volume of 500  $\mu\text{l}$  could be used and the reactions incubated in 1.5 ml centrifuge tubes. The reactions were allowed to develop for 30 min at 37°C followed by 30 min at room temperature, after which 250  $\mu\text{l}$  of the reaction volume was transferred to a 96-well assay plate (Costar 3631); absorbance was measured at 630 nm using a SpectraMax M5

multimode plate reader. Calibration standards were prepared with ammonium chloride in 0.2  $\mu\text{m}$  filtered surface water from Narragansett Bay (Salinity  $\approx$  30–31‰). This water is essentially free of  $\text{NH}_4^+$  and consists of a nutrient matrix otherwise similar to the pore water samples. Pore water samples were also pre-diluted (1:5) with this surface water to reduce artefacts due to hydrogen sulfide and bring  $\text{NH}_4^+$  concentrations within the linear range of the assay (0–100  $\mu\text{M}$ ). Results are reported in Table S1.

#### 2.4. NA measurement by the ARA

N fixation within the sediment samples was measured using the ARA according to Capone (1993). Acetylene was generated in-house by reacting calcium carbide with water, and was collected in a Supel-Inert film gas sampling 1 l bag. Acetylene was added to the flushed tubes containing the sediment to a final concentration of 20% of the total headspace. Following the addition of the acetylene, the content of the tube was fully mixed. The tubes were mixed again every time a gas sample was taken for analysis but were otherwise left undisturbed at 22°C in the dark. Gas samples of 100  $\mu\text{l}$  were taken from the tube headspace with a gas-tight syringe and immediately injected into the Shimadzu 8A gas chromatograph. The column used was stainless steel, 2.5 m long with Haysep T packing 80/100 mesh. Injector and column temperatures were 130 and 100°C, respectively. Gas samples were collected and ethylene concentrations usually measured 0.5, 3, 20, and 24 h after acetylene was added. Rates of acetylene reduction are reported as ethylene production over time normalized by the weight of the sediment (wet) contained in the tubes in units of  $\text{nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ d}^{-1}$ . Several controls were tested over the course of the study including sediment tubes with no acetylene added (to rule out ethylene production from sources other than active nitrogenase), tubes spiked with approximately 100 ppm ethylene but no acetylene (to rule out significant ethylene consumption), and tubes autoclaved prior to acetylene addition (to rule out significant abiotic production of ethylene). In all cases, the controls behaved as expected. Results are reported in Table S1.

#### 2.5. Water quality data and correlation with NA

Relationships between NA and several environmental predictor variables including bottom water

DO, surface water chlorophyll, and bottom water temperature were explored by model fitting. Water quality data from the Narragansett Bay Fixed-Site Monitoring Network (NBFSMN), a multi-agency program led by the Rhode Island Department of Environmental Management Office of Water Resources, were downloaded from [www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php](http://www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php) for the years 2009 to 2012 (NBFSMN 2009, 2010, 2011, 2012) and obtained from the RI DEM program contact for 2013 and 2014 (H. Stoffel pers. comm.). At these fixed sites, measurements of salinity, temperature, chlorophyll, and DO concentrations at the surface (approximately 1 m from the surface) and in the bottom waters (approximately 0.5 m from the sediment) are collected every 15 min from May through October. For the purpose of this work, the fixed-site data were averaged over different time scales to explore short-term and long-term effects of the environmental conditions considered on the measured NA. Linear and quadratic relationships of NA with a single or multiple predictors were examined. Statistical significance of the regression coefficients was tested using standard *t*-tests. Goodness of fit was measured with  $R^2$  and adjusted  $R^2$ . The models were also compared according to fit and parsimony using the standard Bayesian Information Criterion (BIC).

### 3. RESULTS

Sediments were sampled in 2013 and 2014 from 3 areas within Narragansett Bay (Fig. 1): one site in the Providence River estuary (BR), 2 sites in Greenwich Bay (GC and SR), and 2 sites in the middle of the upper west passage (MV and QP). Water quality of these 3 areas is monitored by the NBFSMN. The Providence River estuary and Greenwich Bay are particularly prone to seasonal hypoxia in July and August. Greenwich Bay was the site of a large fish kill in 2003, which was later attributed to severe hypoxia (RIDEM 2003). The west passage area (including MV and QP), by contrast, does not experience severe hypoxia in most years. The sediment surface in all 3 areas is free of macrophytic vegetation. Sites in Greenwich Bay and in the Providence River estuary display obvious signs of sulfate reduction (very dark sediment,  $\text{H}_2\text{S}$  outgassing during sampling). The mid-Bay sites are noticeably less active in terms of sulfate reduction. The sediment at the MV site is rich in shell ash.

The years 2013 and 2014 differed markedly with regards to seasonal hypoxia. Greenwich Bay experienced a severe hypoxic period in 2013 (Fig. 2a). Bot-

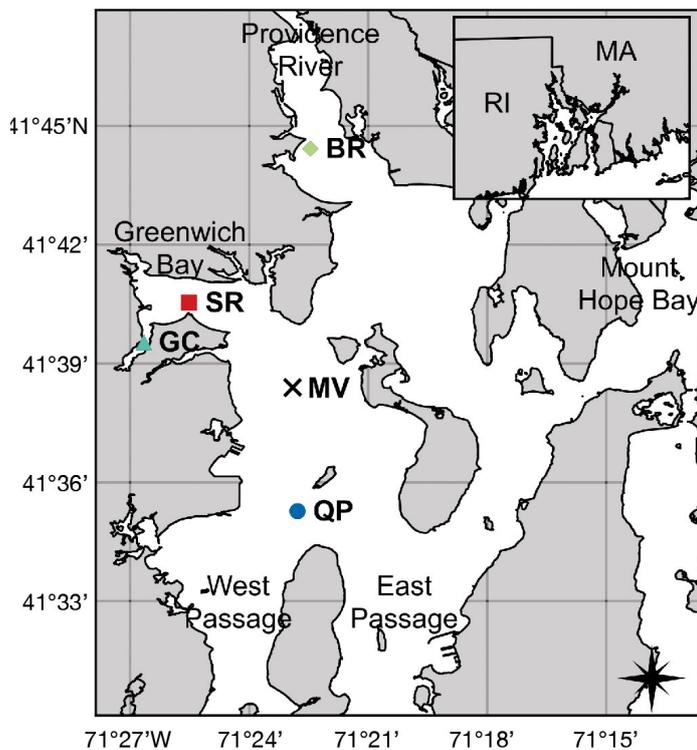


Fig. 1. Narragansett Bay, Rhode Island, showing sites at which sediment sampling occurred: Bullock Reach (BR), Sally Rock (SR), Greenwich Cove (GC), Mount View (MV), and Quonset Point (QP). All sites except GC are locations of Narragansett Bay Fixed-Site Monitoring Network buoys

tom water DO at the fixed monitoring site SR began rapidly declining on 24 June 2013, reaching hypoxic levels by 28 June that persisted until 23 July (Fig. 2a). A particularly severe stretch of low DO of less than  $1 \text{ mg l}^{-1}$  was observed between 7 and 17 July (Fig. 2a). By contrast, in 2014, hypoxic events were limited at SR with only occasional drops in DO below  $2 \text{ mg l}^{-1}$  (Fig. 2a).

### 3.1. Sediment NA activity occurs with low bottom water DO

The relationship between NA, as measured by acetylene reduction, and bottom water DO was examined at all sites sampled (Fig. 2b). In general, a trend was seen in 2013 of increasing NA with decreasing DO, a trend largely driven by DO less than  $2 \text{ mg l}^{-1}$  (Fig. 2b). The highest recorded NA in 2013 (top sediment =  $16 \pm 1 \text{ nmol ethylene g}^{-1} \text{ d}^{-1}$ ; bottom sediment =  $12 \pm 3 \text{ nmol ethylene g}^{-1} \text{ d}^{-1}$ ) was seen at GC at the end of the severe hypoxic period on 19 July, when DO in Greenwich Bay approached  $0 \text{ mg l}^{-1}$  (Fig. 2a,b). NA was also elevated in sediment sampled at GC on 30 July, which had the second lowest *in situ* bottom water DO in 2013 (Fig. 2a,b). In contrast, in 2014, a year when bottom water DO reached no lower than  $1.7 \text{ mg l}^{-1}$ , little variation was observed in NA (be-

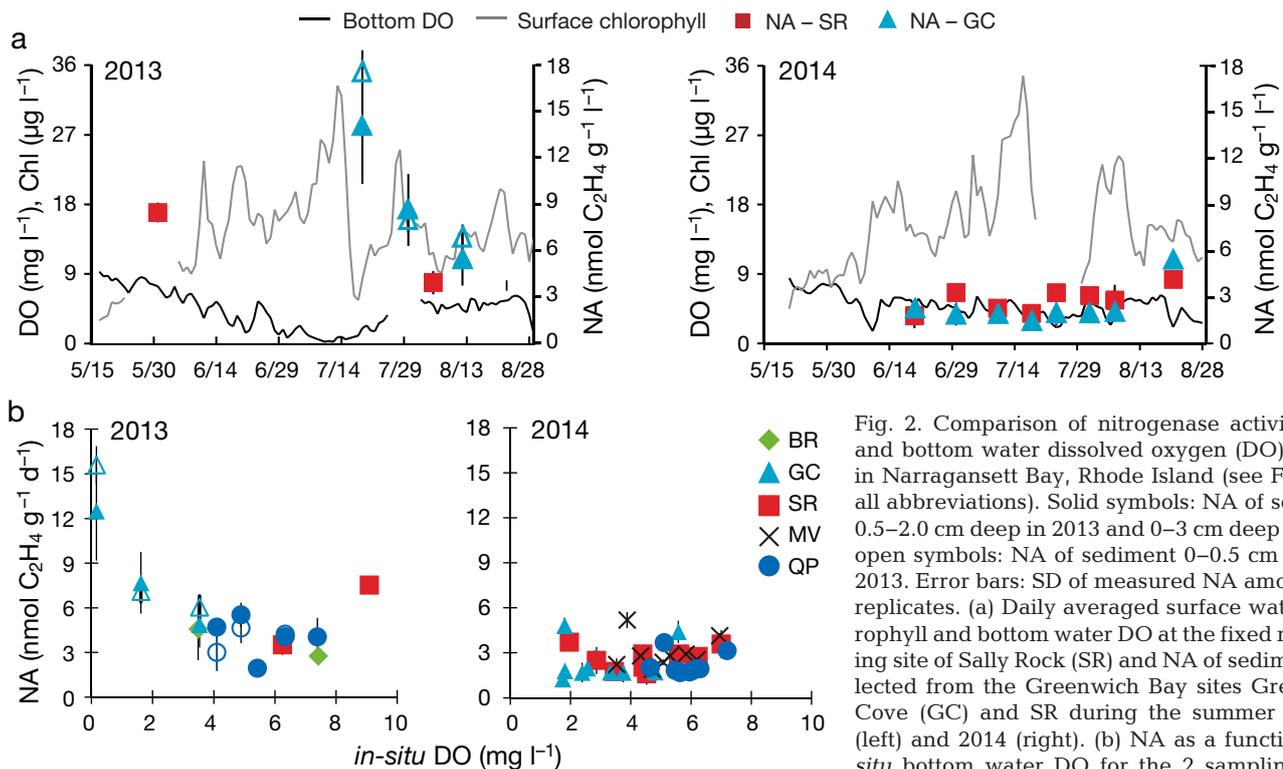


Fig. 2. Comparison of nitrogenase activity (NA) and bottom water dissolved oxygen (DO) at sites in Narragansett Bay, Rhode Island (see Fig. 1 for all abbreviations). Solid symbols: NA of sediment 0.5–2.0 cm deep in 2013 and 0–3 cm deep in 2014; open symbols: NA of sediment 0–0.5 cm deep in 2013. Error bars: SD of measured NA among core replicates. (a) Daily averaged surface water chlorophyll and bottom water DO at the fixed monitoring site of Sally Rock (SR) and NA of sediment collected from the Greenwich Bay sites Greenwich Cove (GC) and SR during the summer of 2013 (left) and 2014 (right). (b) NA as a function of *in situ* bottom water DO for the 2 sampling years

tween  $\sim 2\text{--}4$  nmol ethylene  $\text{g}^{-1} \text{d}^{-1}$  (Fig. 2a,b). Consistent with our observations in 2013, the highest measured NA at SR and GC in 2014 took place during one of these rare dips in DO at SR on 21 August (Fig. 2a). Aside from the high NA values observed at the lowest bottom water DO concentrations in 2013, the NA values were remarkably similar across all sites in both years (Fig. 2b). These NA values averaged  $4.5 \pm 1.6$  and  $2.3 \pm 0.8$  nmol ethylene  $\text{g}^{-1} \text{d}^{-1}$  in 2013 and 2014, respectively, when excluding data with *in situ* DO less than  $2.0 \text{ mg l}^{-1}$ .

A detailed analysis of hypoxia across Narragansett Bay for the years 2001–2006 highlighted the importance of river flows, water column stratification, and chlorophyll concentrations in the process (Codiga et al. 2009). Codiga (2012) found stratification to be most strongly affected by river flows. We also observed a strong correlation between low bottom water DO, high stratification, high surface water temperature, and high surface water chlorophyll concentration in analysis of NBFSMN data from more recent years (2009–2014; Fig. 3). Bottom water DO reaches particularly low levels in the upper parts of the bay at BR and SR when average daily surface water temperature exceeds  $22^\circ\text{C}$  and when average

daily density stratification exceeds  $2 \text{ g l}^{-1}$ . These water characteristics are typically associated with surface water chlorophyll values above  $10\text{--}15 \mu\text{g l}^{-1}$ , which has also been observed previously in Narragansett Bay (Codiga et al. 2009). We also found that warm and low-salinity surface waters generally support elevated levels of primary production as represented by chlorophyll *a* (chl *a*) (Figs. S1 & S2 in the Supplement). The year 2013 was marked by heavy precipitation early in the summer (first 2 weeks in June), followed by calm conditions (low wind) and high air temperatures (late June to late July), which are all conditions that influence the occurrence of hypoxia (Fig. S2). In comparison to 2013, 2014 was marked by moderate precipitation in late spring/early summer and average daily air temperatures that did not exceed  $28^\circ\text{C}$  (Fig. S2).

### 3.2. Low DO and elevated chlorophyll are significantly correlated with elevated NA

We also investigated the temporal relationship between high surface water chlorophyll and occurrences of low DO in relation to NA rates. Low bottom

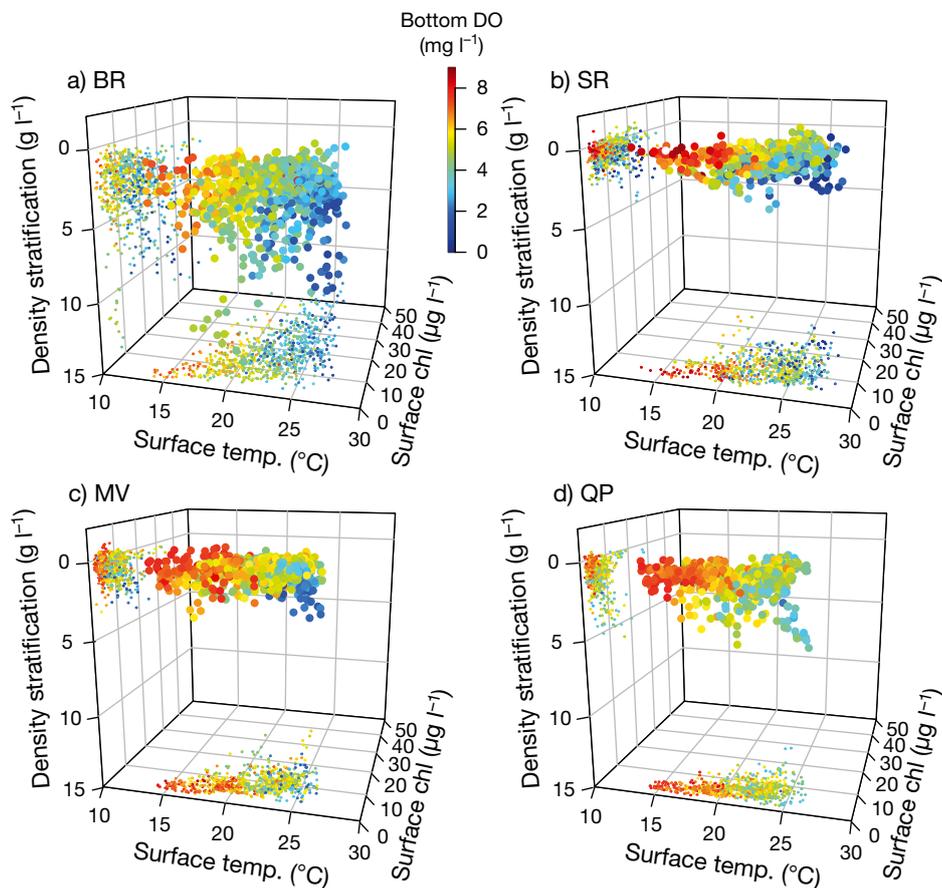


Fig. 3. Bottom water dissolved oxygen (DO) averaged over a given day in relation to surface water temperature, surface water chlorophyll, and the density difference between bottom and surface waters averaged over that same day at study sites in Narragansett Bay, Rhode Island. (a) Bullock Reach (BR), (b) Sally Rock (SR), (c) Mount View (MV), and (d) Quonset Point (QP). Data include those available at the given sites spanning mostly the period of 1 June–31 August for the years 2009–2014. Data are plotted in 3-dimensional space (large dots) and reflected on the bottom and left axes (small dots) to assist visualization in the respective 2-dimensional spaces

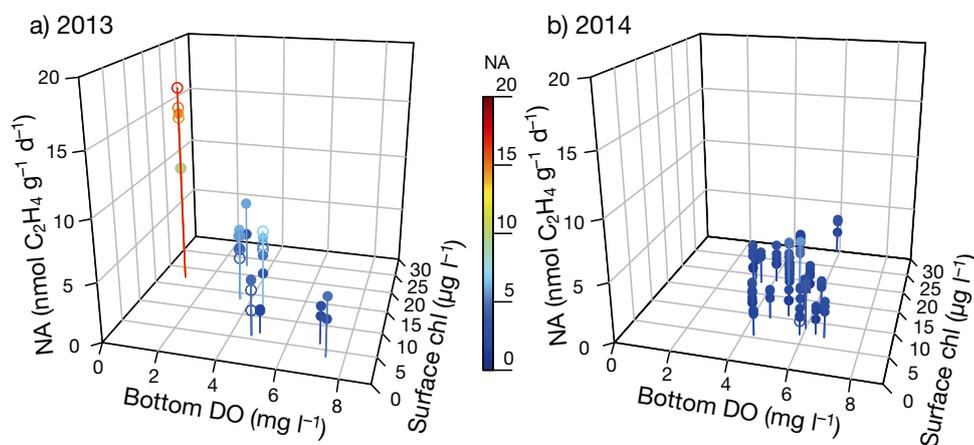


Fig. 4. Nitrogenase activity (NA) in Narragansett Bay, Rhode Island, on a given day (defined as Day 0) in (a) 2013 and (b) 2014 in relation to the fixed site bottom water dissolved oxygen (DO) and surface water chlorophyll averaged over the previous 7 d (from Days -6 to 0)

water DO on a given day may result in part from high surface water chlorophyll in the preceding weeks. Thus, the elevated NA observed at a time when DO was extremely low might also link elevated NA to elevated primary production levels. In fact, we found that the chlorophyll and DO conditions that prevailed in Greenwich Bay during peak NA at GC on 19 July 2013 were unique among all the sediment sampling times tested. The elevated NA observed on 19 July 2013 corresponds to a rare instance of bottom DO averaging  $0.97 \text{ mg l}^{-1}$  and surface chlorophyll averaging  $17.3 \text{ } \mu\text{g l}^{-1}$  over the previous week (Fig. 4), conditions not observed at other sampling sites and times from either year. Correlation analysis of the 2013 NA measurements in relation to bottom water DO and surface water chlorophyll supports a relationship between NA, DO, and chlorophyll (Table 1). Correlation between NA and DO in 2013 was examined with DO averaged over the same day NA was measured (Day 0) and over progressively longer peri-

ods of time preceding that day (1 d prior), 2 d prior, and so on until 13 d prior. The correlation between NA and DO strengthened as the time period of low DO increased, reaching a local maximum in  $R^2$  around 7 d preceding the NA measurement. Table 1 reports the regression outputs for the considered environmental parameters averaged over 1 d (Day 0, current conditions), over 1 wk prior to the NA measurement (Days -6 to 0, the time window where overall correlation with DO was highest) and 1 wk prior to that (Days -13 to -7, a time frame exploring persistent effects over longer time scales). Using these time frames, we found that both bottom water DO and surface water chlorophyll were good predictors of NA in 2013. Considering DO and chlorophyll as single predictors, statistically significant relationships (based on p-values) were found when relating NA to DO at all time scales (Days 0, -6 to 0, -13 to -7) and to chlorophyll at the longer time scales (Days 0 to -6 and -7 to -13). DO was negatively and chlorophyll

Table 1. Linear correlations of nitrogenase activity (NA) and bottom water dissolved oxygen (DO), of NA and surface water chlorophyll (chl), and of DO and chl at various time scales. Although monitoring data is available for most days between 15 May and 15 September of both years, the data used in our model of DO vs. chl was limited to the 2 wk preceding the dates on which NA was measured

Model relationship	2013			2014		
	Slope(s)	$R^2$	p-value	Slope(s)	$R^2$	p-value
$\text{NA} = f(\text{DO}_{\text{Day } 0})$	-1.41	0.31	<0.001	-0.12	0.03	0.097
$\text{NA} = f(\text{DO}_{\text{Day } -6 \text{ to } 0})$	-1.29	0.42	<0.001	0.11	0.01	0.233
$\text{NA} = f(\text{DO}_{\text{Day } -13 \text{ to } -7})$	-1.22	0.51	<0.001	0.20	0.05	0.024
$\text{NA} = f(\text{chl}_{\text{Day } 0})$	0.02	<0.01	0.853	-0.02	0.02	0.245
$\text{NA} = f(\text{chl}_{\text{Day } -6 \text{ to } 0})$	0.40	0.29	<0.001	-0.02	0.02	0.176
$\text{NA} = f(\text{chl}_{\text{Day } -13 \text{ to } -7})$	0.42	0.45	<0.001	-0.01	<0.01	0.366
$\text{NA} = f(\text{DO}_{\text{Day } 0}, \text{chl}_{\text{Day } -6 \text{ to } 0})$	-1.98, 0.02	0.50	0.002, 0.880	-0.15, -0.03	0.05	0.077, 0.082
$\text{NA} = f(\text{DO}_{\text{Day } 0}, \text{chl}_{\text{Day } -13 \text{ to } -7})$	-0.26, 0.39	0.50	0.592, 0.002	-0.19, -0.03	0.06	0.029, 0.087
$\text{NA} = f(\text{DO}_{\text{Day } -6 \text{ to } 0}, \text{chl}_{\text{Day } -13 \text{ to } -7})$	-0.09, 0.46	0.59	0.843, 0.002	0.11, -0.01	0.02	0.314, 0.769
$\text{DO} = f(\text{chl}_{\text{Day } 0})$	-0.13	0.20	<0.001	-0.04	0.05	0.029
$\text{DO} = f(\text{chl}_{\text{Day } -6 \text{ to } 0})$	-0.21	0.56	<0.001	-0.07	0.11	<0.001
$\text{DO} = f(\text{chl}_{\text{Day } -13 \text{ to } -7})$	-0.22	0.57	<0.001	-0.10	0.21	<0.001

positively correlated with NA in 2013. The inclusion of both DO and chlorophyll as predictors in the model resulted in some improvement over the single-predictor models with DO dominating the relationship at Day 0 and chlorophyll dominating at the longer time scales. A challenge of this approach is that both DO and chlorophyll are themselves correlated. This is shown in the bottom section of Table 1 and confirms the inverse relationship between surface chlorophyll and bottom water DO. The model fits between NA, DO and chlorophyll were not significant in 2014 compared to 2013 with p-values ranging from 0.024–0.769 and  $R^2$  not exceeding 0.06 (Table 1). Similar analysis using quadratic regressions (as opposed to linear) improved the fit of many relationships we examined; however, a biological underpinning driving these improvements in fit is difficult to decipher.

### 3.3. N fixation occurs in a background of high $\text{NH}_4^+$ levels

One of the main goals of this work was to explore the influence of bottom water hypoxia on  $\text{N}_2$  fixation. However, since  $\text{NH}_4^+$  concentration may vary at the different sites we studied, and  $\text{NH}_4^+$  has been shown to inhibit  $\text{N}_2$  fixation for some organisms (Daesch & Mortenson 1972, Drozd et al. 1972, Klugkist & Haaker 1984) including the *Geobacteraceae* (Holmes et al. 2004, 2006) known to be active in Narragansett Bay sediments (Brown & Jenkins 2014), we considered its potential effects on NA. The average pore water concentrations of  $\text{NH}_4^+$  were measured at sampling sites in 2013 and in 2014 (Fig. 5, Table S1).

There were large variations in the concentrations of pore water  $\text{NH}_4^+$  at some sites (MV and SR; Table S1) and between sites (QP compared to the other sites; Table S1). Despite these variations in  $\text{NH}_4^+$  concentrations, differences in NA were constrained to a narrow range of values across all sites (especially in 2014). At GC in 2013 when NA was high ( $\sim 7$  nmol ethylene  $\text{g}^{-1} \text{d}^{-1}$ ),  $\text{NH}_4^+$  concentrations averaged 392  $\mu\text{M}$  (Fig. 5, Table S1). In 2014, sediment  $\text{NH}_4^+$  levels at GC ranged between 157–419  $\mu\text{M}$  (Table S1).

### 3.4. Microcosm experiment shows organic matter addition promotes NA

To test the impacts of organic matter and organic N inputs on sediment NA, we conducted limited laboratory microcosm experiments with sediment cores collected from GC and QP. One of our aims was to compare the impact of organic matter additions to mimic deposition from a phytoplankton bloom that contains organic carbon and N (freeze-dried phytoplankton) vs. deposition that is rich in carbon and carbon that may be more refractory (agar). Sediment was the recipient of an initial dose of agar, dried phytoplankton biomass, or nothing (control), and cores were incubated with overlying water at room temperature ( $\sim 22^\circ\text{C}$ , similar to bottom water bay temperature), and subjected to different modulations of oxic/anoxic conditions. In the control incubations, little change in NA was observed with respect to core site or oxic/anoxic condition, and NA values steadily ranged between 3–9 nmol ethylene  $\text{g}^{-1} \text{d}^{-1}$  (Fig. 6). These levels of NA persisted in the

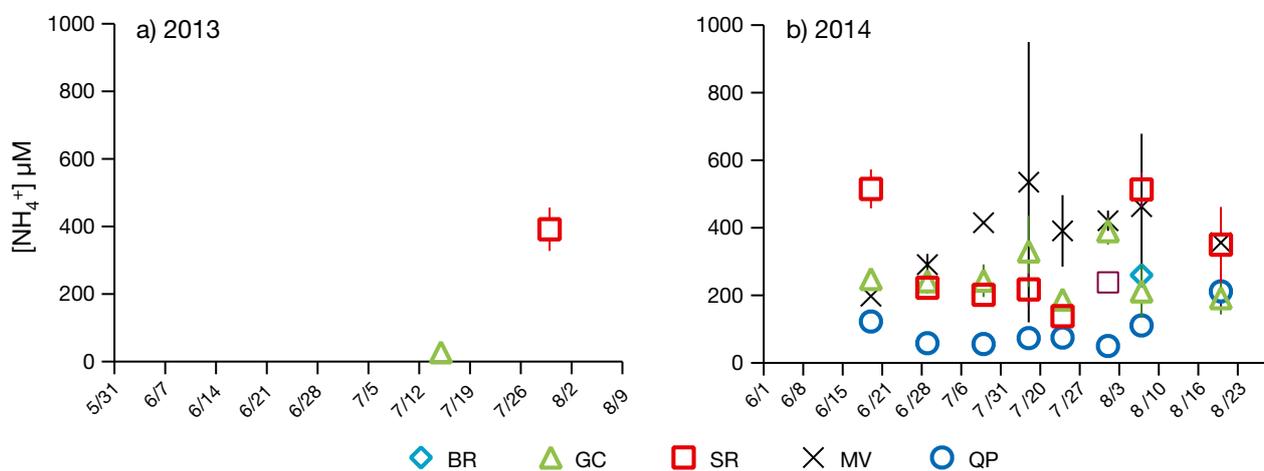


Fig. 5. Average pore water ammonium concentrations in sediments of Bullock Reach (BR), Greenwich Cove (GC), Sally Rock (SR), Mount View (MV), and Quonset Point (QP) in (a) 2013 and (b) 2014. Concentrations are of pore water extracted from sediment 0–2 cm deep in 2013 and 0–3 cm in 2014. Error bars: SD among replicate cores

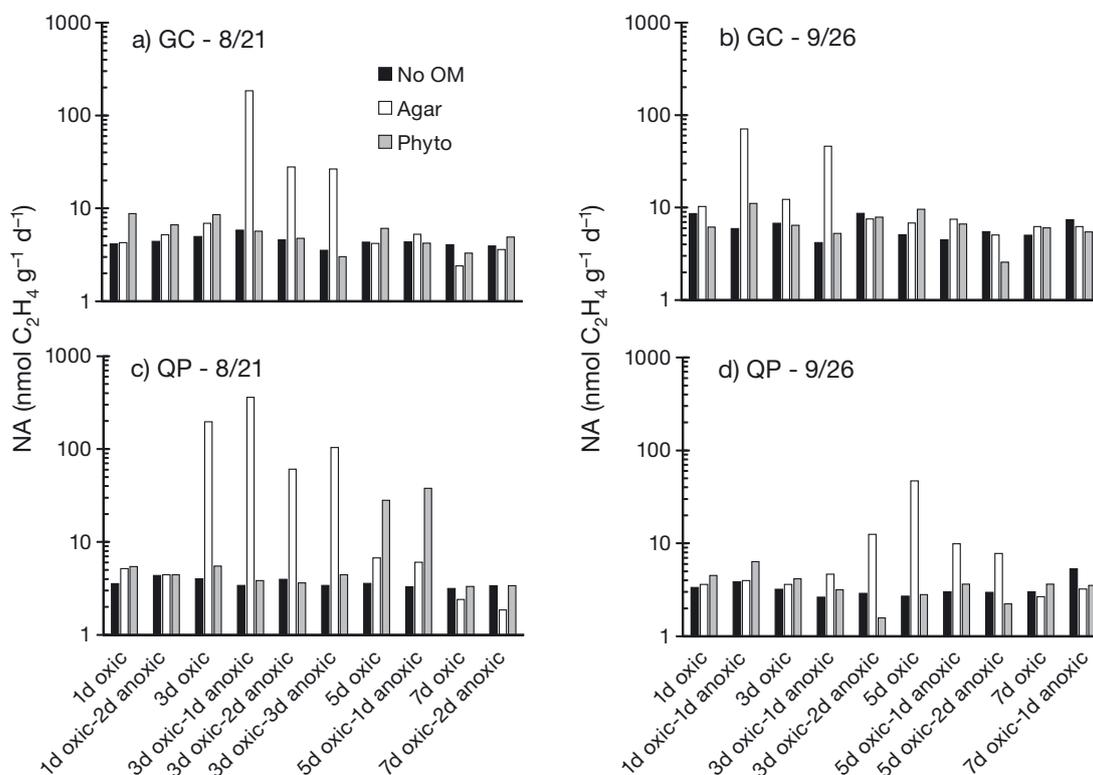


Fig. 6. Nitrogenase activity (NA) of sediment samples incubated under varying oxic periods (1–7 d) followed by varying anoxic periods (1–3 d). Experiments were conducted on cores collected on (a,c) 21 August 2014 and (b,d) 26 September 2014 from the (a,b) Greenwich Cove (GC) and (c,d) Quonset Point (QP) sites. Note the logarithmic scale for NA. Three organic matter treatments were considered: no addition (no OM), 0.25 g agar, and 0.25 g spray-dried phytoplankton (phyto)

control incubations for at least 35 d (data not shown). By comparison, agar addition caused large increases in NA that varied with core collection date and the timing of the oxic/anoxic condition. In general, elevated NA was observed in agar-supplemented sediment that experienced several days of oxic conditions followed by several days of anoxia. For example, the NA of sediment collected at GC on 21 August 2014 increased significantly with the addition of agar and exceeded 100 nmol ethylene  $\text{g}^{-1} \text{d}^{-1}$  when the cores were maintained oxic for 3 d after agar addition, followed by 1–3 d of anoxia (Fig. 6a). While NA in all 4 sets of cores (both sites on both collection dates) responded to the agar addition (Fig. 6), NA in only those from QP collected on 21 August 2014 responded to the phytoplankton addition (Fig. 6c). In this case, a period of 5 d of oxic conditions appeared necessary before NA was stimulated by 0–1 d of anoxia (Fig. 6c).

Overall, in these short incubations, a greater response in NA was observed with the addition of agar or phytoplankton to the sediments collected at both sites on 21 August 2014 compared to 26 September 2014. We also observed that some of the QP cores

amended with agar and phytoplankton displayed NA stimulation even before strict anoxia was initiated, a phenomenon not seen with the GC cores. These observations perhaps resulted from differences in the initial chemical and microbiological state of the sediment cores. Higher sulfide concentrations and higher total carbon and N content have previously been measured in the sediments of GC compared to QP (Ehrlich 2014). Across sediment types and organic matter addition, stimulation of NA was short-lived, with 2 d of additional incubation under oxic conditions past the point where NA was stimulated being sufficient to reduce NA back to control levels. Additionally, at no point in time in any of the cores was NA so low that it became undetectable.

## 4. DISCUSSION

### 4.1. Hypoxia stimulates $\text{N}_2$ fixation in Narragansett Bay

Our data demonstrate that the activity of diazotrophs in Narragansett Bay sediments during sum-

mer broadly falls into 2 categories. During periods when bottom water DO ranges from approximately 2–9 mg l<sup>-1</sup>, NA remains fairly stable with baseline rates between 2–5 nmol ethylene g<sup>-1</sup> d<sup>-1</sup>. During periods of severe hypoxia when DO is much less than 2 mg l<sup>-1</sup> and surface chlorophyll is elevated over the preceding days or weeks, NA can increase significantly compared to baseline levels. The highest measured NA under these conditions approached 16 nmol ethylene g<sup>-1</sup> d<sup>-1</sup>.

The fact that severe hypoxia stimulates diazotrophy is not unexpected. At many sites throughout Narragansett Bay, including those we have sampled in this study, molecular analysis of expressed *nifH* genes (encoding a nitrogenase subunit) has shown that the active diazotrophs are predominantly anaerobes belonging to the *Desulfovibrionaceae* and *Geobacteraceae* (Fulweiler et al. 2013, Brown & Jenkins 2014). Oxygen penetration in estuarine sediments is generally low (Revsbech et al. 1980) and DO in the sediments of Narragansett Bay is essentially absent at depths greater than 1.5 mm at all times during the summer (Ehrlich 2014). However, the establishment of hypoxic conditions in the bottom waters shoals the depth of oxygen penetration and in cases of very severe hypoxia (bottom water DO << 2 mg l<sup>-1</sup> for several days), we would expect the sediments to become completely anoxic. Given that shallow sediments are rich in freshly deposited organic matter, the extension of anoxic conditions into shallow horizons of the sediment would expand the range over which anaerobes can thrive and offer anaerobic diazotrophs a range of nutrients that may otherwise limit their growth and activity.

There are few studies on the impact of oxygen on rates of N fixation in anaerobic diazotrophs, but it is known that anaerobes related to the active diazotrophs in Narragansett Bay (*Desulfovibrio* spp. and *Geobacter* spp.; Brown & Jenkins 2014) can tolerate prolonged O<sub>2</sub> exposure. *Desulfovibrio desulficans* can withstand O<sub>2</sub> exposure for at least 48 h, but remains metabolically inactive (Abdollahi & Wimpenny 1990, Kjeldsen et al. 2005). *D. gigas* engages protective mechanisms against O<sub>2</sub> exposure. They can reduce O<sub>2</sub> to water and produce ATP (Fareleira et al. 1997, Dos Santos et al. 2000) and produce protective proteins against redox damage during this process (Santos et al. 1993). *Geobacter sulfurreducens*, long classified as a strict anaerobe, can also tolerate O<sub>2</sub> exposure for at least 24 h and can actually grow with O<sub>2</sub> as the sole electron acceptor in partial O<sub>2</sub> (<10%; Lin et al. 2004). Thus, anaerobic diazotrophs in estuarine sediments could subsist at redox

boundaries and increase growth and concomitant N fixation upon expansion of an anaerobic horizon in response to hypoxia. Both *Desulfovibrio* (Abdollahi & Wimpenny 1990, Fareleira et al. 1997) and *Geobacter* (Lin et al. 2004) achieve much higher growth in anaerobic conditions, therefore it is anticipated that their contribution to N fixation in sediment systems is greatly enhanced in expanded low oxygen zones. It is also possible that these organisms fix N<sub>2</sub> when exposed to O<sub>2</sub>, albeit at low rates, as mechanisms used for cellular protection against O<sub>2</sub> exposure could also protect their nitrogenase enzyme from O<sub>2</sub> inactivation.

#### 4.2. N fixation occurs in a background of high pore water NH<sub>4</sub><sup>+</sup>

A confounding issue remains in that the deposition of organic matter to benthic sediments results in both carbon and N remineralization processes (Kelly & Nixon 1984) that might influence diazotrophy in opposite ways. On one hand, carbon substrates could be generated that serve as suitable forms of carbon and electron donors to the diazotrophs. On the other hand, NH<sub>4</sub><sup>+</sup> is released, which would presumably satisfy bacterial requirements for nitrogen and repress diazotrophy. Some studies have shown inhibition of N<sub>2</sub> fixation by NH<sub>4</sub><sup>+</sup>, while others have reported active N<sub>2</sub> fixation in sediments containing considerable amounts (Haines et al. 1981, O'Neil & Capone 1989, Tibbles et al. 1994, Welsh et al. 1996, Bertics et al. 2010, 2013). The work presented here provides additional evidence that NA is detectable in estuarine sediments with significant concentrations of pore water NH<sub>4</sub><sup>+</sup>. This contradiction might be resolved by noting that our measurements are of bulk pore water NH<sub>4</sub><sup>+</sup> concentrations covering a span of surface sediment depths (1–3 cm). As others have suggested (Bertics et al. 2010, Fan et al. 2015), actual concentrations could vary significantly over small scales such that microniches may exist where dissolved N concentrations are actually very low. The ability of sulfate reducers, a dominant group of diazotrophs and strict anaerobes in these systems, to subsist in reduced microniches of oxidized marine sediments has previously been documented (Jørgensen 1977). Since we have documented abundances of diazotrophic sulfate reducers in Narragansett Bay surface sediments (Fulweiler et al. 2013, Brown & Jenkins 2014), but not at microscales, it is conceivable that they may exhibit fine-scale (mm) zonal distribution in microniches with low NH<sub>4</sub><sup>+</sup> concentrations.

### 4.3. Diazotrophy in Narragansett Bay is carbon limited

Another factor controlling diazotrophy in Narragansett Bay sediments is the abundance of suitable carbon and electron donors. Indeed, the highest level of NA from field measurements was observed in an area that had experienced an algal bloom in previous weeks. The stimulation of benthic  $N_2$  fixation with the addition of simple (Herbert 1975, Seitzinger & Garber 1987, O'Neil & Capone 1989) as well as complex organic substrates has previously been noted (Tibbles et al. 1994). Heterotrophic  $N_2$  fixation in *Zostera* sea grass beds has been linked to carbon substrate released by the sea grass in the rhizosphere (Welsh et al. 1996). Furthermore, NA increased significantly in our small core incubations with the addition of agar and dried phytoplankton. Our work shows that heterotrophic N fixation in the sediments of Narragansett Bay, as in these other systems, is carbon limited.

In addition to organic matter composition, the amounts of organic matter reaching the benthos could also be important as a stimulant for N fixation. Our core additions of 0.25 g of organic matter, equivalent to approximately  $65 \text{ g m}^{-2}$ , are apparently high relative to the naturally occurring amounts of algal organic matter expected to reach the sediments in upper Narragansett Bay or Greenwich Bay following a typical bloom. However, the added organic matter completely dissolved in the core's overlying water was equivalent to less than 100 mM of carbon and only a small fraction of it was expected to be available and metabolized by diazotrophs. Considering that heterotrophic diazotrophs are commonly grown in pure culture in  $>10 \text{ mM}$  of carbon substrate, we aimed for experimental concentrations that would be high enough to show a significant effect if one was to

be observed. Interestingly, agar stimulated N fixation more than the phytoplankton addition. This may be due to the fact that combined N in the freeze-dried phytoplankton could repress N fixation, while the carbon it contains has a stimulatory impact with a resultant NA rate that is a balance of repression and stimulation. The agar lacks N and more directly tests the addition of a specific carbon source. Additionally, the carbon from the agar addition may be selecting for activity of specific groups of diazotrophs that respond to this specific carbon source. Several strains of agar degrading heterotrophic diazotrophs in the *Vibrionaceae* have been isolated from eel grass bed sediments and shown to reach high rates of N fixation using agar as the sole carbon source (Yang Shieh et al. 1988). It is possible that agar addition specifically stimulates particular groups of diazotrophs. The range of NA activities between plankton vs. agar treatments as well as those achieved in different durations of oxic and anoxic conditions (Fig. 6) raises important questions about the dynamics of organic matter transport, microbial metabolism in the benthos, and possible effects on heterotrophic diazotrophy in estuarine systems. Results from our experiments and previous work adding organic matter to Narragansett Bay sediments (Fulweiler et al. 2008) shows that stimulation of NA from organic inputs can be very short lived. Our microcosms experiments further suggest that transitions between oxic and anoxic conditions could affect NA, perhaps by activating or selecting for different microbial communities that modulate the mechanisms of organic matter breakdown when it reaches the benthos.

Our current hypothesis (Fig. 7) is that the anaerobic diazotrophs in Narragansett Bay sediments are minimally active under most conditions, and active perhaps in N limited microniches, but they can be readily stimulated by sudden inputs of partially oxi-

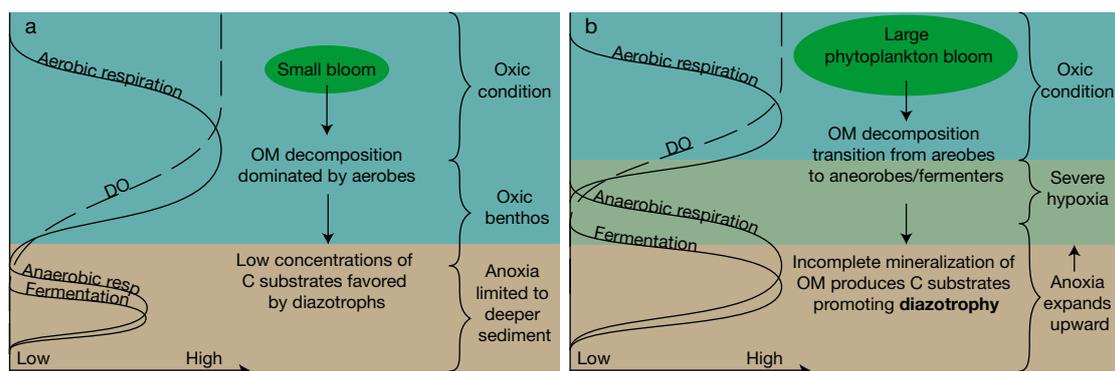


Fig. 7. Hypothetical linkages between surface primary production, water column dissolved oxygen (DO), and several major microbiological processes contributing to (a) low-intensity baseline diazotrophy or (b) enhanced diazotrophy in the estuarine sediments of Narragansett Bay. OM: organic matter; C: carbon

dized carbon substrates originating from the water column above. The extent of this stimulation is also dependent on redox conditions in the sediment. Deviations from baseline rates of  $N_2$  fixation might occur rapidly with changes in prevailing environmental conditions leading to changes in carbon substrate availability. The cascade of organic matter decomposition leading to these substrates is influenced by the DO of the water column and the benthos as organic matter primarily of algal origin moves from the surface down to the sediment. When DO is high in the water column and the benthos (Fig. 7a), organic matter originating at the surface is predominantly decomposed by heterotrophic aerobes, which leaves less labile carbon substrate for diazotrophs in the benthos. However, when hypoxia is severe (Fig. 7b), organic matter is only partially mineralized by aerobes before bacteria performing anaerobic respiration and fermentation further decompose it. These latter forms of metabolism would produce the types of simple organic compounds upon which anaerobic diazotrophs are known to grow. Sulfate-reducing bacteria are commonly grown on lactate, which is a common product of fermentation (Voorouw 1995). Likewise, members of the *Geobacteraceae* are often grown on acetate, ethanol, or other simple carbon substrates such as butanediol and acetoin (Schink 1992).

Although speculative at this time, dynamics of organic matter decomposition could also affect whether or not N present in algal organic matter suppresses  $N_2$  fixation. Although phytoplankton biomass may possess a C:N ratio that would seem to exclude N fixation, selective decomposition of this organic matter could in fact produce pools of substrates with high C:N ratios. As an example, if algal biomass fractions with a high C:N ratio (polysaccharides, lipids) were preferentially degraded over N-rich fractions (proteins, nucleic acids), we would expect a release of carbon-rich metabolites with high C:N ratios that could stimulate anaerobic diazotrophs. In addition, little, if anything, is known regarding how the dynamics of decomposition are affected when bottom water DO approaches anoxic levels.

#### 4.4. Predicting frequency of elevated sedimentary $N_2$ fixation in Narragansett Bay

While we now have a strong baseline value of NA across several sites in Narragansett Bay, the impact of enhanced NA that may occur during severe hypoxia is much less constrained. The data presented

in Figs. 3 & 4 demonstrates that the conditions in Greenwich Bay under which NA was stimulated in July 2013 ( $DO < 2 \text{ mg l}^{-1}$ ,  $chl > 16 \text{ } \mu\text{g l}^{-1}$ ) almost never occur at some locations (MV, QP), and occur with more frequency at others (SR, BR). Using 2009–2014 NBFMSN data, we have determined the frequency over those 6 yr with which a given day (defined as Day 0) was a day when the surface chlorophyll and bottom water DO averaged over the previous 7 d (from Day -6 to 0) met varying conditions of severity for high chlorophyll concentrations and low DO concentrations (Fig. 8). The most frequent occurrence of  $DO < 2 \text{ mg l}^{-1}$  and  $chl > 16 \text{ } \mu\text{g l}^{-1}$  was at SR and reached 33% at most (i.e. 2 of the 6 years analyzed) for a very brief period in July. The BR site had the same conditions ( $DO < 2 \text{ mg l}^{-1}$  and  $chl > 16 \text{ } \mu\text{g l}^{-1}$ ) only 17–20% of the time. Based on this time-series analysis, we would conclude that enhanced NA is rare in most years.

#### 4.5. Comparison of Narragansett Bay sediment $N_2$ fixation rates to those in other benthic systems

A number of assumptions are necessary in order to compare our NA rate measurements to others (Marsho et al. 1975, Haines et al. 1981, Seitzinger & Garber 1987, Howarth et al. 1988, Gardner et al. 2006, Fulweiler et al. 2007, Bertics et al. 2010, Andersson et al. 2014, Fan et al. 2015). Assuming that  $N_2$  fixation is active down to a depth of 5 cm, a wet sediment density of  $1.2 \text{ g ml}^{-1}$  and a ratio of 1:3 relating  $N_2$  fixation to acetylene reduction, we observe that our rate measurements ranging from  $0.81\text{--}17 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ d}^{-1}$  correspond to  $0.17\text{--}3.5 \text{ g m}^{-2} \text{ yr}^{-1}$ . These rates are comparable to those reported for similar environments (Table 2), but are much lower than the peak rates measured by Fulweiler et al. (2007). As noted earlier, hypoxia was widespread in Narragansett Bay in 2006 (Codiga et al. 2009), with bottom water DO reaching lower minima and hypoxic conditions spreading farther south than in 2013. The year 2006 was also marked by heavy precipitation in June, similar to 2013. Several efforts to reduce the impact of wastewater discharges in Narragansett Bay have also been completed since 2006. A biological treatment upgrade to reduce N in the effluent of the largest wastewater treatment facility in the Bay watershed was finished in 2013 and 2 phases of an improvement project to reduce combined sewer overflows (CSO) were completed in 2008 and 2014. Although the primary goal of the CSO project was a reduction of fecal coliform contamination, some re-

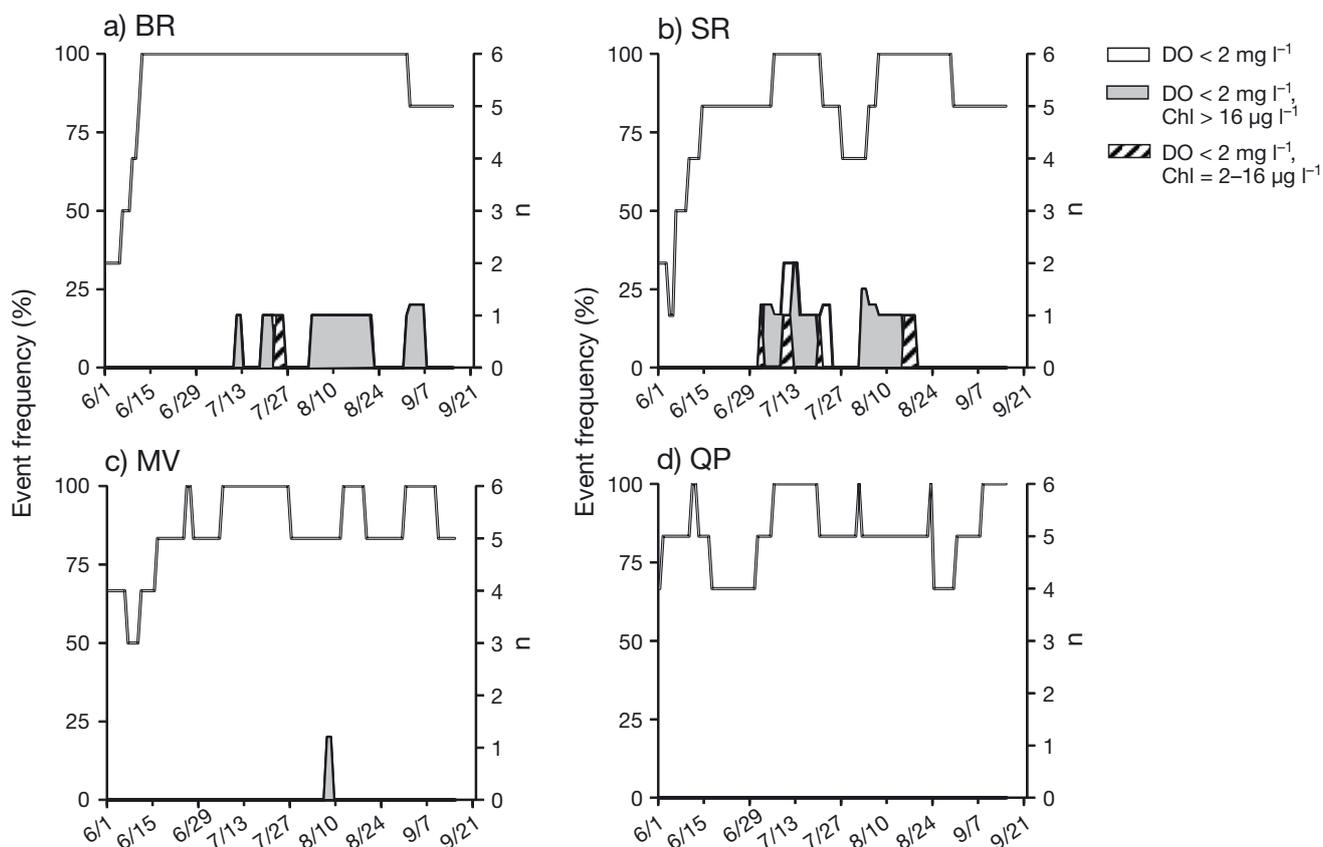


Fig. 8. Frequency of events from 2006–2014 in which the surface chlorophyll and bottom water dissolved oxygen (DO) averaged over the previous 7 d met varying criteria for high chlorophyll and low DO concentrations on a given day at (a) Bullock Reach (BR), (b) Sally Rock (SR), (c) Mount View (MV), and (d) Quonset Point (QP). Data include all available at the given sites from 1 June–15 September (2009–2014). The second y-axis ( $n$ ) denotes the number of years between 2009 and 2014 for which data were available

Table 2.  $N_2$  fixation rates reported for estuarine/coastal sediments. For adjusted rates assumptions were made when necessary using a 3:1 ratio to convert acetylene reduction assay (ARA) rates to  $N_2$  fixation rates, a wet sediment density of  $1.2 \text{ mg l}^{-1}$ , and active  $N_2$  fixation to a depth of 5 cm

Study	Locations	Method	Rates reported	Adjusted rates ( $\text{g N m}^{-2} \text{ yr}^{-1}$ )
Andersson et al. (2014)	Swedish west coast	ARA	$0.03\text{--}3.4 \text{ mmol N m}^{-2} \text{ d}^{-1}$	0.15–17.37
Bertics et al. (2010)	Catalina Harbor	ARA	$0\text{--}5 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ h}^{-1}$	0–20.44
Bertics et al. (2013)	Baltic Sea	ARA	$8\text{--}18 \text{ nmol N cm}^{-2} \text{ d}^{-1}$	0.41–0.92
Fan et al. (2015)	Southern North Sea	Isotopic	$0\text{--}8.1 \text{ nmol N g}^{-1} \text{ d}^{-1}$	0–2.48
Fulweiler et al. (2007)	Narragansett Bay	$N_2/\text{Ar}$	Net fixation rates: $25\text{--}650 \text{ }\mu\text{mol N m}^{-2} \text{ h}^{-1}$	3.07–79.72
Gardner et al. (2006)	Texas estuaries	Isotopic	$0\text{--}97 \text{ }\mu\text{mol N m}^{-2} \text{ h}^{-1}$	0–11.9
Haines et al. (1981)	Alaskan coast	ARA	$0\text{--}1.4 \text{ ng N g}^{-1} \text{ h}^{-1}$	0–0.74
Howarth et al. (1988) (and references therein)	Worldwide coastal seas and estuaries	ARA	$0.002\text{--}1.56 \text{ g N m}^{-2} \text{ yr}^{-1}$	0.002–1.56
Marsho et al. (1975)	Rhode River estuary (Chesapeake Bay)	ARA	Subtidal measured at $20^\circ\text{C}$ : $2.2\text{--}6.8 \text{ ng N g dry wt}^{-1} \text{ h}^{-1}$	0.48–1.49
Seitzinger & Garber (1987)	Narragansett Bay	ARA	$44\text{--}108 \text{ pmol C}_2\text{H}_4 \text{ g}^{-1} \text{ h}^{-1}$	0.21–0.53
Present study	Narragansett Bay	ARA	$0.81\text{--}17 \text{ nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ d}^{-1}$ (baseline = $2.9 \pm 1.4$ )	0.17–3.5 ( $0.58 \pm 0.29$ )

ductions in nutrient loading have taken place as well. The treatment upgrades on the wastewater treatment plants alone have reduced concentrations of inorganic nitrogen bay-wide by close to 50% and caused a decrease in overall surface production (30%), improvements in water clarity, and a significant reduction in summer hypoxia in some areas of the upper bay, including the Providence River estuary but not Greenwich Bay (Oviatt et al. 2017). These changes in nutrient discharge to Narragansett Bay are sure to affect water column and benthic nutrient cycling in the years of this study and in future years, proving a challenge for making direct comparisons of studies before 2008 and after 2014. Even so, net N flux measurements from Narragansett Bay sediments made since 2006 have shown net N loss on most years and only a few instances of modest net N<sub>2</sub> fixation (Fulweiler & Heiss 2014). This suggests that the sediments of Narragansett Bay today are still more likely to behave as net N sinks, which is in agreement with our findings of relatively low rates of N<sub>2</sub> fixation during the summers of 2013 and 2014.

## 5. CONCLUSIONS

Rates of N<sub>2</sub> fixation estimated using ARA for Narragansett Bay sediments overlain with oxygenated waters during the summer months averaged  $2.9 \pm 1.4$  nmol ethylene g<sup>-1</sup> d<sup>-1</sup> in 2013 and 2014. At these rates, N<sub>2</sub> fixation is not believed to contribute significantly to the mass balance of nitrogen in Narragansett Bay, and its sediments would be expected to behave as net N sinks. Spikes in N<sub>2</sub> fixation may occur, however, during infrequent periods of severe hypoxia in conjunction with elevated surface water chlorophyll concentrations in the preceding weeks. Factors likely to control N fixation include bottom water DO concentrations, influx of primary production biomass to the benthos, quality of this biomass, and the dynamics of organic matter decomposition.

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## LITERATURE CITED

- Abdollahi H, Wimpenny JWT (1990) Effects of oxygen on the growth of *Desulfovibrio desulfuricans*. *Microbiology* 136:1025–1030
- ✦ Andersson B, Sundbäck K, Hellman M, Hallin S, Alsterberg C (2014) Nitrogen fixation in shallow water sediments: spatial distribution and controlling factors. *Limnol Oceanogr* 59:1932–1944
- ✦ Bentzon-Tilia M, Traving SJ, Mantikci M, Knudsen-Leerbeck H, Hansen JLS, Markager S, Riemann L (2015) Significant N<sub>2</sub> fixation by heterotrophs, photoheterotrophs and heterocystous cyanobacteria in two temperate estuaries. *ISME J* 9:273–285
- ✦ Bertics VJ, Sohm JA, Treude T, Chow CET, Capone DC, Fuhrman JA, Ziebis W (2010) Burrowing deeper into benthic nitrogen cycling: the impact of bioturbation on nitrogen fixation coupled to sulfate reduction. *Mar Ecol Prog Ser* 409:1–15
- ✦ Bertics VJ, Löscher C, Salonen I, Dale AW, Gier J, Schmitz R, Treude T (2013) Occurrence of benthic microbial nitrogen fixation coupled to sulfate reduction in the seasonally hypoxic Eckernförde Bay, Baltic Sea. *Biogeosciences* 10:1243–1258
- ✦ Bricker SB, Longstaff B, Dennison W, Jones A, Boicourt K, Wicks C, Woerner J (2008) Effects of nutrient enrichment in the nation's estuaries: a decade of change. *Harmful Algae* 8:21–32
- ✦ Brown SM, Jenkins BD (2014) Profiling gene expression to distinguish the likely active diazotrophs from a sea of genetic potential in marine sediments. *Environ Microbiol* 16:3128–3142
- Burdige D (2006) *Geochemistry of marine sediments*. Princeton University Press, Princeton, NJ
- Capone D (1993) Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) *Handbook of methods in aquatic microbial ecology*. Lewis Publishers, Boca Raton, FL, p 621–631
- ✦ Cloern JE, Foster SQ, Kleckner AE (2014) Phytoplankton primary production in the world's estuarine-coastal ecosystems. *Biogeosciences* 11:2477–2501
- Codiga DL (2012) Density stratification in an estuary with complex geometry: driving processes and relationship to hypoxia on monthly to inter-annual timescales. *J Geophys Res Oceans* 117:C12004
- ✦ Codiga DL, Stoffel HE, Deacutis CF, Kiernan S, Oviatt CA (2009) Narragansett Bay hypoxic event characteristics based on fixed-site monitoring network time series: intermittency, geographic distribution, spatial synchronicity, and interannual variability. *Estuaries Coasts* 32: 621–641
- ✦ Cornwell JC, Kemp WM, Kana TM (1999) Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. *Aquat Ecol* 33: 41–54
- ✦ Daesch G, Mortenson LE (1972) Effect of ammonia on the synthesis and function of the N<sub>2</sub>-fixing enzyme system in *Clostridium pasteurianum*. *J Bacteriol* 110:103–109

- Deacutis CF (2008) Evidence of ecological impacts from excess nutrients in upper Narragansett Bay. In: Desbonnet A, Costa-Pierce BA (eds) Science for ecosystem-based management. Springer, New York, NY, p 349–381
- Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. *Science* 321:926–929
- ✦ Dos Santos WG, Pacheco I, Liu MY, Teixeira M, Xavier AV, LeGall J (2000) Purification and characterization of an iron superoxide dismutase and a catalase from the sulfate-reducing bacterium *Desulfovibrio gigas*. *J Bacteriol* 182:796–804
- ✦ Drozd JW, Tubb RS, Postgate JR (1972) A chemostat study of the effect of fixed nitrogen sources on nitrogen fixation, membranes and free amino acids in *Azotobacter chroococcum*. *Microbiology* 73:221–232
- Ehrlich AL (2014) Elucidating the molecular response of microbial nitrogen fixation in estuarine sediments to hypoxia. MSc thesis, University of Rhode Island, South Kingstown, RI
- ✦ Fan H, Bolhuis H, Stal LJ (2015) Drivers of the dynamics of diazotrophs and denitrifiers in North Sea bottom waters and sediments. *Front Microbiol* 6:738
- ✦ Fareleira P, Legall J, Xavier AV, Santos H (1997) Pathways for utilization of carbon reserves in *Desulfovibrio gigas* under fermentative and respiratory conditions. *J Bacteriol* 179:3972–3980
- ✦ Fulweiler RW, Heiss EM (2014) A decade of directly measured sediment N<sub>2</sub> fluxes: What can Narragansett Bay tell us about the global ocean nitrogen budget? *Oceanography (Wash DC)* 27:184–195
- ✦ Fulweiler RW, Nixon SS, Buckley BA, Granger SL (2007) Reversal of the net dinitrogen gas flux in coastal marine sediments. *Nature* 448:180–182
- ✦ Fulweiler RW, Nixon SW, Buckley BA, Granger SL (2008) Net sediment N<sub>2</sub> fluxes in a coastal marine system—experimental manipulations and a conceptual model. *Ecosystems* 11:1168–1180
- ✦ Fulweiler RW, Brown SM, Nixon SW, Jenkins BD (2013) Evidence and a conceptual model for the co-occurrence of nitrogen fixation and denitrification in heterotrophic marine sediments. *Mar Ecol Prog Ser* 482:57–68
- ✦ Gardner WS, McCarthy MJ, An S, Sobolev D, Sell KS, Brock D (2006) Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. *Limnol Oceanogr* 51:558–568
- ✦ Haines JR, Atlas RM, Griffiths RP, Morita RY (1981) Denitrification and nitrogen fixation in Alaskan continental shelf sediments. *Appl Environ Microbiol* 41:412–421
- ✦ Herbert RA (1975) Heterotrophic nitrogen fixation in shallow estuarine sediments. *J Exp Mar Biol Ecol* 18:215–225
- ✦ Herbert RA (1999) Nitrogen cycling in coastal marine ecosystems. *FEMS Microbiol Rev* 23:563–590
- ✦ Holmes DE, Nevin KP, Lovley DR (2004) In situ Expression of *nifD* in *Geobacteraceae* in subsurface sediments. *Appl Environ Microbiol* 70:7251–7259
- ✦ Holmes DE, Chaudhuri SK, Nevin KP, Mehta T and others (2006) Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. *Environ Microbiol* 8:1805–1815
- Howarth RW, Marino R, Lane J, Cole JJ (1988) Nitrogen fixation in freshwater, estuarine, and marine ecosystems: 1. Rates and importance. *Limnol Oceanogr* 33:669–687
- ✦ Howarth R, Chan F, Conley DJ, Garnier J, Doney SC, Marino R, Billen G (2011) Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. *Front Ecol Environ* 9:18–26
- ✦ Jørgensen B (1977) Bacterial sulfate reduction within reduced microniches of oxidized marine sediments. *Mar Biol* 41:7–17
- ✦ Joye SB, Hollibaugh JT (1995) Influence of sulfide inhibition of nitrification on nitrogen regeneration in sediments. *Science* 270:623–625
- ✦ Kelly JR, Nixon SW (1984) Experimental studies of the effect of organic deposition on the metabolism of a coastal marine bottom community. *Mar Ecol Prog Ser* 17:157–169
- ✦ Kemp W, Testa J, Conley D, Gilbert D, Hagy J (2009) Temporal responses of coastal hypoxia to nutrient loading and physical controls. *Biogeosciences* 6:2985–3008
- ✦ Kjeldsen KU, Joulain C, Ingvorsen K (2005) Effects of oxygen exposure on respiratory activities of *Desulfovibrio desulfuricans* strain DvO1 isolated from activated sludge. *FEMS Microbiol Ecol* 53:275–284
- ✦ Klugkist J, Haaker H (1984) Inhibition of nitrogenase activity by ammonium chloride in *Azotobacter vinelandii*. *J Bacteriol* 157:148–151
- Koroleff F (1983) Determination of ammonia. In: Grasshoff K, Ehrhardt M, Kremling K (eds) Methods of seawater analysis, 2nd edn. Wiley-VCH, Weinheim, p 150–157
- ✦ Lin WC, Coppi MV, Lovley DR (2004) *Geobacter sulfurreducens* can grow with oxygen as a terminal electron acceptor. *Appl Environ Microbiol* 70:2525–2528
- ✦ Marsho TV, Burchard RP, Fleming R (1975) Nitrogen fixation in the Rhode River estuary of Chesapeake Bay. *Can J Microbiol* 21:1348–1356
- NBFSMN (Narragansett Bay Fixed-Site Monitoring Network) (2009) 2009 datasets. Rhode Island Department of Environmental Management, Office of Water Resources. [www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php](http://www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php)
- NBFSMN (2010) 2010 datasets. Rhode Island Department of Environmental Management, Office of Water Resources. [www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php](http://www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php)
- NBFSMN (2011) 2011 datasets. Rhode Island Department of Environmental Management, Office of Water Resources. [www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php](http://www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php)
- NBFSMN (2012) 2012 datasets. Rhode Island Department of Environmental Management, Office of Water Resources. [www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php](http://www.dem.ri.gov/programs/emergencyresponse/bart/netdata.php)
- ✦ Newell SE, Pritchard KR, Foster SQ, Fulweiler RW (2016) Molecular evidence for sediment nitrogen fixation in a temperate New England estuary. *PeerJ* 4:e1615
- ✦ Nixon S, Granger S, Nowicki B (1995) An assessment of the annual mass balance of carbon, nitrogen, and phosphorus in Narragansett Bay. *Biogeochemistry* 31:15–61
- ✦ O’Neil JM, Capone DG (1989) Nitrogenase activity in tropical carbonate marine sediments. *Mar Ecol Prog Ser* 56:145–156
- ✦ Oviatt C, Smith L, Krumholz J, Coupland C and others (2017) Managed nutrient reduction impacts on nutrient concentrations, water clarity, primary production, and hypoxia in a north temperate estuary. *Estuar Coast Shelf Sci* 199:25–34
- ✦ Rabalais N, Diaz R, Levin L, Turner R, Gilbert D, Zhang J (2010) Dynamics and distribution of natural and human-caused hypoxia. *Biogeosciences* 7:585–619

- ✦ Revsbech NP, Sorensen J, Blackburn TH, Lomholt JP (1980) Distribution of oxygen in marine sediments measured with microelectrodes. *Limnol Oceanogr* 25:403–411
- RIDEM (Rhode Island Department of Environmental Management) (2003) The Greenwich Bay fish kill—August 2003: causes, impacts and responses. Rhode Island Department of Environmental Management, Narragansett, RI
- Saarman E, Prell WL, Murray DW, Deacutis CF (2008) Summer bottom water dissolved oxygen in upper Narragansett Bay. In: Desbonnet A, Costa-Pierce BA (eds) *Science for ecosystem-based management*. Springer, New York, NY, p 325–347
- ✦ Santos H, Fareleira P, Xavier AV, Chen L, Liu MY, LeGall J (1993) Aerobic metabolism of carbon reserves by the 'obligate anaerobe' *Desulfovibrio gigas*. *Biochem Biophys Res Commun* 195:551–557
- Schink B (1992) The genus *Pelobacter*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) *The Prokaryotes*. Springer, New York, NY, p 3393–3399
- ✦ Seitzinger SP, Garber JH (1987) Nitrogen fixation and  $^{15}\text{N}_2$  calibration of the acetylene reduction assay in coastal marine sediments. *Mar Ecol Prog Ser* 37:65–73
- ✦ Thajudeen J, Yousuf J, Veetil VP, Varghese S, Singh A, Abdulla MH (2017) Nitrogen fixing bacterial diversity in a tropical estuarine sediments. *World J Microbiol Biotechnol* 33:41
- ✦ Tibbles B, Lucas M, Coyne V, Newton S (1994) Nitrogenase activity in marine sediments from a temperate saltmarsh lagoon: modulation by complex polysaccharides, ammonium and oxygen. *J Exp Mar Biol Ecol* 184:1–20
- ✦ Voordouw G (1995) The genus *Desulfovibrio*: the centennial. *Appl Environ Microbiol* 61:2813
- Welsh DT, Bourguès S, de Wit R, Herbert RA (1996) Seasonal variation in rates of heterotrophic nitrogen fixation (acetylene reduction) in *Zostera noltii* meadows and uncolonised sediments of the Bassin d'Arcachon, southwest France. *Hydrobiologia* 329:161–174
- Yang Shieh W, Simidu U, Maruyama Y (1988) Nitrogen fixation by marine agar-degrading bacteria. *J Gen Microbiol* 134:1821–1825

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