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Long-term nutrient addition differentially alters community composition and diversity of genes that control nitrous oxide flux from salt marsh sediments

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ABSTRACT

Enrichment of natural waters, soils, and sediments by inorganic nutrients, including nitrogen, is occurring at an increasing rate and has fundamentally altered global biogeochemical cycles. Salt marshes are critical for the removal of land-derived nitrogen before it enters coastal waters. This is accomplished via multiple microbially mediated pathways, including denitrification. Many of these pathways, however, are also a source of the greenhouse gas nitrous oxide (N_2O). We used clone libraries and quantative PCR (qPCR) to examine the effect of fertilization on the diversity and abundance of two functional genes associated with denitrification and N_2O production (*norB* and *nosZ*) in experimental plots at the Great Sippewissett Salt Marsh (Falmouth, MA, USA) that have been enriched with nutrients for over 40 years. Our data showed distinct *nosZ* and *norB* community structures at different nitrogen loads, especially at the highest level of fertilization. Furthermore, calculations of the Shannon Diversity Index and Chao1 Richness Estimator indicated that *nosZ* gene diversity and richness increased with increased nitrogen supply, however no such relationship existed with regard to richness and diversity of the *norB* gene. Results from qPCR demonstrated that *nosZ* gene abundance was an order of magnitude lower in the extra-highly fertilized plots compared to the other plots, but the abundance of *norB* was not affected by fertilization. The majority of sequences obtained from the marsh plots had no close cultured relatives and they were divergent from previously sequenced *norB* and *nosZ* fragments. Despite their divergence from any cultured representatives, most of the *norB* and *nosZ* sequences appeared to be from members of the Alpha- and Betaproteobacteria, suggesting that these classes are particularly important in salt marsh nitrogen cycling. Our results suggest that both *norB* and *nosZ* containing microbes are affected by fertilization and that the Great Sippewissett Marsh may harbor distinct clades of novel denitrifying microorganisms that are responsible for both the production and removal of N_2O.

1. Introduction

Estuaries are among the most productive ecosystems on Earth (Nixon et al., 1986). As interfaces between the land and the sea, estuaries receive a number of exogenous inputs from human activity, including excess nitrogen additions. The fate of anthropogenic nitrogen is particularly important in coastal systems, as coastal primary producers are most often nitrogen limited (Boynton and Kemp, 2008). Excess nitrogen input to estuaries may cause widespread or localized eutrophication, which can have severe effects on estuarine ecology (Galloway et al., 2008). Salt marshes are highly effective at the removal of bioavailable nitrogen through incorporation of nitrogen into plant biomass and trapping of nitrogen within sediments (Valiela et al., 1973; Brin et al., 2010). Marshes also convert inorganic nitrogen to nitrogenous gases via coupled nitrification-denitrification, direct denitrification, and, to a lesser extent, anaerobic ammonium oxidation (anammox; Hopkinson and Giblin, 2008). In both natural waters and sediments, the cycling of nitrogen is microbially mediated, however the effect of anthropogenic nutrient loading on microbial communities in coastal marine systems is poorly understood.

The microbially mediated processes that serve to remove nitrogen (N) from marsh sediments may also promote the production of the potent greenhouse gas nitrous oxide (N_2O). Atmospheric

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concentrations of N_2O are increasing at a rate of approximately 0.3% per year, and much of this increase is related to the release of anthropogenic nitrogen to the environment (Forster et al., 2007). Isotopic analysis of N_2O in the open ocean suggests that it is generated primarily by ammonia oxidizing archaea (Santoro et al., 2011), but the source of N_2O from estuarine systems remains unclear. Unlike open ocean systems, rates of denitrification in estuarine sediments and salt marshes are high (Hopkinson and Giblin, 2008) and since N_2O is a common byproduct of denitrification, this metabolic process could contribute to high rates of N_2O production observed in coastal waters (Wrage et al., 2001). N_2O could also result from methane or ammonia oxidizing bacteria in surface sediments (Simon and Klotz, 2013) or from detoxification of cellular nitric oxide (Stein, 2011). Examining how increasing nutrient supply alters the microbes associated with N_2O fluxes will provide important context for understanding controls on these greenhouse gas fluxes.

Under low oxygen conditions, such as those that exist in salt marsh sediments, N_2O is most commonly produced through either nitrifier denitrification or direct denitrification pathways (Wrage et al., 2001; Arp and Stein, 2003). In both cases, the concentration of N_2O is controlled by two enzymes: nitric oxide reductase (Nor), which generates N_2O by reducing highly destructive nitric oxide (NO; Spiro, 2007), and nitrous oxide reductase (Nos), which reduces N_2O to N_2. Nitric oxide reduction is mediated by the enzymes encoded by the *nor* gene cluster, in particular, the *norB* gene. *norB* is widespread, possessing orthologs in all domains of life (Kraft et al., 2011), and is divided into two-types (1) *cnorB* (cytochrome electron donor) and (2) *qnorB* (quinol electron donor; Hendriks et al., 2000). Canonical denitrifiers, as well as many nitrifiers, possess *cnorB* and are able to derive energy from the reduction of NO. Microbes that possess *qnorB* generally use the proteins encoded by the *qnor* gene cluster as a means of NO detoxification or possibly NO scavenging (Hendriks et al., 2000).

Once N_2O is produced it either escapes from the system or can be reduced to N_2 by nitrous oxide reductase, encoded by the *nosZ* gene. Up to a third of cultured denitrifiers do not possess the *nosZ* gene and many more do not express the *nosZ* gene during denitrification, thereby resulting in incomplete denitrification and the flux of N_2O (Jones et al., 2008). Several taxa of microbes also possess the *nosZ* gene but lack the ability to perform complete denitrification (Jones et al., 2013). These N_2O 'scavengers' derive a small amount of energy from N_2O reduction and have recently been found to be abundant and diverse in soil environments (Sanford et al., 2012; Jones et al., 2013). The relative response to perturbations by microbes that contain the *norB* and *nosZ* genes may ultimately control whether coastal sediments are net sources or net sinks of N_2O.

In this study we assessed the effects of long-term fertilization on the diversity of two genes that directly control N_2O fluxes (*norB* and *nosZ*). We used the experimental plots of the Great Sippewissett Salt Marsh (Falmouth, MA; Valiela et al., 1973) to test the effect of fertilization on the diversity, abundance, and community composition of *norB* and *nosZ* bearing denitrifiers. Because our interest was primarily those organisms that derive energy from the reduction of NO to N_2O or N_2, we focused on defining the community structure of microbes that contain *cnorB* rather than *qnorB*, as well as those containing *nosZ*. We hypothesized that both genes would be affected by fertilization, however due to the facultative nature of the reduction of N_2O and the substantial proportion of denitrifiers that lack the *nosZ* gene (Jones et al., 2008), the *nosZ* gene would demonstrate increased richness, diversity, and abundance in the highly fertilized plots. Additionally due to the highly toxic nature of NO, we would expect the *norB* gene to be more highly conserved and thus have lower genetic diversity and be less responsive to perturbation than the *nosZ* gene.

2. Methods

2.1. Site description

Our study was conducted in the fertilized plots (Valiela et al., 1973, 1975) of the Great Sippewissett Salt Marsh in Falmouth, MA, USA (Fig. 1). Fertilization of the plots has occurred since 1971. Circular 10 m^2 diameter plots are fertilized biweekly throughout the growing season using commercially available mixed NPK fertilizer (10% N, 6% P, 4% K by weight) at three different levels of fertilization in duplicate plots with the following loads: LF: 0.85 g N m^{-2} wk^{-1}, HF: 2.52 g N m^{-2} wk^{-1}, and XF: 7.46 g N m^{-2} wk^{-1}. Two control plots (C) remain unfertilized. Each plot is centered on a tidal creek to allow equivalent flooding of the low marsh, which is dominated by the tall ecotype of the cordgrass *Spartina alterniflora*. Areas of each plot above mean high tide are dominated by the short ecotype of *Spartina alterniflora* (in C plots), *Distichlis spicata* (in XF plots), or a combination of the two (Fox et al., 2012). More information about the establishment and maintenance of the plots, as well as the distribution of vegetation can be found in Valiela et al. (1973, 1975) and Fox et al. (2012).

2.2. Sample collection and DNA extraction

Duplicate samples from each plot were collected in September 2009 using cutoff sterile 25 cc syringes. In each plot the top 1–2 cm of marsh surface sediment underlying the tall ecotype of *Spartina alterniflora* was collected and the duplicate cores were thoroughly mixed with a sterile metal spatula in a 50-ml single use centrifuge tube. Subsamples of the homogenized sediments were then aliquoted into cryovials, and frozen in liquid nitrogen for transportation to the lab. Sediment was stored at −80 °C until extraction. DNA was extracted in triplicate using the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was verified via electrophoresis on a 1.5% agarose gel stained with ethidium bromide. DNA concentrations were measured fluorometrically on a NanoDrop 2000c (ThermoScientific, Waltham, MA, USA).

2.3. PCR amplification, cloning, and sequencing

Fragments of the *norB* gene were amplified using the norB2F/6R primer set (Casciotti and Ward, 2005), corresponding to a 395 bp fragment. Triplicate 25 μl reactions were carried out using the following reaction chemistry: 1× High Fidelity Phusion Buffer and 0.5 U Phusion polymerase (New England BioLabs, Ipswich, MA, USA), 0.8 mM dNTPs, 2.0 μM of each primer, 0.7–1.4 ng of DNA, and an additional 1.5 mM $MgCl_2$. Fragments were amplified using the following cycling conditions: an initial denaturation at 98 °C for 2m30s, followed by 6 cycles of 98 °C for 30s, 59.5 °C for 30s (−0.5 °C touchdown per cycle), and 72 °C for 15s. An additional 29 cycles were run using the following conditions: 98 °C for 10s, 56.5 °C for 30s, 72 °C for 10s, and ending with a final extension at 72 °C for 5m. Triplicate reactions were assessed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Target bands were excised and purified using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).

Fragments of the *nosZ* gene were amplified using the primer set nosZ1F/2R (Henry et al., 2006) resulting in a 799 bp product. *nosZ* fragments were amplified in triplicate using the following reaction chemistry at a total volume of 25 μl: 1× High Fidelity Phusion Buffer, 1.54 mM dNTPs, 0.77 μM of each primer, 0.5 U Phusion polymerase, 0.1–0.5 ng of DNA, and 0.38 mM additional $MgCl_2$. All *nosZ* PCR reactions were performed with the following cycling conditions: an initial denaturing at 98 °C for 2m, followed by 30

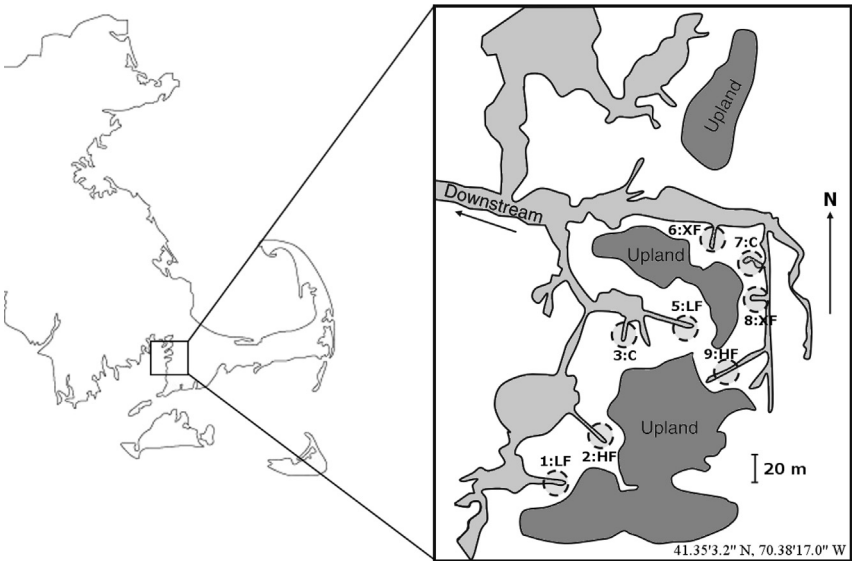


Fig. 1. A map of the Great Sippewissett Salt Marsh, Falmouth, MA with the eight experimental plots marked. C: no fertilizer added, LF: low fertilization, HF: high fertilization, and XF: extra high fertilization. Figure modified from Fox et al. (2012).

cycles of 98 °C for 10s and 72 °C for 1m, with a final extension for 5m at 72 °C. After evaluation by gel electrophoresis, PCR products were pooled for each plot and purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA).

Purified PCR products were ligated into vectors using the Invitrogen TOPO TA Cloning Kit (Invitrogen, Grand Island, NY, USA) and transformed into Invitrogen's One-Shot chemically competent *E. coli* cells following the manufacturer's instructions. For each gene, 100-200 clones were selected per fertilization treatment (50–100 per plot), screened by PCR and electrophoresis, and sequenced bidirectionally at the Massachusetts General Hospital DNA Core Facility (Cambridge, MA, USA) using the M13 primer set (Vieira and Messing, 1982). Sequences were aligned, trimmed, and checked for quality in MEGA 5.0 (Tamura et al., 2011). We additionally checked the sequences for chimeras in Mothur (Schloss et al., 2009) using UCHIME (Edgar et al., 2011).

2.4. Quantitative PCR

Quantitative PCR (qPCR) was used to enumerate the abundance of both genes in the eight marsh plots. We performed *norB* qPCR with the norB2F/6R primer set (Casciotti and Ward, 2005). In a total volume of 25 μl we added 10 μl of 5 Prime HotMasterMix (5 Prime, Hamburg, Germany), 1 μl of 25× Syber Green (Life Technologies, Grand Island, NY, USA), 0.3 μl reference dye, 1 μl of 3 ng μl^{-1} DNA template, and 1 μM of each primer. *norB* qPCR was performed on an Agilent MX3005p qPCR system with the following cycling conditions: an initial denaturation at 94 °C for 5m followed by 35 cycles of 94 °C for 45s, 54 °C for 45s, and 72 °C for 60s.

We used the nosZ2F/2R primer set (Henry et al., 2006) to perform *nosZ* qPCR. In a 20 μl reaction we added 10 μl SYBR®Green Brilliant III Ultra-Fast Master Mix (Agilent Technologies, Santa Clara, CA, USA), 0.3 μl reference dye, 1 μl of 3 ng μl^{-1} DNA template, 0.6 μl BSA (300 μg mL^{-1}), and 1 μM of each primer. The *nosZ* qPCR reaction was performed on an Agilent MX3005p qPCR system following the protocol from Henry et al. (2006): an initial denaturation at 94 °C for 15m followed by 40 cycles of 95 °C for 15s, 60 °C for 30s, 72 °C for 30s, and a data acquisition step at 80 °C for 15s.

Standards for both genes were prepared from cloned gene fragments, quantified with Quant-iT™ PicoGreen® dsDNA Assay (Life Technologies, Grand Island, NY, USA), and standard curves were diluted over seven orders of magnitude. All samples were run in triplicate reactions with triplicate standard curves and triplicate no template controls. Amplification efficiencies were 85% and 92% for *norB* and *nosZ* respectively. Reaction stringency for both genes was verified with melt curves and the resulting PCR product was examined via gel electrophoresis to confirm proper product formation.

2.5. Statistical and sequence analysis

483 *norB* and 515 *nosZ* sequences passed quality filtering and were included in the final analysis. We used Mothur to calculate the Shannon Diversity Index, Chao1 Richness Estimator, and Abundance-Based Coverage Estimator (ACE). For each gene we assessed the differences in community structure as a function of treatment by performing principal coordinate analyses (PCoAs) on Bray–Curtis dissimilarity values derived from Hellinger transformed count data using the Vegan package (Oksanen et al., 2011) in R (R Core Team, 2012). We used analysis of similarity (ANOSIM) in Mothur to determine the degree of community similarity among the eight plots. Finally, we used R to calculate linear regressions to test for the effect of fertilization on operational taxonomic unit (OTU) richness, diversity, and gene abundance, and on the ordination obtained by the PCoA analysis.

For phylogenetic analyses, we used Mothur to cluster DNA sequences at 85% similarity at the nucleic acid level and BLASTn to find the closest identity of each OTU. OTUs were aligned with ClustalW and maximum likelihood trees were constructed using MEGA 5.0 (Tamura et al., 2011). We determined the confidence in tree topology using a bootstrap analysis with 500 restarts. Weighted UniFrac analysis (Lozupone et al., 2006) was performed on both genes to test for the effect of nutrient enrichment on the phylogenetic grouping of the communities. Sequences were deposited in GenBank under the accession numbers: KF705687–KF706140 and KF706141–KF706340.

3. Results

3.1. Diversity, richness, and abundance

We identified 41 *norB* and 64 *nosZ* unique OTUs from 483 and 515 clones in each library respectively. The number of unique OTUs

Table 1
Total number of clones, number of unique OTUs, and calculated Shannon diversity, Chao1 and ACE richness estimators for eight salt marsh plots. The 95% confidence intervals, calculated from random subsamplings of the OTU table using Mothur, are indicated parenthetically.

Plot	Total # of clones	Unique OTUs	Shannon	Chao1	ACE
norB					
3 (C)	50	10	2.01 (1.8–2.2)	11 (10–23)	12 (10–24)
7 (C)	41	8	1.77 (1.6–2.0)	8 (8–17)	9 (8–14)
1 (LF)	88	14	2.21 (1.9–2.5)	16 (14–31)	19 (15–36)
5 (LF)	66	11	1.92 (1.6–2.2)	12 (11–24)	19 (13–37)
2 (HF)	41	13	2.07 (1.9–2.3)	13 (0–13)	13 (13–18)
9 (HF)	54	7	1.12 (0.9–1.4)	8 (7–18)	11 (8–17)
6 (XF)	70	11	2.19 (2.0–2.3)	12 (11–25)	12 (11–19)
8 (XF)	73	9	1.64 (1.4–1.9)	10 (9–17)	10 (9–19)
nosZ					
3 (C)	47	14	2.06 (1.8–2.4)	24 (16–68)	38 (25–69)
7 (C)	77	11	1.85 (1.6–2.1)	12 (11–26)	14 (11–27)
1 (LF)	56	10	1.79 (1.5–2.0)	25 (13–78)	61 (36–111)
5 (LF)	71	15	2.02 (1.7–2.3)	16 (15–24)	17 (16–21)
2 (HF)	59	14	1.52 (1.2–1.8)	28 (17–81)	48 (30–89)
9 (HF)	75	19	2.15 (1.8–2.5)	30 (22–63)	63 (41–109)
6 (XF)	65	21	2.57 (2.3–2.8)	43 (27–108)	52(35–90)
8 (XF)	65	18	2.39 (2.1–2.6)	27 (20–58)	31 (21–68)

in each plot was variable, ranging from 7 to 14 for *norB* and 10 to 21 for *nosZ* (Table 1). The number of unique *nosZ* OTUs increased as a function of fertilization, but no such relationship was found with the *norB* sequences (Fig. 2A) ($p = 0.86$). We also calculated the Shannon Diversity Index and the ACE and Chao1 richness estimators for each marsh plot (Table 1). Shannon diversity and Chao1 richness estimator of *nosZ* also increased with increasing nitrogen supply (Fig. 2B and C), although the predictive power of these regressions is moderately low (Prairie, 1996). Chao1 richness and diversity of *norB*, however, did not display any trend with fertilization as values fell within a narrow range for both Chao1 (8–16.5) and ACE (10.75–18.81) richness estimators.

As with the diversity and richness estimators, there was no trend in the abundance of the *norB* gene as a function of nutrient supply, with *norB* copy numbers ranging from 1.10×10^8 to 6.01×10^8 per gram of sediment (Fig. 2D). qPCR of the *nosZ* gene, however, revealed a strong effect of fertilization with *nosZ* copy number declining as a function of fertilization. Copy numbers of the *nosZ* gene ranged from 3.95×10^7 to 5.25×10^7 in C, LF, and HF plots, but was an order of magnitude less abundant, with 4.5×10^6 and 7.1×10^6 copies in the XF plots (Fig. 2D).

3.2. norB and nosZ phylogeny and UniFrac analysis

A BLASTn search revealed that the *norB* sequences from this study had >10% sequence divergence from all *norB* sequences in GenBank (Table S1), suggesting that the environmental distribution of this gene is not well documented. Of the 41 unique OTUs recovered, only two (OTUs 1 and 3) were present in all eight plots (Table S1). The most abundant OTU was present 140 times in the clone library and its abundance did not change as a function of external nitrogen supply. Similarly OTU 3, also present in all the plots and with a total of 53 clones, did not vary in abundance as a function of nutrient enrichment. In contrast to OTUs 1 and 3, OTU 2, present 74 times, was notably absent from the two most highly fertilized plots, though it was present in all other treatments.

Overall, the 10 most abundant *norB* OTUs (accounting for nearly 80% ($n = 381$) of the clones recovered) shared, on average, only an 82% sequence identity to cultured representatives present in GenBank. Ten of the 42 unique *norB* OTUs (OTUs 33–42) were singletons, and, as with the most abundant OTUs, were highly divergent (>15%) from previously reported *norB* fragments. *norB* OTUs appeared to be most closely affiliated with members of the phylum proteobacteria, in particular the α and β proteobacteria (Table S1). Phylogenetic analysis of the *norB* OTUs resulted in most sequences being assigned to one of five major clades, four of which (II, III, IV, V) were distinct from cultured denitrifiers (Fig. 3). These highly distinct clades harbored seven of the ten most abundant OTUs, accounting for ~87% of all the *norB* fragments recovered. The remaining OTUs grouped more tightly with known denitrifier *norB* fragments.

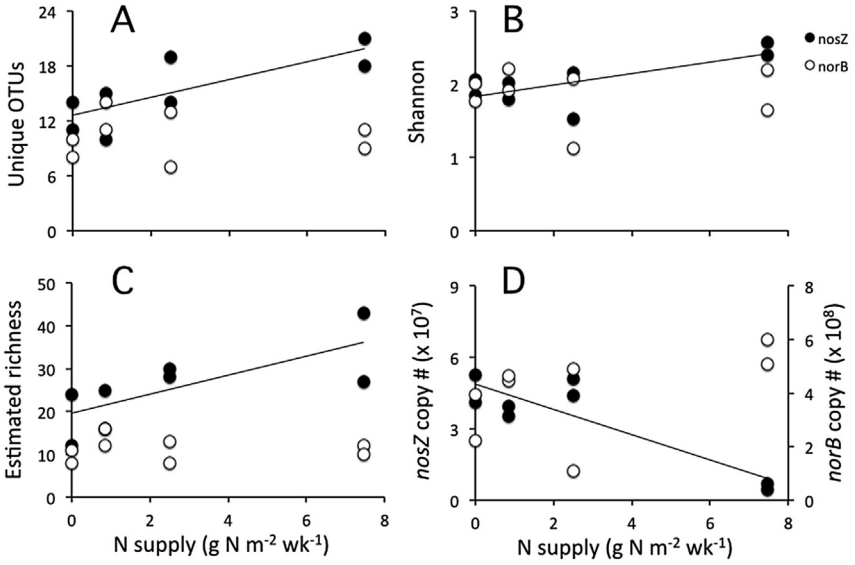


Fig. 2. Contrasting patterns in the diversity and abundance of *norB* and *nosZ* genes as a function of exogenous nutrient supply. The number of unique *nosZ* operational taxonomic units (OTUs) increases with exogenous nitrogen supply (A: $F = 9.4$, $p = 0.022$, $R^2 = 0.66$) but the number of unique *norB* OTUs does not. Similarly, the diversity (B: $F = 6.5$, $p = 0.043$, $R^2 = 0.52$) and Chao1 estimated richness (C: $F = 7.0$, $p = 0.038$, $R^2 = 0.54$) of *nosZ* OTUs also increases with exogenous nitrogen supply, though there is no change evident with the *norB* gene. Finally, the abundance of the *nosZ* gene decreases as a function of nitrogen supply (D: $F = 22.03$, $p = 0.003$, $R^2 = 0.79$), while *norB* abundance does not change. Regression lines are fit only to the *nosZ* data, as *norB* regressions were not significant.

Similarly, a BLASTn search revealed that *nosZ* fragments from this study had high sequence divergence from *nosZ* sequences in GenBank (Table S2). Of the 64 unique *nosZ* OTUs, only OTU 1, the most abundant OTU ($n = 126$) was present in all 8 plots. The 10 most abundant OTUs, accounting for 73% ($n = 376$) of all the *nosZ* clones we sequenced had, on average, only a 77% identity to a cultured organism that contained *nosZ*. The *nosZ* clone library also contained 22 singletons, which had, on average, 78% sequence identity to cultured representatives. Phylogenetic analysis demonstrates that our sequences grouped into six main clades. Clade I, which contained 13 of the 64 OTUs, formed a clade with previously known proteobacterial denitrifiers such as *Roseobacter* sp. and *Marinobacter* sp. Clades II-VI, which contained 41 of the 64 OTUs, clustered separately from cultured denitrifiers (Fig. 4). All of the ten most abundant OTUs, accounting for ~70% of the sequences recovered, grouped separately from known denitrifiers in clades II and III, except for OTU 8 which formed a separate branch off of clade II. The remaining ten OTUs clustered with known denitrifiers primarily from the α-proteobacteria.

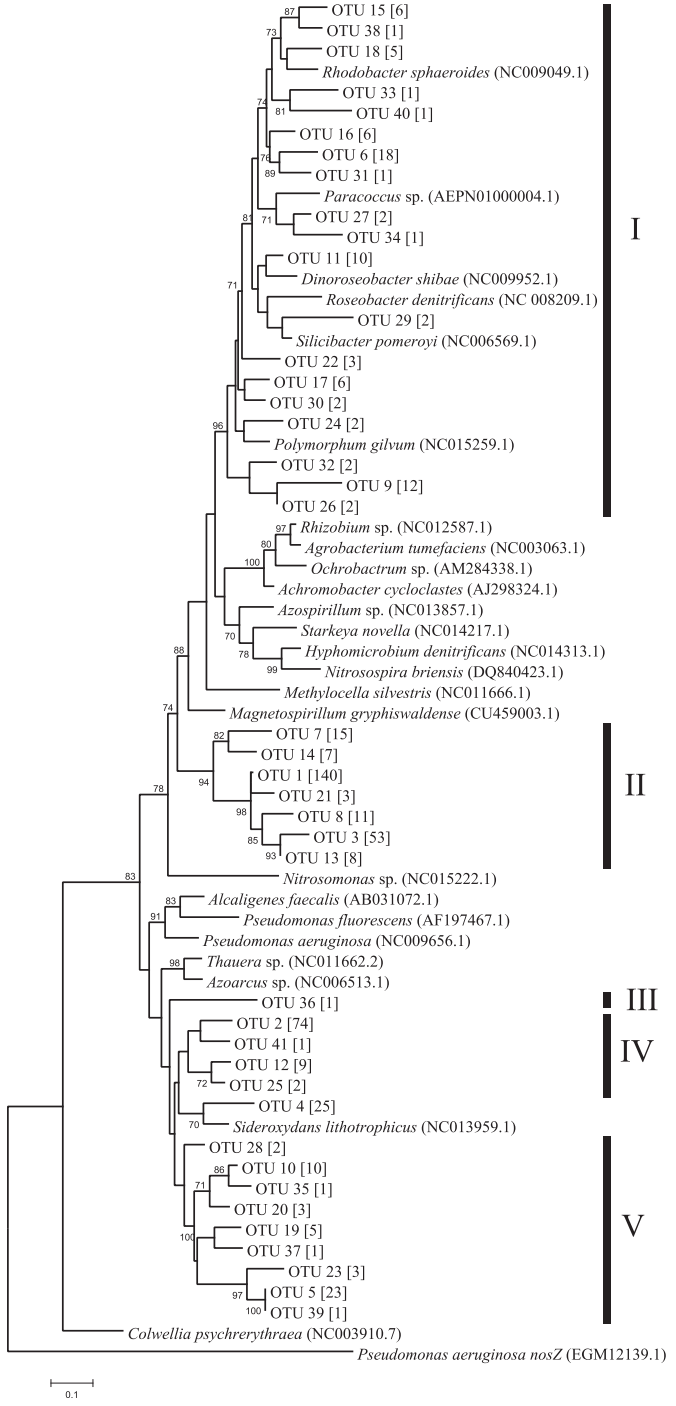


Fig. 3. Maximum-likelihood phylogenetic tree based on an amino acid alignment of the 395bp *norB* fragments sequenced from Sippewissett Marsh sediments. The tree contains all 41 *norB* OTUs that have been clustered at 85% sequence similarity and 25 reference sequences obtained from GenBank (accession numbers indicated in parenthesis). The 799bp *nosZ* fragment of *Pseudomonas aeruginosa* was used as an out-group for this analysis. Bootstrap values, based on 500 restarts, which are greater than 70, are shown. The number of time each OTU appeared in the study is indicated in brackets next to each sequence name. Scale bar indicates 10% sequence divergence.

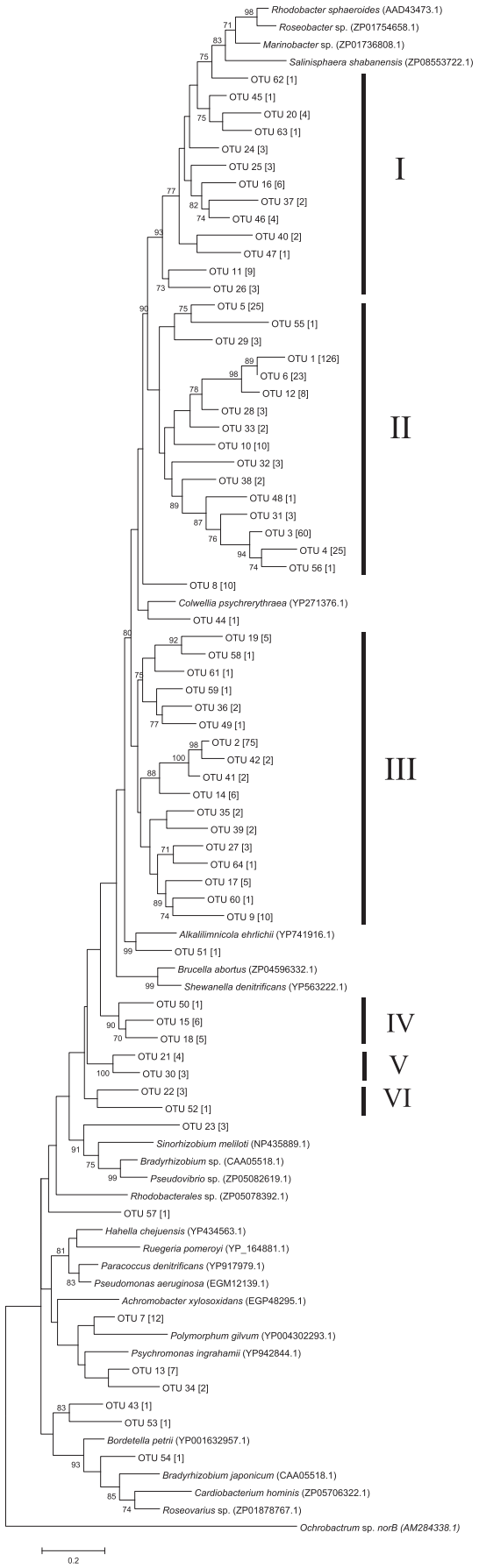
We used weighted UniFrac (Lozupone et al., 2006) to examine the phylogenetic similarity of the community members within the marsh plots and among the different treatments. Weighted UniFrac uses a phylogenetic tree containing all sequences from the dataset and, based on the branch lengths among OTUs that are found in different abundances among samples, calculates a dissimilarity index. Therefore, this dissimilarity index differs from other metrics because it includes a measure of phylogenetic relatedness. The resulting dissimilarities are used in Monte Carlo simulations to create a distribution and calculate a *p*-value that indicates whether or not there are significant differences among samples. As this is a permutation-based analysis it is robust to the low level of replication in this study. UniFrac results, however, indicated that the specific phylogeny of the *norB* and *nosZ* sequences played no significant role in the observed differences among the fertilization treatments.

3.3. Similarities among marsh plots

We performed a principal coordinate analysis (PCoA) on Hellinger transformed Bray–Curtis dissimilarity values for both genes to determine the community similarity among the plots. Bray–Curtis is a resemblance measure that we use to compare the composition of microbial communities present in sediments from two different locations based on the abundance and diversity of taxa present at each location. We calculate a matrix of all pairwise combinations of samples and visualize the matrix of community dissimilarities using a PCoA (Fig. 5), in which the more dissimilar two locations are, the further apart they are distributed in the figure. In coordination with the PCoA visualization we used Analysis of Similarities (ANOSIM) to determine whether there were statistically significant differences in community structure among treatments. Treatments that are statistically distinguished by ANOSIM are clustered together by colored ellipses (Fig. 5).

The distribution of clusters reveals ecologically relevant patterns. Data for both genes revealed distinct differences in community structure among levels of fertilization (Fig. 5). The *norB* XF plots (Fig. 5A, MP6 and MP8; red points) had communities that were differentiated along the primary axis (which explained 35.6% of the variance in the PCoA) from the remaining 6 plots (ANOSIM, $r = 0.02$, $p < 0.001$). Among the remaining plots there was no effect of fertilization and there was no pattern of differentiation along the secondary axis. As determined by SIMPER analysis, and as indicated by the green numbers (representing the ordination of the different OTUs within the PCoA), the separation of XF from the other plots along the primary axis was driven by OTUs 4, 5, 6, and 7 (Fig. 5A). Collectively these OTUs were present 47 times in the two XF plots but only 34 times in the remaining six plots (Table S1).

PCoA of the *nosZ* gene indicated two distinct communities grouped by fertilization level (Fig. 5B). The highly fertilized marsh plots (MP6 and MP8, red points) formed one distinct cluster



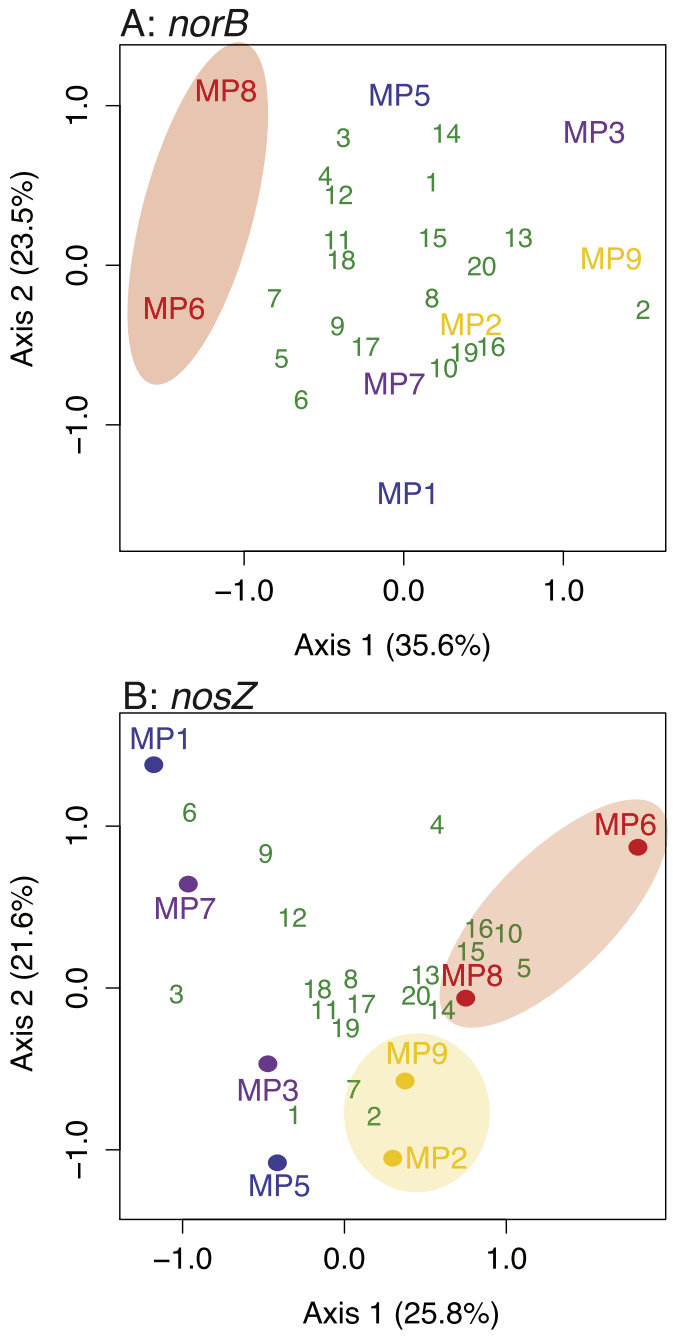


Fig. 5. Principal coordinate analysis based on Bray–Curtis dissimilarity values for *norB* (a) and *nosZ* (b). Color code in the plots are denoted by red: XF, orange: HF, blue: LF, and purple: C. Green numbers represent the ordination of OTUs in the sample space and indicates which OTUs drive the dissimilarity pattern. The ellipses correspond to the clusters identified as significantly different by ANOSIM analysis.

Fig. 4. Maximum-likelihood phylogenetic tree based on an amino acid alignment of the 799bp *nosZ* fragments sequenced from Sippewissett Marsh sediments. The tree contains all 41 *nosZ* OTUs that have been clustered at 85% sequence similarity and 24 reference sequences obtained from GenBank (accession numbers indicated in parenthesis). The 395bp *norB* fragment of *Orchrobactrum* sp. was used as an out-group for this analysis. Bootstrap values, based on 500 restarts, which are greater than 70, are shown. The number of time each OTU appeared in the study is indicated in brackets next to each sequence name. Scale bar indicates 10% sequence divergence.

(ANOSIM, $r = 0.20$ to 0.3, $p < 0.001$), which was driven primarily by increased abundances in OTUs 4, 5, 10, 15, and 16 (Table S2). These OTUs were present 56 times in the two XF plots, compared to only 16 times in the remaining six plots. Similarly, the HF plots, (MP2 and MP9, yellow points) clustered together, driven primarily by high abundances of OTUs 1, 2, and 7 (Table S2). Both the XF and HF plots were differentiated along the primary axis, which explained 25.8% of the variance in the PCoA, from the C and LF plots. Additionally, the HF and XF plots appear to be differentiated along the secondary axis, suggesting that additional environmental drivers may be acting at the highest levels of fertilization.

To test whether the supply of exogenous nitrogen was responsible for the differentiation along the primary axis of the two microbial denitrifier populations represented by the *norB* and *nosZ* genes, we regressed the axis 1 values from Fig. 5 against the nitrogen supply to the marsh plots (Fig. 6). The regression of both the *nosZ* (solid line) and *norB* (dashed line) ordination values varied significantly with nitrogen supply, indicating that the differences in the microbial communities observed with both genes (Fig. 5) is likely either a direct or indirect result of the added nitrogen.

4. Discussion

Salt marshes perform a wide range of ecosystem services, including removing excess nutrients derived from anthropogenic sources (Valiela and Cole, 2002). The ability of salt marshes to remove excess N is important for mitigating many of the deleterious effects of eutrophication including estuarine anoxia/hypoxia (Rabalais et al., 2002; Osterman et al., 2008), the development of harmful algal blooms (Paerl, 1997), and the disruption of local food webs and the commercial fisheries that they support (Deegan, 2002). Despite their importance, salt marsh loss has accelerated over the past several decades and part of this decline may be due to increased coastal nutrient additions (Deegan et al., 2007, 2012; Turner et al., 2009). Salt marshes have long been known to ameliorate the negative effects of nutrient addition through very high rates of denitrification (Hammersely and Howes, 2005; Koop-Jakobsen and Giblin, 2010). Whether the high rates of denitrification make marshes a net source or sink of N_2O remains an area of active research.

Nitrous oxide is biologically produced through both nitrification and denitrification, both of which are active processes in salt marsh sediments. N_2O can also result from many microbes that are not directly involved in N cycling, as a result of the need to detoxify NO

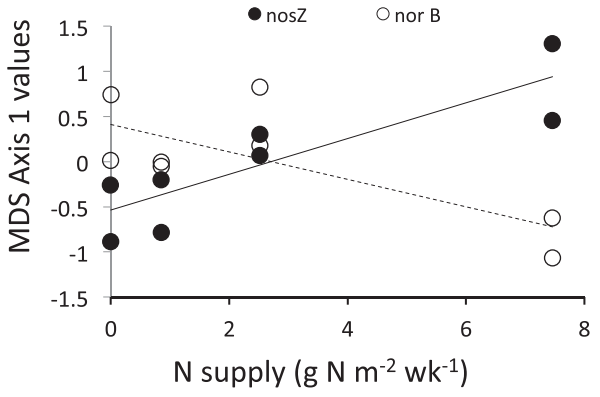


Fig. 6. The role of nutrient supply in structuring the *nosZ* and *norB* gene containing microbial community. The ordination values from the primary axis in Fig. 5 are regressed against exogenous nitrogen supply. Both *nosZ* ($F = 17.53$, $p = 0.005$, $R^2 = 0.74$) and *norB* ($F = 7.0432$, $p = 0.034$, $R^2 = 0.55$) ordinations vary as a function of nitrogen supply.

(Stein, 2011), however, our focus is on those bacteria directly involved in nitrification and denitrification. Nitrification rates in surface sediments of marshes are often tightly coupled with rates of denitrification so disentangling which process is responsible for the preponderance of N_2O production is challenging. Furthermore, some nitrifiers are capable of nitrifier denitrification, which can also result in N_2O production (Casciotti and Ward, 2005). N_2O production is enzymatically controlled by the activity of *nor* and *nos* genes, and *nor* genes are present, but distinct, in both canonical denitrifiers and nitrifier denitrifiers. Our results suggest that at the highest levels of fertilization there was a shift in the structure of the *norB* containing microbial community, but there was no evidence from the sequence data that nitrifier denitrifiers were responsible for this shift. Although the marsh plots with the highest levels of fertilization had distinct *norB*-containing denitrifiers, fertilization did not affect the taxonomic richness, diversity, or abundance of this gene.

The response of the *nosZ* containing microbes to fertilization was much stronger than the response of the *norB* containing microbes. In the canonical denitrification pathway, the step that yields the least energy is the reduction of N_2O to N_2 (Zumft, 1997), which is facilitated by the enzyme encoded by the *nosZ* gene. The decrease in *nosZ* abundance at the highest rates of fertilization (Fig. 2D) suggests that the excess supply of nitrogen selects for denitrifiers that lack this final step in the enzymatic pathway, perhaps due to the higher oxygen sensitivity of the *nosZ* gene relative to *norB* and *nirS* genes (Zumft, 1997). Fertilization within the Sippewissett plots resulted in changes in redox gradients within sediment underlying tall *Spartina alterniflora* (Howes et al., 1981) as well as changes in plot elevation relative to mean sea level (Fox et al., 2012), which could explain the observed decrease in *nosZ* bearing microorganisms. Despite a decrease in the abundance of *nosZ*, we observed an increase in the taxonomic richness and diversity of this gene as a function of fertilization (Fig. 2A–C). Previous work on the diversity and abundance of the *nirS* gene, another key gene in the denitrification pathway, also indicated that there was a greater diversity of specialist denitrifiers in the highly fertilized plots, and suggested that the increased supply of nitrogen led to an increase in niche space (Bowen et al., 2013). The increased diversity and richness of the *nosZ* gene observed here supports this conclusion and suggests that fertilization increases the functional capacity to remove N_2O, although the abundance of *nosZ* suggests that this capacity may not be realized.

The differing response to fertilization by the two different genes suggests that there is greater modularity in the denitrification process than is typically recognized. Historically, denitrifying microbes were thought to remove nitrogen via a step-wise reduction of nitrate (NO_3^-), through several tightly coupled intermediates [nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O)], to N_2 (Zumft, 1997). Data presented here, along with other evidence from the literature, however, demonstrates that this process can be highly modular, with some taxa using the full enzymatic machinery to reduce NO_3^- to N_2, and other taxa using only a portion of the needed components (Simon and Klotz, 2013). The distribution and environmental regulation of microbial taxa containing different modules for fixed nitrogen removal is relevant at an ecosystem scale because it can result in either production or consumption of N_2O (Jones et al., 2014). Our data suggest that increased nitrogen supply does not enhance bacteria that produce N_2O but may reduce the abundance of bacteria capable of removing N_2O, which could exacerbate the global warming potential of marsh sediments.

Short-term N additions to salt marsh sediments lend credence to the hypothesis that fertilization inhibits bacteria containing the *nosZ* gene. In one experiment short-term additions caused a shift in the marsh from a N_2O sink to a N_2O source (Moseman-Valtierra

et al., 2011), although the genetic underpinnings of this shift were not examined. Our results suggest that the high levels of fertilization in the XF plots, coupled with the very low copy number of the *nosZ* gene, may result in net flux of N_2O out of the marsh sediment, as has been seen in other studies (Blackmer and Bremner, 1978; Firestone et al., 1979; Avrahami and Bohannan, 2009). Decreased abundance, however, does not necessarily imply decreased overall activity of the *nosZ* bearing community as some low abundance members of the community could have high rates of activity, which could be assessed via analysis of mRNA.

Evidence from soils suggests that *nosZ* abundance is generally one or more orders of magnitude lower in abundance than either the *nir* or *nor* genes (Henry et al., 2006; Babić et al., 2008; Hallin et al., 2009) due to the considerable number of denitrifying bacteria that lack the *nosZ* gene (Jones et al., 2008). In soil mesocosms, Philippot et al. (2011) demonstrated that increases in the number of denitrifying bacteria lacking the *nosZ* gene resulted in increased nitrous oxide emissions. Additionally, Jones et al. (2014) recently reported that higher *nosZ* abundance and diversity was strongly correlated with the ability of soils to be a N_2O sink, and that much of the sink capacity of soils was a result of a newly identified clade of microorganisms that are capable only of the reduction of N_2O to N_2 (Sanford et al., 2012; Jones et al., 2014). How this non-denitrifying *nosZ* bearing clade responds to fertilization, and the source/sink capacity of coastal marshes is an important avenue for future investigation.

Despite the importance of these genes in regulating N_2O fluxes, the sequence analysis of *norB* and *nosZ* genes from the marsh plots indicates a large degree of divergence from the cultured denitrifiers represented in GenBank (Tables S1 and S2, Figs. 3 and 4). This high divergence from cultured denitrifiers has been previously shown for *norB* (Braker and Tedije, 2003; Casciotti and Ward, 2005) and *nosZ* (Scala and Kerkoff, 1998; Stres et al., 2004; Henry et al., 2006; Horn et al., 2006; Jones et al., 2013), although these studies did not examine the effect of N enrichment on the diversity and abundance of these genes. Four of the five *nosZ* OTUs that were most important in differentiating the highly fertilized plots from the remaining plots in this study (OTUs 4, 5, 15, and 16) were most similar to *nosZ* genes identified along a salinity gradient from intertidal sediments to arable lands (accession numbers FJ227165, FJ227217, FJ227262, FJ227270), however none had a percent identity greater than 85% at the nucleic acid level (Table S2). The fifth OTU that was important in differentiating community structure was OTU 10, which shared a 98% identity to a *nosZ* gene identified in German soil (accession number AY95772). The nearest identity to the *norB* sequences derived here were all closest matches to cultured representatives (Table S1), this however is primarily due to the lack of environmental *norB* sequences in the NCBI database and demonstrates our lack of knowledge about the distribution of this gene in the environment. Our inability to closely link the *norB* and *nosZ* sequences derived here to cultured denitrifiers demonstrates the need for increased focus on culturing of these critically important organisms. Increasing the number of denitrifying bacteria in culture or co-culture will better allow us to determine their physiology and response to perturbations, which in turn, will improve our understanding of the microbial underpinnings of marsh geochemistry.

Clone libraries provide a relatively shallow glimpse of the *norB* and *nosZ* containing microbes in marine sediments and the data here should be interpreted as representative only of the dominant clades of microbes containing these genes. Low abundance microbes that contain the two genes involved in this study would most certainly be missed by these methods. Similar conclusions, that community structure of denitrifiers was affected at high levels of fertilization, was also demonstrated via pyrosequencing of the *nirS* gene, suggesting that the clone library results are robust (Bowen et al., 2013). In addition, an *in silico* comparison between a clone library study (Francis et al., 2013) and pyrosequencing output from the same samples, suggests that clone libraries are capable of revealing meaningful ecological patterns (Bowen et al., in review). Other techniques with lower resolution than pyrosequencing, such as terminal restriction fragment length polymorphisms (T-RFLPs), have also been shown to produce comparable results to high-throughput sequencing methodologies (van Dorst et al., 2014). Our result, however, reveal both ecologically meaningful patterns in response to nutrient enrichment and they fill a gap in the diversity of these two genes in GenBank, as it appears that salt marshes harbor previously unidentified genetic diversity, diversity that could play in important role in whether marshes are a source or sink of N_2O.

In conclusion, we demonstrated that *norB* and *nosZ* containing communities are affected by nitrogen supply in the Great Sippewissett Salt Marsh. The abundance of the *norB* gene did not change as a function of fertilization while *nosZ* abundance declined as rates of fertilizer supply increased. Both genes responded to long-term fertilization by a shift in microbial community composition at the highest levels of fertilization but the effect of fertilization was most dramatic with the *nosZ* containing microbes, as they also experienced an increase in richness and diversity as a result of long-term N enrichment. The changes in abundance and community composition of the *nosZ* gene due to long-term fertilization may contribute to the marsh's ability to be a source or sink of N_2O. Greater diversity of the *nosZ* gene suggests that there is increased functional capacity of the sediments to remove N_2O, but the low abundance of sequences in the XF plots suggests that capacity may not be realized. Finally, our sequences displayed considerable divergence from previously sequenced fragments and from cultured denitrifiers, suggesting that the Great Sippewissett Marsh may harbor unique and novel communities of denitrifying microbes that play an important role in protecting coastal waters.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ecss.2014.12.014.

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