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Effect of Eastern Oysters (*Crassostrea virginica*) and Seasonality on Nitrite Reductase Gene Abundance (*nirS*, *nirK*, *nrfA*) in an Urban Estuary

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Abstract The influence of oysters on nitrogen (N) cycling has received increased research attention. Previous work focused on fluxes of N solutes and gases, but the effects on microbes responsible for N transformations are unknown. In May 2010, we deployed eastern oysters (*Crassostrea virginica*) in mesh cages above sand-filled boxes at four sites across a nutrient gradient in Jamaica Bay, New York City. In fall and winter, we used quantitative PCR to measure abundance of 16S rRNA and nitrite reductase genes for denitrification (*nirS* and *nirK*) and dissimilatory nitrate reduction to ammonium (*nrfA*) in sediment. We measured water column nutrients and chlorophyll *a*, sediment C:N and organic matter (OM), exchangeable ammonium (NH_4^+), ammonification, nitrification, and denitrification potential (DNP). Oysters did not affect gene abundance in fall, when we predicted that their influence would be strongest, or in winter. However, gene abundance was significantly different among sites and seasons. Factors which explained 16S rRNA, *nirS*, and *nirK* gene abundance included sediment OM, water column N, and chlorophyll *a*, similar to previous research. Abundance of *nrfA* was lower than that of *nir* genes and positively related to sediment C:N, suggesting OM lability may drive the balance between *nir* and *nrfA*. Finally, *nirS* and *nirK* abundance was unrelated to DNP, which is consistent with variable results from the literature. More studies that combine molecular techniques with N transformation rates in the context of oyster reefs are needed. Results will advance models which predict the ecosystem effects of reef conservation and restoration under variable environmental conditions.

Keywords Denitrification · Eutrophication · Biodeposits · Bivalves · Oyster reef habitat

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Introduction

Approximately 85 % of oyster reefs have been lost globally (Beck et al. 2011), attributed to overfishing, cultural eutrophication, shoreline alteration, habitat destruction, and disease (Cerco and Noel 2007; Levinton et al. 2011). Oyster reef conservation and restoration is ongoing at many locations worldwide because oysters provide multiple ecosystem services such as water filtration, increased water clarity, enhanced nutrient cycling, structured habitat, and shoreline stabilization (Beck et al. 2011; Grabowski et al. 2012; Grabowski and Peterson 2007). Oysters and other bivalves can enhance water filtration and sediment nutrient cycling via feeding and biodeposits (i.e., feces and pseudofeces) (Kellogg et al. 2013; Newell et al. 2002). Oysters filter particles from the water column and sort the material on their gills. Particles deemed unpalatable are rejected as pseudofeces, which are aggregated with mucus and expelled (Newell and Langdon 1996). Feces are formed after nutritious particles are ingested, digested, and defecated (Langdon and Newell 1996). Biodeposits can sink up to 40 times faster than unaggregated particles (Newell et al. 2005) and contain 2–3 times more

carbon (C) and nitrogen (N). Thus, oysters can deliver C and N to sediment microbes, where elements are assimilated or transformed (Newell et al. 2005).

Denitrification, the microbially mediated anaerobic respiration of nitrate (NO_3^-) to nitrous oxide and dinitrogen (N_2) gas, represents a loss of biologically active N from the aquatic environment. Denitrification is primarily controlled by the availability of organic C, NO_3^-, and favorable redox conditions (Groffman et al. 2006; Tiedje et al. 1989). In many marine habitats, nitrification (i.e., the autotrophic oxidation of ammonium NH_4^+ to NO_3^-) and denitrification are coupled, where the NO_3^- produced by nitrification is used as a denitrification substrate (Herbert 1999; Wallenstein et al. 2006). However, in eutrophic habitats, nitrification and denitrification may become decoupled as coupled nitrification-denitrification requires adjacent oxic-anoxic microsites, and instead, water column NO_3^- may serve as the electron acceptor for sediment C oxidation (i.e., direct denitrification) (Seitzinger et al. 2006). Recent evidence has been equivocal, showing that oysters can affect denitrification through coupled and direct pathways (Kellogg et al. 2013; Piehler and A. R. Smyth 2011; Smyth et al. 2013a), that oysters have no effect on denitrification or denitrification potential (Higgins et al. 2013; Hoellein and Zarnoch 2014), or that effects vary with environmental conditions and oyster feeding (Hoellein et al. 2015).

Dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) competes with denitrification for NO_3^-, but rather than removing N from an ecosystem as inert N_2, DNRA retains N in the form of biologically active NH_4^+ (Giblin et al. 2013). The balance between N_2 removal and NH_4^+ retention is important because NH_4^+ retention is a positive feedback which can exacerbate eutrophication. Recent measurements suggest that the abundance of bacterial genes required for denitrification outnumbers the genes needed for DNRA in a variety of aquatic ecosystems (Dong et al. 2009; Huang et al. 2011; Morrissey et al. 2013), although this pattern can be reversed in tropical marine sediments (Dong et al. 2011; Dunn et al. 2013). DNRA may be more favorable than denitrification under conditions with low NO_3^- availability and high sediment C due to more efficient use of electrons, because DNRA transfers eight electrons per mole of NO_3^- while denitrification transfers five (Burgin and Hamilton 2007; Tiedje et al. 1989). In addition, DNRA appears less sensitive to O_2 fluctuations than denitrification (Fazzolari et al. 1998). Sediment underlying mussel aquaculture has elevated rates of DNRA (Nizzoli et al. 2006). Research on oysters' role in sediment N cycling has focused on denitrification rather than DNRA. However, Smyth et al. (2013b) showed that DNRA at oyster reefs was higher than other habitats, but represented a relatively minor component of overall NO_3^- flux.

Oysters' influence on dissimilatory N cycling has been examined by measuring changes in the chemical substrates and products of microbial N transformations (Higgins et al. 2013; Hoellein and Zarnoch 2014; Hoellein et al. 2015; Kellogg et al. 2013; Smyth et al. 2013a, b), while oysters' effects on the abundance of microbial genes responsible for sediment N transformations have not been measured. Measurements of microbial gene abundance should facilitate a greater understanding of environmental factors which drive ecosystem processes (Morrissey et al. 2013; Wallenstein et al. 2006), including the effect of oysters. The four microbial enzymes that mediate denitrification are nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (Zumft 1997), while a single nitrite reductase, encoded by the *nrfA* gene, is required for DNRA (Smith et al. 2007). Not all denitrifiers contain the suite of genes required for complete denitrification, and nitrite reductase genes (*nir*) are commonly used to indicate the presence of denitrifying bacteria (Braker et al. 1998; Wallenstein et al. 2006). The two functionally homologous *nir* genes, *nirS* and *nirK*, are mutually exclusive and are thought to exist primarily in single copies (Coyne et al. 1989). The relative abundance of genes for denitrification and DNRA should reflect the microbial capacity for each metabolic pathway in the sediment, thereby indicating the relative potential of the microbial community to conduct dissimilatory NO_3^- transformations to remove or recycle N.

We conducted an experimental deployment of oysters in Jamaica Bay, New York City (NYC), USA, at four sites which spanned a gradient of eutrophication. This was a large experiment spanning 17 months, multiple treatments for oyster density, and numerous measurements of sediment biogeochemistry (Hoellein and Zarnoch 2014). The microbial gene abundance data presented in the current study was measured from only a subset of dates and treatments for the larger study. In fall and winter, we measured the abundance of *nirS*, *nirK*, *nrfA*, and 16S ribosomal RNA (rRNA) gene sequences in sediment exposed to the highest density of oysters (125 individuals m^{-2}) and control sediment (no oysters). In addition, we collected data for water column physicochemical characteristics and sediment biogeochemistry. We selected fall because temperature and food availability supported oyster feeding and biodeposition, and data from the larger experiment showed fall was the only time when oysters increased denitrification potential (Hoellein and Zarnoch 2014). Thus, we predicted that oysters would increase abundance of all four gene types in fall, and have no effect in winter (when oysters are not actively feeding). We expected that the abundance of all four genes would be higher in fall relative to winter, and gene abundance would be positively correlated with water column nutrients and sediment organic matter (OM). We also predicted that *nir* gene abundance would be correlated with DNP. Finally, we expected that *nirS* gene sequences will be more abundant than *nirK* gene sequences, and both *nir* gene sequences would outnumber *nrfA* gene sequences.

Methods

Study Site

Jamaica Bay is on the south shore of western Long Island in NYC, connected to the Atlantic Ocean by the Rockaway inlet. This 5260-ha bay is highly urbanized and the watershed population is approximately 2.5 million (Wigand et al. 2014). Jamaica Bay receives anthropogenic N from wastewater effluent, combined sewer overflows, and stormwater (Benotti et al. 2007; Wigand et al. 2014). The four study sites were Floyd Bennett Field, Wildlife Refuge, Motts Basin, and Spring Creek. Floyd Bennett Field was the least eutrophic due to its close proximity to the ocean. The most eutrophic site was Spring Creek (SC), situated in the eastern part of the bay with reduced flushing and downstream from the effluent of a water pollution control plant. Motts Basin and Wildlife Refuge received less wastewater inputs and were classified as moderately eutrophic in the context of this study.

Populations of the eastern oyster (*Crassostrea virginica*) in NYC greatly declined in the nineteenth century (Franz 1982). Considered "functionally extinct" in the Hudson-Raritan Estuary of New York and New Jersey (Black 1981), rebuilding oyster reefs has been a primary objective of many regional restoration projects, including Jamaica Bay. Although subject to low recruitment, sedimentation, disease, and contamination by heavy metals (Franz 1982; Levinton et al. 2011), oyster restoration has been promoted to increase biodiversity and reduce anthropogenic N concentrations in NYC waters.

Experimental Design

A comprehensive description of the experimental design, map of the study site, sampling procedures, and biogeochemical methods and results can be found in Hoellein and Zarnoch (2014). Briefly, at each site, 660 cm^2 plastic sediment boxes were filled with 11 kg of playground sand (Paverstone Company, Atlanta, GA). For the oyster treatment, sediment boxes were fitted with 27.5×30×10 cm mesh bags (mesh size= 16 mm) on top, which were filled with 5-year-old oysters at a density of 125 individuals m^{-2}. Oysters were purchased from Frank M. Flower and Sons in Bayville, NY, USA. Control boxes were fitted with empty mesh bags. Each box was connected to a trotline (i.e., a heavy rope), anchored parallel to the shore. Depth of the boxes was 1–1.5 m (mean low water depth), and boxes were accessible by wading only at low spring tide each month. The boxes were deployed in May 2010 and sediment biogeochemistry was measured every other month through November 2011 as part of the larger study (Hoellein and Zarnoch 2014). Sand had very little organic matter and was purchased from the same company at the same time. We did not measure gene abundance in the sand before deployment. We assume that any "founder effect" of microbes in sand was minimal and equivalent across sites.

For this study, we sampled sediment from control and oyster boxes for two different months at each site (i.e., a subset of dates for the larger study). We sampled Wildlife Refuge and Floyd Bennett Field in December 2010 and Spring Creek and Motts Basin in January 2011 (winter). We sampled Wildlife Refuge and Floyd Bennett Field in October 2011 and Spring Creek and Motts Basin in September 2011 (fall). We selected fall because temperature, food availability, and feeding measurements showed high rates at that time period (Table 1; Zarnoch, unpublished data), and it was one of the few periods in which oysters significantly increased DNP (Table 2; Hoellein and Zarnoch 2014). Winter dates were selected to contrast with fall, as oysters do not actively feed at temperatures <7 °C (Galstoff 1964) and had no effects on DNP.

On each collection date, sediment was collected from three replicate control and oyster boxes (N=6 $site^{-1}$). Boxes were carried by hand to the shoreline for processing. We removed the mesh bags from the top of each box. A composite sediment sample was collected from the top 2–3 cm of sediment from three random locations within each box using a 28-cm^2 corer and a trowel (total volume=150 ml sediment per box). Within 2 h, mesh bags were reattached to sediment boxes and boxes were redeployed. After redeploying the boxes, we collected bulk water samples for use in denitrification potential (DNP; acetylene block method) and nitrification (nitrapyrin inhibition) assays and filtered water for measuring nutrient concentrations and chlorophyll *a*. For detailed methods on experiment maintenance, sampling, and laboratory assays, see Hoellein and Zarnoch (2014).

Sediment Biogeochemistry and DNA Extraction

Sediment samples were stored in a cooler on ice and in a laboratory refrigerator (4 °C) until processing. We collected sediment for bacterial DNA extraction simultaneous with sediment biogeochemistry measurements. To do so, we filled sterile 1.5 ml microcentrifuge tubes approximately half full using sterile tools (N=3 per treatment per site on each date) and stored the tubes at −80 °C. We extracted bacterial DNA from three replicates per box using the UltraClean Soil DNA isolation kit (MoBio, Carlsbad, CA). DNA was extracted from 0.5 to 1 g of sediment. The 1.5-ml microcentrifuge tubes containing the samples were weighed prior to extraction and the bead beating solution was added directly to the sample tube. The MoBio DNA Clean-Up Kit (MoBio, Carlsbad, CA) was used to remove PCR inhibitors from the extracted sediment bacterial DNA.

Genomic DNA was used for *nirS*, *nirK*, and 16S rRNA gene standards for absolute quantitation in quantitative polymerase chain reaction (qPCR). The organism used to generate *nirS* gene standard curves was *Pseudomonas stutzeri* (ATCC

Table 1 Mean (±SE) physiochemical measurements for sampling sites in fall and winter

	Floyd Bennett		Wildlife Refuge		Motts Basin		Spring Creek	
	Winter	Fall	Winter	Fall	Winter	Fall	Winter	Fall
Temp	2.1 (0.4)	21.5 (0.2)	2.0 (0.4)	24.2 (0.2)	0.3 (0.1)	24.0 (0.2)	1.7 (0.8)	22.7 (0.3)
Chl *a*	5 (1)	37 (6)	4 (1)	27 (8)	3 (1)	57 (11)	5 (1)	15 (4)
NO_3^-	118 (37)	78 (51)	228 (20)	144 (13)	135 (18)	152 (34)	245 (22)	142 (84)
NO_2^-	17 (3)	27.5 (3)	40 (3)	66 (5)	21 (2)	35 (9)	37 (4)	16 (1)
NH_4^+	105 (38)	180 (33)	245 (15)	186 (28)	29 (7)	215 (17)	301 (27)	175 (12.3)
SRP	15 (6)	52 (20)	30 (4)	78 (12)	BD	58 (8)	25 (2)	81 (20)
DIN	315 (74)	258 (64)	513 (39)	649 (46)	185 (26)	448 (37)	584 (51)	248 (27)

Units for temperature are degree Celsius, units for Chl *a* are micrograms per liter, and units for solutes are micrograms N or P per liter

Temp temperature, *Chl a* chlorophyll *a*, NO_3^- water column nitrate, NO_2^- water column nitrite, NH_4^+ water column ammonium, *SRP* water column soluble reactive phosphorus, *DIN* water column dissolved inorganic nitrogen ($NH_4^+ + NO_3^- + NO_2^-$), *BD* below detection

17588). For *nirK* and 16S rRNA genes, the organism was *Pseudomonas protegens* (ATCC BAA-477). Overnight liquid cultures (nutrient broth for *P. stutzeri* and tryptic soy broth for *P. protegens*) were used for genomic DNA extraction (Wizard Genomic DNA purification kit, Promega, Madison, WI). Plasmid DNA was used for *nrfA* gene standards. Genomic DNA extracted from *Escherichia coli* strain MC4100 (Wang and Gunsalus 2000) and the *nrfA* gene was amplified via PCR using primers nrfA6F and nrfA6R with conditions from Takeuchi (2006). PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Life Technologies, Grand Island, NY). We isolated plasmid DNA containing a single copy of the *nrfA* gene using the Wizard Plus SV miniprep DNA purification kit (Promega, Madison, WI). All sample and standard DNA concentrations were determined by spectrophotometry at 260 nm (NanoDrop 2000 UV–vis spectrophotometer, Thermo Scientific, Hanover Park, IL).

Table 2 Results (*p* values) from a two-way ANOVA by site (four locations) and oyster treatment (oysters vs. control) and for measurements of sediment biogeochemistry and gene abundance, where values $p \leq 0.05$ are in italics

	Fall			Winter		
	Site	Oyster	Interaction	Site	Oyster	Interaction
Sediment biogeochemistry						
%OM	*0.017*	0.698	0.980	*<0.001*	0.674	0.689
C:N	0.257	0.277	0.271	0.084	0.103	*0.001*
xNH_4^+	*0.037*	0.858	0.997	0.087	0.579	0.596
NH_4^+ Min	*0.011*	0.417	0.829	*0.004*	0.181	0.090
NIT	0.070	0.722	0.767	0.524	0.722	0.362
DNP	0.241	*<0.001*	*0.035*	*0.023*	0.678	0.656
Gene abundance						
nirS	*<0.001*	0.856	0.111	*0.006*	0.203	0.360
nirK	*<0.001*	0.946	0.380	*0.028*	0.299	0.329
nrfA	*0.019*	0.600	0.101	*0.003*	0.340	0.540
16S rRNA	*0.008*	0.776	0.887	*0.002*	0.167	0.851

%OM percent organic matter, *C:N* carbon to nitrogen ratio, xNH_4^+ exchangeable ammonium, NH_4^+ *Min* ammonium mineralization, *NIT* nitrification, *DNP* denitrification potential

Quantitative PCR

We used qPCR to determine gene abundance, and all reactions were carried out in a StepOnePlus Real Time PCR system (Life Technologies, Grand Island, NY). After measuring DNA concentration, serially diluted standard DNA was used to generate qPCR standard curves. The qPCR reaction mixtures consisted of 5 μl Power SYBR® Green Master Mix (AmpliTaq Gold® DNA Polymerase, Power SYBR® Green PCR buffer, deoxynucleoside triphosphate mix with dUTP, SYBR Green I, ROX, and 5 mM $MgCl_2$), 1 μl forward primer (final concentration 0.3 μM), 1 μl reverse primer (final concentration 0.3 μM), 2 μl water, and 1 μl sample DNA. Triplicate reactions were analyzed for each sample. The *nirS* primers used were cd3aF (GTSAACGTSAAGGARACSGG) and Rc3d (GASTTCGGRTGSGTCTTGA) (Throback et al. 2004). Primers F1aCu (GASTTCGGRTGSGTCTTGA) and R3Cu (GCCTCGATCAGRTTGTGGTT) (Throback et al. 2004) were used for *nirK*. Cycling conditions adapted from Hallin et al. (2009) for both *nir* genes were as follows: 1 cycle of 95 °C for 10 min for initial denaturation and 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, primer extension at 72 °C for 60 s, and holding at 80 °C for 15 s, followed by plate reading. Primers nrfA6F (GAYT GCCAYATGCCRAAAGT) and nrfA6R (GCBKCTTT YGCTTCRAAGTG) were used for *nrfA* (Takeuchi 2006). Cycling conditions for *nrfA* quantification were as follows: 1 cycle of 95 °C for 10 min for initial denaturation and 50 cycles of denaturation at 95 °C for 15 s, primer annealing at 54.5 °C for 30 s, primer extension at 72 °C for 60 s, and

holding at 76 °C for 10 s, followed by plate reading (Lam et al. 2009). Universal primers 341 F (CCTACGGGAGGCAGCAG) and 534R (ATTACCGCGGCTGCTGG) were used to target the 16S rRNA gene (Muyzer et al. 1996). Cycling conditions for 16S rRNA were as follows: 1 cycle of 95 °C for 10 min for initial denaturation and 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C for 30 s, primer extension at 72 °C for 30 s, and holding at 80 °C for 15 s, followed by plate reading (López-Gutiérrez et al. 2004).

Genomic DNA was serially diluted over approximately 4 orders of magnitude and analyzed in triplicate to generate standard curves for *nirS*, *nirK*, and 16S rRNA gene abundance. For *nirS*, genomic DNA from *P. stutzeri* (ATCC 17588), which contains a single *nirS* copy (NCBI Accession: NC015740.1), was isolated and serially diluted to construct the standard curve covering an approximate range of 10^2–10^6 *nirS* copies μl^{-1}. The *nirK* and 16S rRNA gene standard curves were generated from isolated genomic *P. protegens* DNA which also has a fully sequenced genome with a single copy of *nirK* and 5 copies of the 16S rRNA gene (NCBI Accession: NC004129). The *nirK* gene standard curves covered an approximate range of 10^2–10^6 *nirK* copies μl^{-1} and 16S rRNA gene curves covered a range of 10^4–10^8 16S copies μl^{-1}. Gene copy numbers in serially diluted DNA standards were determined by measuring DNA concentration as described above, converting nanogram DNA per microliter to genome copy number, and determining gene copy numbers in DNA standards based on the number of *nirS*, *nirK*, or 16S rRNA genes per genome. The multiple copies of the 16S rRNA gene in *P. protegens* were taken into account when calculating gene copy numbers used to generate the standard curve.

Plasmids containing cloned *nrfA* genes were used to generate *nrfA* standard curves. The standard curves for *nrfA* were constructed using a TOPO-TA pCR-4 (Invitrogen, Grand Island, NY) generated plasmid with a single copy of *nrfA* per plasmid. The *nrfA* gene was isolated from *E. coli* MC4100 (Accession: HG738867.1). The number of *nrfA* gene copies in *E. coli* MC4100 is unknown (Wang and Gunsalus 2000), so we used plasmid DNA to ensure a known copy number per plasmid. The *nrfA* standard curves were generated using the constructed *nrfA* plasmid DNA and covered a range of 10^1–10^5 *nrfA* copies μl^{-1} with no samples falling outside of this range. The limits of detection for *nirS*, *nirK*, and *nrfA* were 50, 20, and 10 copies μl^{-1} consecutively. The slopes of all standard curves were used to calculate qPCR efficiency (qPCR efficiency $= 10^{(-1/\text{slope})} - 1$). All genes were cloned and sequenced for confirmation of primer specificity.

Two independent qPCR reactions were carried out for every gene and sample. Efficiencies ranged from 82 to 100 % (*nirS* mean=91 %, *nirK* mean=92 %, *nrfA* mean=82 %, 16S rRNA mean=93 %). Gene copy numbers were normalized to DNA extraction yield as DNA concentration varied between samples (Banerjee and Siciliano 2012). Gene copy numbers were converted to gene copies per gram sediment (wet weight) according to dilution factors. We followed the convention from other environmental studies which report gene copies per gram sediment, while accounting for variable DNA extraction efficiency across samples as has been done previously (Peng et al. 2013; Warneke et al. 2011). Three nontemplate controls were analyzed with every qPCR reaction and yielded no or negligible amplification. Finally, reaction specificity was analyzed with a melt curve at the end of every qPCR reaction and periodic checks for correct amplicon sizes on 2 % agarose gels.

Statistical Analysis

We used two-way analysis of variance (ANOVA) to compare gene abundance and biogeochemistry by oyster treatment and site for each season independently. Significant ANOVA results were followed by Tukey's multiple comparison test. If the interaction term was significant, we used a *t* test to measure oyster effect at each site individually, with a Bonferonni correction (i.e., $p = 0.05/n$, where n = number of comparisons). We used multiple linear regression to assess relationships among gene abundance, water chemistry, sediment characteristics, and N transformation rates. Multiple linear regressions were completed using a forward-stepping method for each of the four genes individually, where the dependent variable was gene abundance and the independent variables were all other environmental factors. Finally, principal components analysis (PCA) was used to analyze relationships among samples for our entire data matrix. All statistics were completed using R (R Development Core Team 2009), and PCA was completed using the package FactoMineR. Models were tested for normality using the Shapiro-Wilk normality test, examining the normal probability plots, and checking plots of residuals. If needed, data were log transformed to meet the assumptions of normality.

Results

Water Column Physicochemistry

Physicochemical measurements in the water column at each study site and date are summarized in Table 1. As expected, temperature in fall was >20 °C and was <5 °C in winter across all four sites (Table 1). At all sites, chlorophyll *a* was also lower in winter (3–5 $\mu g\ l^{-1}$) relative to fall (15–57 $\mu g\ l^{-1}$). However, water column nutrients were variable by season and site. For example, NO_3^- and NH_4^+ concentrations were highest at Spring Creek in winter (245 and 301 $\mu g\ N\ l^{-1}$, respectively) and SRP concentration was highest at Spring Creek in fall (81 $\mu g\ P\ l^{-1}$). In contrast, nitrite (NO_2^-)

concentrations and dissolved inorganic N (DIN) were highest at Wildlife Refuge in the fall (66 and 649 μg N l^{-1}, respectively; Table 1).

Sediment Biogeochemistry and Gene Abundance

There were few significant effects of oysters on sediment biogeochemistry, but significant differences among sites for almost all measurements (Table 2). In both fall and winter, sediment OM was lowest at Floyd Bennett Field (Fig. 1a and b). In contrast, NH_4^+ mineralization was highest at Floyd Bennett Field in both seasons (Fig. 1c and d). There was no difference among sites for nitrification in either season (Fig. 1e and f). Oysters showed no effect on DNP in winter (Fig. 1h). However, there was a significant interaction between site and oyster presence in fall (Table 2; Fig. 1g), where there was no oyster effect in Motts Basin (*t* test, $p=0.403$), but there was a significant effect in Spring Creek (*t* test, $p=0.010$) and Wildlife Refuge (*t* test, $p=0.009$; Fig. 1g). Fall samples for DNP in Floyd Bennett Field oyster sediment were lost. Exchangeable NH_4^+ was different among sites in fall (ANOVA $p=0.037$), but not in winter (ANOVA $p=0.087$). Finally, there were no differences in sediment C:N in fall and a significant interaction between oyster and site for C:N in winter (Table 2).

As expected, the abundance of 16S rRNA genes was higher than the abundance of *nirS*, *nirK*, and *nrfA* genes in both seasons (Fig. 2). Mean abundance for 16S rRNA was $1.4(10^8)$–$2.8(10^9)$ copies g $sediment^{-1}$, *nirS* abundance was $5.2(10^5)$–$1.2(10^7)$ copies g $sediment^{-1}$, *nirK* abundance was $1.1(10^5)$–$3.4(10^6)$ copies g $sediment^{-1}$, and *nrfA* abundance

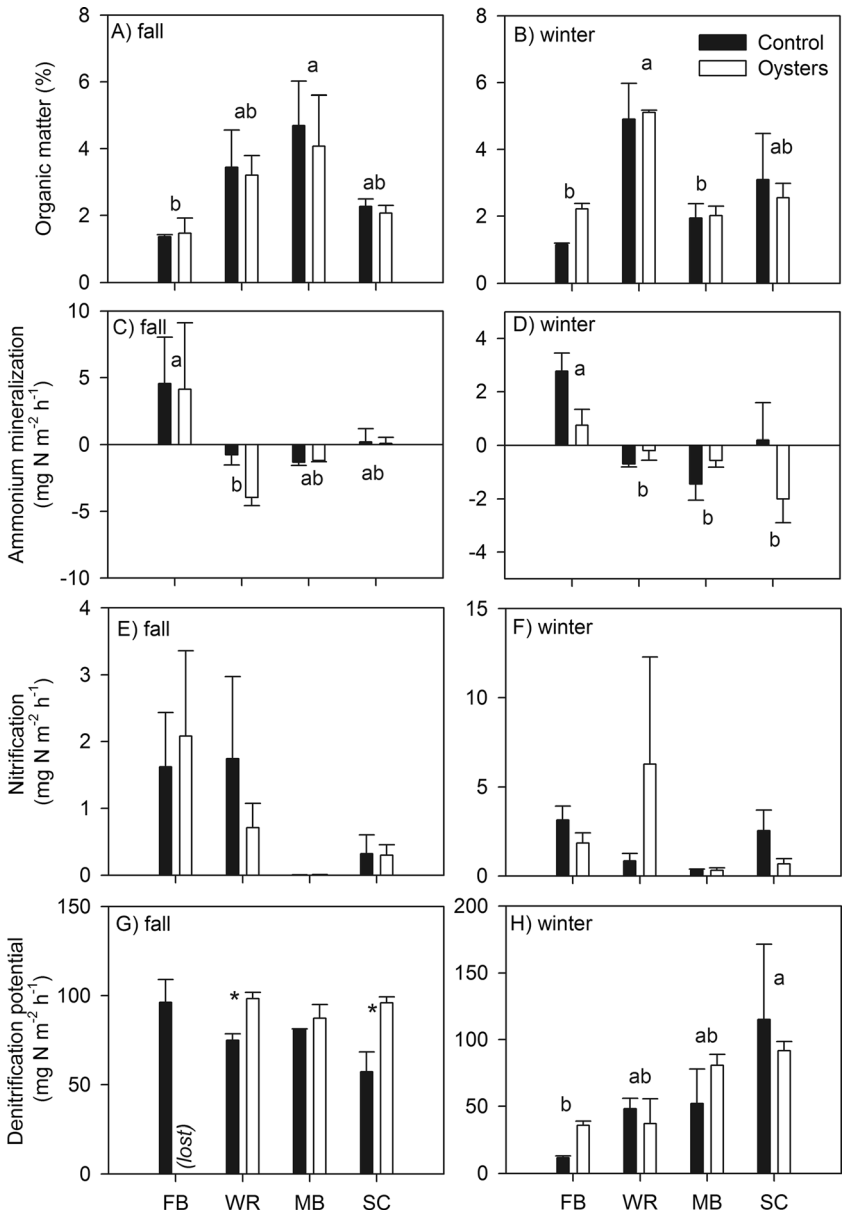

Fig. 1 Mean (±SE) sediment biogeochemistry measurements from Floyd Bennett Field (FB), Wildlife Refuge (WR), Motts Basin (MB), and Spring Creek (SC) for organic matter in **a** fall and **b** winter, ammonium (NH_4^+) mineralization in **c** fall and **d** winter, nitrification in **e** fall and **f** winter, and denitrification potential in **g** fall and **h** winter. *Small letters* indicate significant differences detected by Tukey's tests among sites within each panel, following two-way ANOVA showing a significant site effect and no oyster effect. *Asterisk* indicates significant difference in DNP between oyster and control sediment at a site, performed after a significant interaction factor in the two-way ANOVA and significant where $p \leq 0.017$ (Bonferroni adjusted as 0.05/3) (see Table 2)

was lowest at 2.4(10^3)–6.5(10^4) copies g sediment^{-1} (Fig. 2). On average, *nirS* gene abundance was 5 times greater than *nirK* and 300 times greater than *nrfA* (Fig. 2).

The abundance of all four genes was significantly different among sites in fall and winter; however, oysters had no effect on gene abundance (Table 2; Fig. 2). In fall, 16S rRNA, *nirS*, and *nirK* gene abundances showed similar patterns, where Motts Basin had the highest abundance for all three genes (Fig. 2a-g). Abundance of *nrfA* genes in fall was highest at Floyd Bennett Field and Wildlife Refuge (Fig. 2e). In winter, Wildlife Refuge and Spring Creek had the highest abundance of *nirS*, *nirK*, and 16S rRNA gene copies (Fig. 2b-f), while Wildlife Refuge had the highest abundance of *nrfA* gene copies (Fig. 2f).

We used the number of copies of each functional gene relative to the number of copies for the 16S rRNA gene to estimate the relative composition of the microbial community. Mean (±SE) *nirS* abundance was 1.5 (±1.1)% in fall and 1.78 (±2.23)% in winter, where Motts Basin was highest in both seasons (Supplemental Fig. 1A, B). Mean (±SE) *nirK* abundance was 0.46 (±0.40)% in fall and 0.34 (±0.44)% in winter, where Motts Basin was highest in fall (0.68 %) and Spring Creek was highest in winter (0.48 %; Supplemental Fig. 1C, D). Finally, mean (±SE) *nrfA* abundance was lower than for *nirS* and *nirK*, at 0.01 % (±0.01 %) in fall and 0.01 % (±0.02 %) in winter. Floyd Bennett had the highest percentage of *nrfA* in fall (0.02 %) and Motts Basin in winter (0.02 %; Supplemental Fig. 1E, F). We note that this relative abundance metric is a general estimate as individual cells may contain 1–

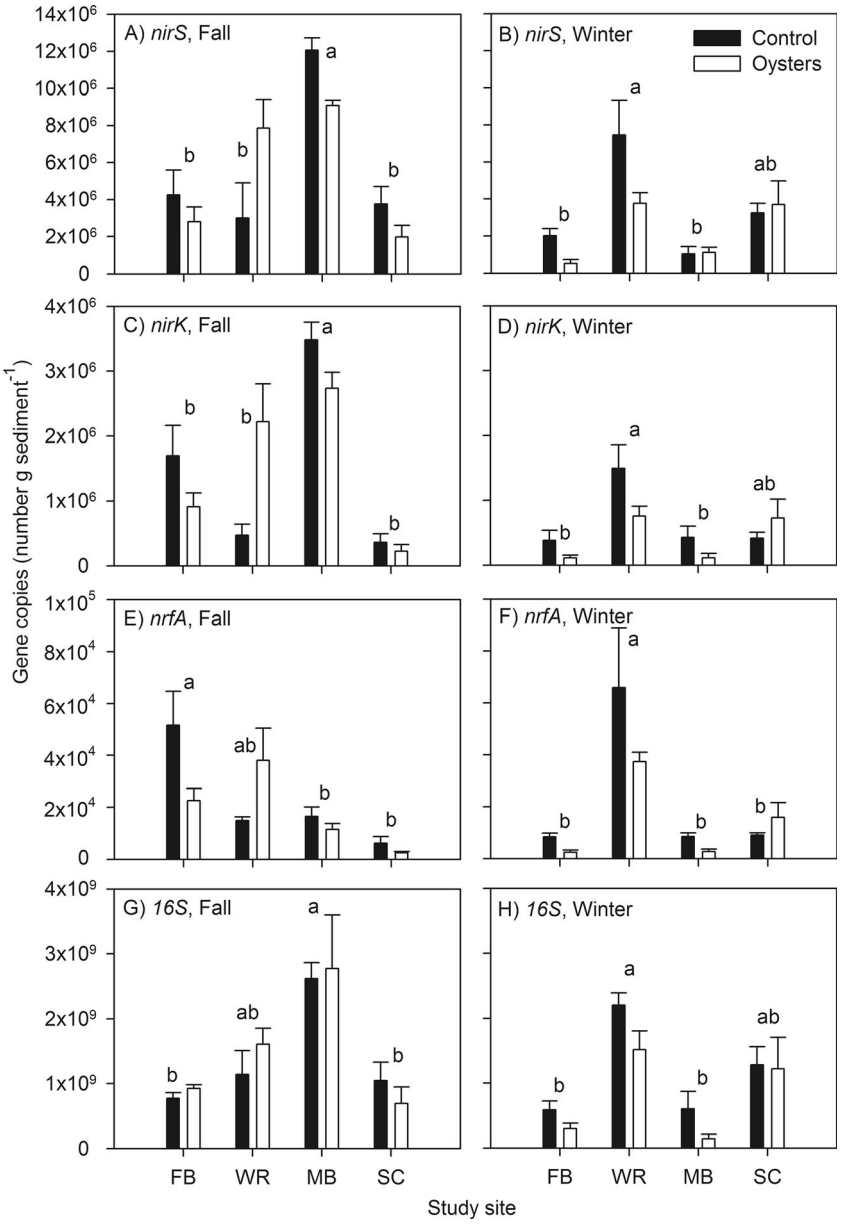

Fig. 2 Mean (±SE) gene copy numbers per gram sediment from Floyd Bennett Field (FB), Wildlife Refuge (WR), Motts Basin (MB), and Spring Creek (SC) for n*irS* in **a** fall and **b** winter, *nirK* in **c** fall and **d** winter, *nrfA* in **e** fall and **f** winter, and 16S rRNA in **g** fall and **h** winter. *Small letters* indicate significant differences detected by Tukey's tests among sites within each panel, following a two-way ANOVA showing a significant site effect and no oyster effect (see Table 2)

15 copies of the 16S rRNA gene per genome (Kembel et al. 2012).

Principal Components Analysis

The first component of the PCA (PC1) explained 33.9 % of the variation in the data and the second component (PC2) explained 17.5 % (total variation in PC1 and PC2=51.4 %; Fig. 3). PC1 had a significant positive relationship with *nirS*, *nirK*, and 16S rRNA gene abundances and water chemistry measurements including SRP, NO_2^-, and NH_4^+ concentrations and water column chlorophyll *a* (Table 3; Fig. 3a). Sediment OM and water column NO_3^- concentration showed a positive correlation with PC2, and temperature was negatively correlated with PC2 (Table 3; Fig. 3a). Finally, *nrfA* abundance was significantly related to the third principal component, which explained 12.1 % of the variation in the data (Table 3). Nitrification and NH_4^+ mineralization were also positively related to PC3, while DNP was negatively related to PC3 (Table 3).

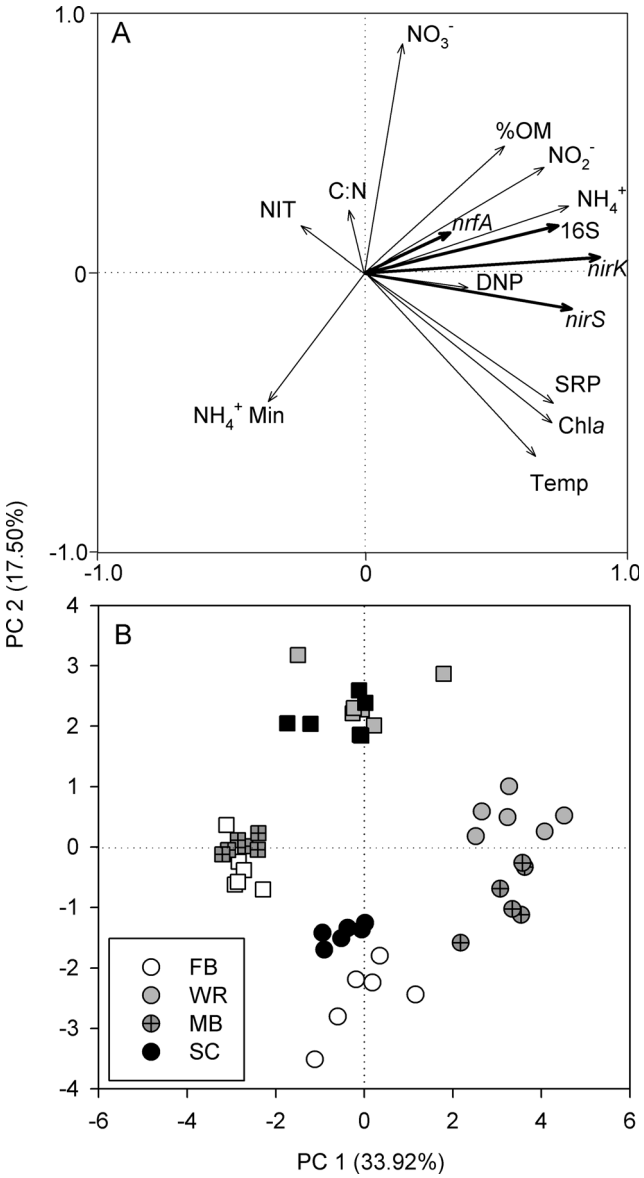

Fig. 3 **a** PCA factor map for variables included in the PCA and **b** scatterplot of component scores for each sediment sample from Floyd Bennett Field (FB), Wildlife Refuge (WR), Motts Basin (MB), and Spring Creek (SC) from fall and winter (n=48). *Circles* indicate data from fall, *squares* indicate data from winter

Data clustered in the PCA scatterplot diagram based on season and site (Fig. 3b). Data from fall scored positively on PC1 and negatively on PC2. In fall, Floyd Bennett Field and Spring Creek samples formed clusters that scored negatively on PC2, attributed to lower NO_3^- and chlorophyll *a* concentrations, while Motts Basin and Wildlife Refuge scored positively on PC1 based on high gene abundance and water column NO_2^-. In winter, data also formed two distinct clusters. Spring Creek and Wildlife Refuge scored high on PC2 due to high NO_3^- concentrations, while Floyd Bennett Field and Motts Basin scored low on PC1 due to relatively low abundance of the four genes measured.

Table 3 Loading matrix of correlation coefficients for variables including gene abundance, sediment biogeochemistry, and water column physicochemistry with principal components (PC) 1, 2, and 3

	PC1	PC2	PC3
Gene abundance			
nirS	*0.355*	0.050	0.080
nirK	*0.365*	−0.059	0.095
16S rRNA	*0.295*	0.089	0.051
nrfA	0.138	0.095	*0.426*
Sediment biogeochemistry			
OM%	0.207	*0.317*	0.077
C:N	−0.061	0.163	0.064
NH_4^+ Min	−0.149	−0.265	*0.414*
NIT	−0.097	0.142	*0.603*
DNP	0.171	−0.038	*−0.485*
Water column physicochemistry			
Temp	0.31	*−0.391*	0.033
Chl *a*	*0.322*	−0.313	0.077
SRP	*0.315*	−0.233	0.008
NO_3^-	0.034	*0.545*	−0.090
NO_2^-	*0.315*	0.305	0.045
NH_4^+	*0.355*	0.217	0.015

Values are considered significant at ≥0.3 and≤−0.3, which are marked in italics

%OM percent organic matter, *C:N* carbon to nitrogen ratio, *xNH_4^+* exchangeable ammonium, *NH_4^+ Min* ammonium mineralization, *NIT* nitrification, *DNP* denitrification potential, *Temp* temperature, *Chl a* chlorophyll *a*, *SRP* soluble reactive phosphorus, *NO_3^-* water column nitrate, *NO_2^-* water column nitrite, *NH_4^+* water column ammonium

Multiple Linear Regressions

Relationships between gene abundance and environmental characteristics were quantified using multiple linear regression (MLR) for each gene. For *nirS* gene abundance, the MLR model accounted for 71 % of the variance in the data. Significant factors included sediment OM, chlorophyll *a*, exchangeable NH_4^+, and SRP (Table 4). Sediment OM, water column chlorophyll *a*, and SRP had a positive relationship with *nirS* gene abundance, but the relationship with exchangeable NH_4^+ was negative, and sediment OM explained the most variance (50 %). The MLR model generated for *nirK* gene abundance accounted for 79 % of the variation in the data. Significant factors were chlorophyll *a*, temperature, and exchangeable NH_4^+ (Table 4). Chlorophyll *a* and temperature both had positive relationships with *nirK* gene abundance, while exchangeable NH_4^+ was negatively related. Here, chlorophyll *a* explained the most variance in *nirK* gene abundance (61 %). For *nrfA* gene abundance, the MLR model explained 61 % of the variance. Significant factors were C:N, NO_2^-, NH_4^+, chlorophyll *a*, and NO_3^- (Table 4). Variables negatively related to *nrfA* gene abundance were chlorophyll *a* and NH_4^+, while C:N, NO_2^-, and NO_3^- were positively related to *nrfA* gene abundance. C:N explained the most variance in this case (12 %). The last MLR model accounted for 52 % of the variance in 16S rRNA gene abundance. Significant factors were sediment OM, NH_4^+, NO_3^-, and NH_4^+ mineralization (Table 4), where OM accounted for 28 % of the variance, and all relationships were positive.

Discussion

No Evidence for Oyster Effects on Gene Abundance

Our primary goal was to test the hypothesis that oysters would increase the abundance of denitrifying and DNRA genes in underlying sediments in fall. However, no significant effect of oysters on *nirS*, *nirK*, or *nrfA* gene abundances was found, despite finding a significant effect of oysters on DNP at two sites in fall. Overall, gene abundance results are consistent with the larger project which showed relatively few effects of oysters on N dynamics in Jamaica Bay (Hoellein and Zarnoch 2014), despite the longer sampling periods and more oyster treatments (i.e., low and medium density) in the larger study.

No previous research has quantified the effects of oysters on the abundance of denitrifying bacteria, but a growing body of work suggests the effect of oysters on denitrification (Hoellein et al. 2015; Kellogg et al. 2013; Smyth et al. 2013a), and sediment OM (Azandégbé et al. 2012; Hoellein and Zarnoch 2014) is context dependent (Kellogg et al. 2014). Because *nirS* and 16S rRNA gene abundances were correlated with sediment OM at these sites (Table 4), and oysters

Table 4 Forward-stepping multiple regression results for nitrite reductase and 16S rRNA gene abundances

Dependent variable	Independent variable(s)	R^2	*p* value
nirS	%OM	0.497	<0.001
	%OM, Chl *a*	0.576	<0.001
	%OM, Chl *a*, xNH_4^+	0.666	<0.001
	%OM (+), Chl *a* (+), xNH_4^+ (−), SRP (+)	*0.705*	<0.001
nirK	Chl *a*	0.614	<0.001
	Chl *a*, temp	0.725	<0.001
	Chl *a* (+), temp (+), xNH_4^+ (−)	*0.785*	<0.001
nrfA	C:N	0.116	0.027
	C:N, NO_2^-	0.163	0.031
	C:N, NO_2^-, NH_4^+	0.264	0.008
	C:N, NO_2^-, NH_4^+, Chl *a*	0.431	<0.001
	C:N (+), NO_2^- (+), NH_4^+ (−), Chl *a* (−), NO_3^- (+)	0.614	<0.001
16S rRNA	%OM	0.280	<0.001
	%OM, NH_4^+	0.416	<0.001
	%OM, NH_4^+, NO_3^-	0.474	<0.001
	%OM (+), NH_4^+(+), NO_3^- (+), NH_4 Min (+)	*0.518*	<0.001

The direction of relationship between the independent and dependent variables are shown in the final step of each model and the final R^2 for each regression is in italics. All models were checked for normality with Shapiro-Wilk test

NO_3^- water column nitrate, NO_2^- water column nitrite, NH_4^+ water column ammonium, NH_4 *Min* ammonium mineralization, *SRP* water column soluble reactive phosphorus, *DIN* water column dissolved inorganic nitrogen, *Chl a* water column chlorophyll *a*, *temp* temperature, *%OM* sediment organic matter, *C:N* sediment carbon to nitrogen ratio, xNH_4^+ sediment exchangeable ammonium

increased sediment OM (Hoellein and Zarnoch 2014), we suggest that there is *potential* for oysters to affect denitrification and other microbially mediated processes at these sites. Of the two seasons selected for this analysis, oysters increased DNP in the fall, although oysters had no effect on sediment N biogeochemistry when data from all dates were considered together (Hoellein and Zarnoch 2014). Thus, the influence of oysters on sediment processes may be periodic, reflecting spatial and temporal changes in limiting chemical substrates and conditions (i.e., NO_3^-, C, or redox). If this occurs for short time periods, the effect of oysters could be detected as a change in DNP with no corresponding effects on gene abundance. Overall, a larger suite of tools may be required to fully understand the effect of oysters on N cycling, including a diversity of measurements for microbial communities and biogeochemical transformations (Kellogg et al. 2014).

The experimental approach of sediment boxes with overlying oysters was designed to reveal the influence of oyster biodeposits (i.e., feces and pseudofeces) on microbially mediated biogeochemical transformations in underlying sediment in this eutrophic ecosystem. Our research for the larger project showed that oysters increased sediment organic matter (Hoellein and Zarnoch 2014). However, we note that experimental conditions likely differ from sediment at restored oyster reefs. Natural reefs contain shell hash in sediments, alter local hydrology, and contain many reef-associated organisms (e.g., fish and invertebrates), all of which may affect microbial communities and processes (Hoellein et al. 2015; Kellogg et al. 2014). Restored oyster reefs in NYC waters are relatively new and construction is ongoing. The results from this experimental approach will guide hypotheses for how restored reefs will affect N cycling at those sites and in eutrophic coastal environments elsewhere. In particular, oyster-mediated denitrification should be expected to be site and season specific, and microbial gene abundance and N cycling rates may be decoupled (Baxter et al. 2013; Beman 2014).

Sediment Characteristics, not Water Column NO_3^-, Explained *nirS*, *nirK*, and 16S rRNA Gene Abundance

Despite the lack of oyster effects on gene abundance, significant variation by season and site allowed us to explore which environmental factors affect abundance of denitrifying and DNRA bacteria. For example, sediment OM showed strong, positive relationships with *nirS* and 16S rRNA gene abundances (Table 4; Fig. 3). Similarly, the factor which explained the most variation in *nirK* abundance was water column chlorophyll *a*, which can serve as a labile OM source after settling to the sediment surface (Eyre and Ferguson 2005; Fulweiler et al. 2013). Results are consistent with previous research showing that *nirS* and *nirK* gene abundances were related to OM across ecosystem types including estuaries and soils (Abell et al. 2013; Banerjee and Siciliano 2012; Barrett et al. 2013; Kandeler et al. 2006; Levy-Booth and Winder 2010).

In addition to sediment OM, we hypothesized that water column NO_3^- concentrations would be positively related to functional gene abundance since DNP was related to water column NO_3^- (Hoellein and Zarnoch 2014). However, we found no relationship between NO_3^- concentrations and abundance of *nirS*, *nirK*, or *nrfA* genes. This pattern has been documented in estuaries elsewhere (Beman 2014) and in other ecosystems. For example, Kandeler et al. (2006) found that soil NO_3^- availability did not explain any variation in denitrification gene abundance, while OM was the most important factor. The same pattern was documented for sediment from agricultural streams (Baxter et al. 2013). In forest soils, *nirS* and *nirK* abundances were correlated with OM but not NO_3^- levels (Levy-Booth and Winder 2010). Finally, a synthesis of studies on denitrification using molecular methods concluded that NO_3^- concentration was a proximal control on denitrification rates, but did not have a strong effect on denitrifier community composition (Wallenstein et al. 2006).

Abundance of *nirS* was greater than *nirK*, and abundance of both genes was comparable to the results from other estuaries (Bowen et al. 2014; Bulow et al. 2008; Mosier and Francis 2010; Smith et al. 2007). The predominance of *nirS* may be caused by a variety of factors, and previous work has focused on how oxygen (O_2) affects the *nirS* to *nirK* ratio. In environments conditionally exposed to O_2 (e.g., agricultural soils), *nirK* prevails over *nirS*, while *nirS* is more abundant in continuously anoxic environments (Desnues et al. 2007; Knapp et al. 2009). The cytochrome cd_1 nitrite reductase encoded by *nirS* can catalyze two reactions: the single electron reduction of NO_2^- to NO and the four-electron reduction of O_2 to H_2O (Einsle and Kroneck 2004). In contrast, the copper-containing nitrite reductase encoded by *nirK* carries out only the reduction of NO_2^- to NO (Einsle and Kroneck 2004). These enzymatic differences contribute to the ability of *nirK*-containing organisms to denitrify in the presence of higher O_2 compared to *nirS*-containing organisms. The higher O_2 thresholds for *nirK* bacteria may enhance their capacity to compete with aerobic heterotrophs in environments like agricultural soils (Priemé et al. 2002). In contrast, the *nirS* gene could be better suited for aquatic habitats and was more abundant than the *nirK* gene in Jamaica Bay and several other marine environments (Beman 2014; Desnues et al. 2007; Huang et al. 2011; Mosier and Francis 2010).

The abundance of 16S rRNA genes was orders of magnitude higher than the *nirS*, *nirK*, and *nrfA* genes, and sediment OM alone accounted for 28 % of the variance in 16S rRNA gene abundance. The results suggest that heterotrophic microbes dominate the bacterial community at these sites. Other studies which quantify gene abundance and related environmental factors have found that elevated C increases 16S rRNA gene abundance across terrestrial and aquatic environments

(Abell et al. 2013; Kandeler et al. 2006). Abundance of the 16S rRNA genes was also positively related to DIN (NH_4^+ and NO_x^-), which after OM accounted for an additional 19.8 % of variation. DIN was unrelated to functional gene abundance (i.e., *nirS*, *nirK*, *nrfA*), however, suggesting that DIN availability was strongly correlated with the growth of the general community, rather than the growth of microbes which carry out denitrification and DNRA.

Abundance of *nrfA* Was Lower than That of *nirS* and *nirK* and Related to Different Factors

As predicted, abundance of *nrfA* was much lower than that of *nir* genes, which has implications for predicting the magnitude of rates of denitrification and DNRA in Jamaica Bay (Song et al. 2014). The abundance of the *nrfA* gene is lower than that of functional genes for denitrification in many aquatic habitats (Dong et al. 2009; Huang et al. 2011; Smith et al. 2007), and we speculate from this pattern that rates of DNRA potential are lower than denitrification potential. In Jamaica Bay, this would indicate that less NO_3^- is recycled within the ecosystem as NH_4^+ than is removed in the form of N gases, at least from the context of dissimilatory NO_3^- cycling. This is a positive finding from a management perspective because greater capacity for DNRA than denitrification would enhance the effects of eutrophication. However, we did not measure DNRA rates in this study, and support for this conclusion is reduced by the lack of relationship between DNP and *nirS* and *nirK* gene abundance. Previous research has shown that the balance between the two rates can shift toward DNRA in conditions with high sediment sulfide (which inhibits *nosZ* but enhances DNRA), high salinity, and warmer temperature (Burgin and Hamilton 2007; Dunn et al. 2013; Gardner and McCarthy 2009). Recent evidence from Smyth et al. (2013b) also showed that while DNRA was higher in oyster reefs relative to other estuary habitats, DNRA represented a relatively minor component of total NO_3^- flux across habitat types in summer and winter.

The major controlling factors of *nrfA* gene abundance in our study were sediment C:N (positive) and water column chlorophyll *a* (negative; Table 3), which may be attributed in part to competitive relationships between microbes which carry out denitrification and those which complete DNRA. Tiedje et al. (1989) hypothesized that the availability of labile C favors denitrification over DNRA because of enzymatic efficiencies. Abundance of the *nrfA* gene was also positively correlated with C:N in an urban river (Huang et al. 2011), and *nirS* gene abundance decreased with experimental additions of recalcitrant OM to sediments while *nrfA* gene abundance was unaffected (Morrissey et al. 2013). Our results are similar, as *nrfA* gene abundance was unrelated to sediment OM but positively related to C:N, while *nirS*, *nirK*, and 16S rRNA gene abundances showed positive relationships with OM and no relationship with C:N. Song et al. (2014), however, showed a positive correlation between sediment organic content and *nrfA* gene abundance and DNRA rates in a lagoonal estuary. Therefore, we note that more research is needed to correlate DNRA gene abundance with DNRA rates and to

Table 5 Summary of results from studies which have measured the relationship between denitrification gene abundance (qPCR) and denitrification potential (DNP; acetylene block), across ecosystem types

Gene	Relationship with DNP	Strength	Ecosystem	Citation
nirS and *nirK*	None	–	Estuary	This study
nirS and *nirK*	None	–	Estuary	Beman (2014)
nosZ	None	–	Stream	Baxter et al. (2013)
nirS	None	–	Wetland	Song et al. (2010)
nirK	None	–	Agricultural field	Dandie et al. (2008)
nirS and *nirK*	None	–	Tropical soil	Djigal et al. (2010)
nirS and *nirK*	Positive *nirS* None *nirK*	*nirS* ρ=0.57	Estuary	Mosier and Francis (2010)
nirS and *nirK*	Positive	*nirS* r=0.46 *nirK* r=0.40	Wetland	Correa-Galeote et al. (2013)
nirS and *nirK*	Positive	r=0.73	Forest soil	Petersen et al. (2012)
nirS and *nirK*	Positive	*nirS* r=0.34 *nirK* r=0.27	Arctic soil	Banerjee and Siciliano (2012)
nirS and *nirK*	Positive *nirK* None *nirS*	*nirK* R^2=0.40	Agricultural field	Attard et al. (2011)
nirS and *nirK*	Positive *nirK* None *nirS*	*nirK* R^2=0.28	Tropical soil	Baudoin et al. (2009)
nirS and *nirK*	Positive	*nirS* r=0.36 *nirK* r=0.31	Agricultural field	Enwall et al. (2010)

Results from correlation are indicated as r or ρ values, linear regression results are shown as R^2 values, and "–" indicates no significant relationship

measure competition among bacteria which carry out dissimilatory NO_3^- cycling in urban coastal environments. In particular, we suggest that future research would benefit from including measurements in all seasons.

No Relationship Between *nirS* or *nirK* with Denitrification Potential

Contrary to our hypothesis, *nir* gene abundance and DNP were unrelated. This was unexpected because DNP and gene abundance should both reflect the capacity for the microbial community to conduct denitrification. However, this pattern has been documented in some previous research. To facilitate comparison of results from this project to similar studies in the literature, we composed a summary table of results from published research which measured the relationship between *nirS* and/or *nirK* and DNP using the same methods (i.e., acetylene block and qPCR). An assessment of the summary confirms variability in the results among studies. Like this study, no relationship between gene abundance and DNP was found in research spanning estuaries, streams, wetlands, and soils. However, several other studies found a positive relationship with *nir* genes and DNP of varying strength, and some studies show a positive relationship with one *nir* gene and not the other (Table 5).

Several factors may explain decoupling of gene abundance and DNP, including the DNP method, the use of DNA instead of RNA, limitations of PCR primers, and not accounting for all denitrification products and genes. We used chloramphenicol in our 4-h acetylene block measurements, which prevents de novo synthesis of new enzymes (Smith and Tiedje 1979). However, methodological artifacts of the acetylene block measurements are well documented (Groffman et al. 2006) and include inhibition of nitrification and slow diffusion of acetylene. In estuaries, studies using other methods to measure denitrification have found correlations between *nirS* and *nirK* gene abundances (Dong et al. 2009; Morales et al. 2010). We measured DNA abundance, which may not reflect enzyme production or distinguish between live and dormant cells. Instead, the number of mRNA copies of each gene represents active transcription and may provide a better relationship with rate measurements (Freeman et al. 1999; Nolan et al. 2006). There are some limitations to the *nirK* and *nrfA* primer sets. Most *nirK* primers are based on class I copper nitrite reductase (CuNIR) genes from α-Proteobacteria and do not amplify class II and III *nirK* sequences such as archaeal *nirK* (Braker et al. 1998; Green et al. 2010). There are only few *nrfA* sequences available in nucleotide databases that originate from pathogenic strains and not environmental samples (Giacomucci et al. 2011). However, the *nirK* and *nrfA* primers were among the most widely used to quantify bacteria conducting denitrification and DNRA at the time of our study. Finally, many microbial denitrifiers do not contain the entire suite of enzymes and may therefore contain *nir* genes but do not conduct the other steps of denitrification (Wallenstein et al. 2006; Zumft 1997). Thus, measuring the abundance of multiple genes and fluxes of solutes and N gases may be required to more fully explain the relationship between gene abundance and denitrification.

Conclusion

Data on microbial communities are often absent from models that predict ecosystem effects of eutrophication or restoration, including for oyster reefs (Kellogg et al. 2014), in part because of the challenge in linking microbial processes to communities. In addition, aspects of microbial community composition may be extraneous to measurements of microbially derived products (e.g., denitrification), which are the desired outcome. However, further attempts to relate aspects of microbial communities (i.e., gene abundance, gene expression, or community composition) with microbial processes in the context of eutrophication or restoration are important. First, these data will add to basic research on microbial ecology, helping to build our understanding of how communities regulate processes. Second, restoration and eutrophication present environmental conditions that can be used to experimentally quantify relationships between microbial communities and processes in new ways. Finally, microbial ecology is a rapidly developing field. Aspects of microbial communities which generate no relationship to measurement of microbial processes at present may be reexamined from a different perspective as new data emerge, or help justify new or expanded approaches to data collection.

Our experiment contained results from multiple treatments, sites, and seasons within an urban coastal ecosystem and revealed which environmental factors drive the abundance of genes responsible for critical sediment N transformations. These data may help to predict under what conditions oysters most likely effect microbially mediated N cycling at this site and similar ecosystems elsewhere. Further explorations of the relationship between microbes and N transformation rates will benefit from multiple, simultaneous measurements (e.g., combining ^{15}N tracers, RNA abundance, and a suite of N cycling genes) across a variety of environmental conditions and time periods, including additional seasons. Results will serve to develop stronger predictive relationships between microbial gene abundance, ecosystem processes, sediment characteristics, and physicochemical conditions, thereby maximizing the potential for oyster reef restoration to benefit ecosystem health.

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