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ORIGINAL ARTICLE

Nitrogen and phosphorus enrichment alter the composition of ammonia-oxidizing bacteria in salt marsh sediments

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Ammonia oxidation is a central process in the nitrogen cycle. Particularly in marine and estuarine environments, few experiments have been conducted to tease apart the factors influencing their abundance and composition. To investigate the effect of nitrogen and phosphorus availability on ammonia-oxidizing bacteria (AOB), we conducted a nutrient enrichment experiment in a Maine salt marsh and sampled sediment communities in three seasons over 2 years. We assessed community composition using terminal restriction fragment length polymorphism analysis and sequencing of cloned fragments of the ammonia monooxygenase (*amoA*) gene. Almost all of the *amo*A sequences fell within the marine and estuarine-specific *Nitrosospira*-like clade. Applied separately, nitrogen and phosphorus significantly altered AOB composition; however, together the nutrients had an interactive effect, and composition did not change. In contrast, nutrient enrichment did not alter AOB abundance. Furthermore, the response of AOB composition to nutrient enrichment varied over time. We conclude that closely related taxa within the marine/estuarine-specific *Nitrosospira*-like clade vary in their preference for nutrient concentrations, and this preference may depend on other temporally variable abiotic factors. Finally, AOB composition was highly variable within and across years even in untreated plots. Further studies are needed to test how these different aspects of compositional variability in AOB communities influence nitrogen cycling.

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Introduction

Ammonia-oxidizing bacteria (AOB) and archaea (AOA) convert ammonium to nitrite, the first step in nitrification. Depending on the environmental conditions, nitrification can positively or negatively affect nitrogen retention in ecosystems (Kowalchuk *et al.*, 2000; Templer *et al.*, 2008; von Schiller *et al.*, 2009). At the same time, the composition of nitrifying microorganisms can influence nitrification rates (Bottomley *et al.*, 2004; Balser and Firestone, 2005; Bernhard *et al.*, 2007). Therefore, knowledge about the interplay between nitrifiers and their environment is key to understanding the nitrification process.

Environmental conditions clearly affect the abundance and genetic composition of nitrifying communities. Studies from a variety of habitats document that AOB communities vary over space (Kowalchuk and Stephen, 2001) and that this variation is correlated with a variety of factors, including salinity (Stehr *et al.*, 1995; Sahan and Muyzer, 2008), temperature (Avrahami and Conrad, 2005; Fierer *et al.*, 2009), nutrients (Webster *et al.*, 2002; Mosier and Francis, 2008), plant composition (Briones *et al.*, 2002; Mintie *et al.*, 2003; Dollhopf *et al.*, 2005), soil moisture (Hastings *et al.*, 2000) and pH (Kowalchuk *et al.*, 2000; Nicol *et al.*, 2008). Field experiments confirm that pH (Stephen *et al.*, 1998; Backman *et al.*, 2003) and nitrogen availability (Phillips *et al.*, 2000; Webster *et al.*, 2002) can directly influence AOB composition and/or abundance. Furthermore, experiments show that multiple variables interact to affect AOB composition. For instance, in laboratory microcosms, temperature, soil moisture and ammonium concentration interact to alter AOB composition (Avrahami *et al.*, 2003; Avrahami and Bohannan, 2009). In the field, nitrate and CO_2 enrichment altered AOB composition and abundance in a Californian grassland, and these effects depended on temperature and precipitation (Horz *et al.*, 2004).

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The goal of this study was to test whether increased nitrogen and phosphorus (or the interaction of both nutrients) influence AOB composition and abundance in a New England salt marsh. Terrestrial runoff from industrial sources, sewage, storm water and agricultural sources increases nitrogen (N) and phosphorus (P) concentrations in coastal ecosystems (Conley *et al.*, 2009). In New England, nearly 80% of salt marshes in this region have been altered dramatically by human activities (Bromberg and Bertness, 2005). Field experiments in this region show that nitrogen enrichment alters plant composition (Levine *et al.*, 1998; Emery *et al.*, 2001) and reduces the diversity of native plant species while promoting the invasion of non-native plant species (Bertness *et al.*, 2002).

We fertilized plots with nitrogen (ammonium nitrate) and phosphorus (triple superphosphate) for 4 years in a factorial, replicated experiment (Crain, 2007). We sampled the sediments three times a year in the third and fourth years of fertilization to address two questions: Does AOB composition and abundance respond to the addition of N, P and/or the combination of both? And, if so, does the response vary over time?

To assay the AOB community, we analyzed the *amoA* gene, which codes for the α-subunit of the ammonia monoxygenase enzyme. This protein subunit is responsible for catalyzing the first step in the conversion of ammonia to nitrite, the oxidation of ammonia to hydroxylamine. Specifically, we targeted *amoA* from the β-proteobacteria (Rotthauwe *et al.*, 1997), which are thought to be more dominant than γ-proteobacteria in marine sediments (Nold *et al.*, 2000).

Although the discovery of abundant and diverse AOA suggests that archaea also contribute significantly to nitrification in natural ecosystems (Francis *et al.*, 2005; Konneke *et al.*, 2005; Leininger *et al.*, 2006), the relative contribution of AOB versus AOA to nitrification in marine and estuarine sediments remains unresolved. A number of recent studies suggest that AOA dominate in marine habitats (Wuchter *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008). In contrast, Mosier and Francis, (2008) found that copies of β-proteobacterial *amoA* were two orders of magnitude higher than archaeal *amoA* in high-salinity estuarine sediments. In salt marshes specifically, a report from southeastern Connecticut found a high abundance of 16S rRNA genes related to the ammonia-oxidizing '*Candidatus* Nitrosopumilus maritimus' isolate (Nelson *et al.*, 2009). Thus, although the study here focuses solely on AOB, further study on AOA is needed to expand the picture of the salt marsh nitrifying community.

Materials and methods

Experimental design

The experiment was conducted in a mid-tidal *Spartina patens*-dominated salt marsh in Scarborough Marsh, Maine. The study site (70 19′ 38.33' W, 43 32′ 27.81 N) experiences semidiurnal tides with maximum amplitude over 3.8 m and was selected to represent typical euhaline (>25 ppt NaCl) marshes of Northern New England with characteristic plant composition (Ewanchuk and Bertness, 2004). Plant cover in the study area was almost exclusively *S. patens,* with rare occurrences of *S. alterniflora, Salicornia maritima* and *Distichlis spicata.* Twenty 0.25 m^2 plots were flagged within a 10×20 m area of marsh and assigned randomly to one of four nutrient treatments: control (C), nitrogen enrichment (N), phosphorus enrichment (P) or nitrogen and phosphorus enrichment (NP). To encourage slow release within the rooting zone, nutrients were administered through four 50 ml centrifuge tubes that were drilled with 12 holes (1 mm each), placed 15 cm apart in a square centered in the plot, and sunk flush to the substrate. Nutrients were added in pelletized form to each centrifuge tube as 10 g ammonium nitrate (34% N) and 5 g triple superphosphate (46%P). NP treatments received both nutrients in the above quantities and controls received empty centrifuge tubes. Nutrients were added three times during the plant-growing season (in May, June and July), resulting in a supplement of 163 $g\,m^{-2}$ per year N and 110 $g\,m^{-2}$ per year P for 3 years (2002–2004).

Sediment nutrients

To assess sediment nutrient availability, a 10 cm diameter sediment core was collected from each plot in July 2004. The sediment was stored on ice in the field and at −20 °C in the laboratory. The cores were sifted through a 1 mm mesh to remove root material, and one gram was extracted in a solution of 2 M KCL and 0.5 M $NaCOH_3$. Ammonium and phosphate concentrations in the extracts were measured with Hach colorimetric kits (Loveland, CO, USA). Another gram of sifted sediment was dried and weighed to determine moisture content.

The nutrient treatments increased nitrogen as ammonium (analysis of variance: $F = 5.96$; $P = 0.006$) and phosphate ($F = 7.10$; $P = 0.003$) in the treatment plots. Nitrogen as ammonium averaged $7.5 \pm 2\,\mu g\,g^{-1}$ dry sediment in N-enriched plots, and phosphate averaged $8.0 \pm 5.6\,\mu g\,g^{-1}$ in P-enriched plots. In contrast, in the control plots, ammonium averaged $3.9 \pm 0.4\,\mu g\,g^{-1}$ and phosphate, $0.8 \pm 0.4\,\mu g\,g^{-1}$ dry sediment.

AOB composition

Sediments were sampled in May, July and October of 2003 and 2004. In May and October 2003, samples were collected only from the control and NP enrichment. From each plot, a 1 cm diameter sediment core was collected to a depth of 1 cm. The cores were stored on ice in the field and within 12 h returned to the lab for storage at −80 °C. For DNA extraction, we removed the top 0.5 gram of

the sediment core. Community DNA was extracted from this sample using the FastDNA spin kit for soils (Qbiogene, Inc., Montreal, Canada) following the instructions of the manufacturer.

The *amoA* gene (460 bp) was amplified using forward primer amoA-1F (5′-GGGGTTTCTACTGG TGGT-3′) 5′-labeled with 6-FAM and reverse primer amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) (Rotthauwe *et al.*, 1997). PCR was performed in triplicate with 25 μl Pre-Mix F (Epicentre Biotechnologies, Madison, WI, USA), 0.5 ul of each primer (12.5 μM), 2.5 U of MasterAmp Taq polymerase (Epicentre) and 2 μl of 1:10 diluted community DNA with the same reaction conditions as Horz *et al.*, 2000. The triplicate PCR products were pooled, purified using the QIAquick PCR purification kit (Qiagen Inc, Valencia, CA, USA), and quantified using ethidium bromide staining.

Restriction digestion with *Aci*I of 200 ng of the purified products was performed for 4 h according to the protocol of the supplier (New England Biolabs, Ipswich, MA, USA). After digestion, samples were desalinated using a QIAquick nucleotide removal kit (Qiagen Inc) and sent to the Genomics Technology Support Facility (Michigan State University) for analysis. Peak areas were quantified by GeneScan analysis software (Applied Biosystems, Carlsbad, CA, USA), and samples were used in further analyses only if they had cumulative peak heights greater than 10 000 fluorescent units (Marsh, 1999). To minimize peak noise, only peaks greater than 0.5% of the total fluorescent units in the sample were recorded (Kennedy *et al.*, 2005). We repeated our analyses using absolute peak height cutoff of 50, 100 or 500 fluorescent units, and our general results did not change (data not shown).

The terminal restriction fragment length polymorphism (T-RFLP) approach carries the risk that pseudo-terminal restriction fragments are incorrectly identified as unique fragments (Egert and Friedrich, 2003). To avoid such fragments, we took a number of precautions. First, we digested with a large amount of enzyme (20 U) to avoid incomplete digestion. We also performed digestions on dilution series of DNA to confirm that the banding patterns were repeatable across DNA concentrations. We determined the digestion time (4 h) on the basis of initial experiments with a series of digestion times (1–16 h); further digestion time after 4 h did not change banding patterns. We confirmed that our protocol produced the expected fragment from *Nitrosomonas europea* (ATCC 19718). Finally, 25 of the samples were digested in duplicate with independent PCR reactions and enzyme digestion to confirm the repeatability of the fragment patterns.

AOB abundance

The same primer pair used for T-RFLP (amoA-1F and amoA-2R) was used to quantify the abundance of *amo*A genes from all the treatment plots in 2004. Each quantitative PCR reaction contained Green Supermix (BioRad, Hercules, CA, USA), 0.5 μM of each primer, 5 μl of diluted DNA template in a final volume of 25 μl and was carried out in a BioRad iCycler with the following parameters (modified from Horz *et al.*, 2004): 15 min at 94 °C; 40 cycles of 45 s at 94 °C, 15 s at 55 °C, and 30 s at 72 °C; plate read at 82.5 °C; final elongation at 72 °C for 7 min following a melting curve 30–98 °C by increasing 0.4 °C in each cycle (Horz *et al.*, 2004). A standard curve was generated using a plasmid containing the *amo*A gene from *N. europea* (ATCC 19718). The plasmid was quantified several times using a NanoDrop (Wilmington, DE, USA) spectrophotometer. An average of the reading was taken to calculate the amounts needed to produce a standard curve ranging from 50 to 500 000 copies of the *amo*A gene. The reactions were carried out in triplicate; all 60 (20 plots over 3 months) samples from a year were amplified together with the standard curve in three separate reactions. The average of these triplicate reactions for each sample was used in the analyses.

We determined the appropriate template dilution on the basis of initial quantitative PCR experiments comparing copy numbers from 1:10, 1:50, 1:100, 1:500 and 1:1000 dilutions of a select group of samples. We found that the dilution greatly altered the estimated copy number, presumably because of PCR inhibitors in the samples (data not shown). The highest copy numbers were obtained in the 1:50 and 1:100 dilutions; higher dilutions yielded inconsistent amplification. Thus, we used 1:100 dilutions for all sample reactions. Two of the 60 samples did not yield consistent amplification and were removed from the analysis.

Phylogenetic analysis

To characterize the phylogenetic diversity of the *amoA* gene in our samples, we created a clone library for each treatment. To capture some of the spatial and temporal heterogeneity within a treatment, we pooled the *amoA* PCR products of one randomly selected plot from each of the three sampling months in 2004. Pooled amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc) and cloned into *Escherichia coli* using the TOPO-TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA). In all, 96 clones per library were sequenced by Gennassiance Pharmaceuticals (New Haven, CT, USA).

We aligned the DNA sequences in protein space using Geneious Pro 4.6 (Biomatters, Auckland, New Zealand) and used Dnadist in the Phylip version 3.68 package (Felsenstein, 2005) followed by DOTUR (Schloss and Handelsman, 2005) to define operational taxonomic units (OTUs) as sequences that shared $\geqslant$99% nucleotide similarity. We then constructed phylogenetic trees of sequences representing each OTU and sequences from other studies for reference. The trees were constructed in Phylip

using Dnadist followed by neighbor-joining and maximum likelihood analysis for both nucleotide and amino-acid sequences using the default parameters. The overall topology of all the trees was similar, and therefore we only report the amino-acid neighbor-joining tree. Bootstrap analyses were performed using 100 replicates. To compare the cloned sequences with the observed T-RFLP patterns, we performed *in silico* digestion of all sequences with *Aci*I restriction enzyme using Geneious.

To illustrate the fine-scale differences among the sequences between treatments, we also constructed a phylogenetic tree with all the sequences (that is, not using OTU representatives) using the RAxML algorithm and Web servers (Stamatakis *et al.*, 2008). We report the best-scoring maximum likelihood tree with bootstrap values using Interactive Tree of Life to display the results (Letunic and Bork, 2007).

Statistical analysis

To summarize AOB composition in the plots at each sampling time, we performed a principal component analysis of the T-RFLP patterns among our samples (Le Progiciel R, version 4.0). We used the Jaccard index (Magurran, 1988) to calculate compositional differences between each of the samples. This index only uses information about the presence and absence of each fragment, as we were hesitant to use chromatogram peak heights as an indicator of taxon abundance.

To test whether AOB composition varied across treatments and over time, we performed repeated measures analysis of variance (JMP software, SAS, Cary, NC) on the principal coordinates (PCOs) that explained the majority of the variation in our data. (Shapiro-Wilk tests suggest that the PCO values are normally distributed.) We also used repeated measures analysis of variance to test whether AOB richness (as indicated by the number of T-RFLP bands) or AOB abundance (from the quantitative PCR assay) was altered by nutrient treatment over time. We used ANOSIM (Primer 5 software, PRIMER-E Ltd, Ivybridge, UK; Clarke and Warwick, 2001) to test whether sequence composition of the clone libraries varied by nutrient treatment. ANOSIM *post hoc* comparisons were used to determine which treatments were responsible for overall treatment differences.

Results

Overall AOB composition

We detected 49 distinct terminal restriction fragments across all samples. Most fragments were detected in multiple samples; four were observed in all samples, and only four were unique to one sample. To confirm the repeatability of the fragment patterns, 25 samples were replicated with independent PCR amplification and restriction digestion. The T-RFLP patterns were highly correlated between replicates (average R^2 between band lengths of replicated samples $= 88.9 \pm 0.09$ s.d.).

The 342-clone library sequences were grouped into 27 OTUs defined at $\geqslant 99\%$ nucleotide similarity. In comparison, only 8 OTUs were defined at a 95% similarity cutoff. All but two of the 99% OTUs fell within the marine/estuarine *Nitrosospira*-like clade (for example, cluster B in Francis *et al* 2003) (Figure 1). This cluster consists of environmental *amoA* sequences from various marine environments (Francis *et al.*, 2003; Bernhard *et al.*, 2005; Molina *et al.*, 2007; Kim *et al.*, 2008; Mosier and Francis, 2008). The other two OTUs are closely related to cultured *N. europea* and environmental sequences from various environments such as the Baltic Sea water column and an ANAMMOX bioreactor (Kim *et al.*, 2008; Bae H, Paul T and Jung JY, unpublished). Both of these OTUs were represented by only one sequence each, and both were detected in the phosphorus addition treatment. These sequence data have been submitted to GenBank under accession numbers GQ454451–GQ454792.

All the fragment sizes predicted by *in silico* digestion of the sequences (Figure 1) were observed in the T-RFLP analysis. The most commonly predicted fragment (128 bp) was also one of the most abundant found in a marsh within Plum Island Sound Estuary, MA (Bernhard *et al.*, 2005). Although closely related sequences tend to have similar predicted fragment sizes, the same size can be found in other clades (for example, sequences with 128 bp are found in the *N. marina* cluster, the *Nitrosospira* cluster and the *Nitrosospira*-like cluster; Figure 1). Therefore, we cannot extrapolate changes in phylogenetic composition from the T-RFLP results.

Effects of N and P on AOB composition

Comparison of the T-RFLP patterns across treatments shows that AOB communities responded to N and P enrichment. The first five principal components explained 50% of variability in AOB community composition (Table 1). N and P had significant main effects on the second principal coordinate (PCO2), which explained 9.3% of variation in the T-RFLP patterns. Further, N and P addition interacted to affect AOB composition along PCO2 (7.4% variation) and PCO3 (7.0% variation), and marginally along PCO4 (Figure 2; Table 1). The addition of N and P together resulted in a smaller change in AOB composition than expected from the N and P only treatments. As a result, composition in the NP treatment was more similar to the control treatment than to the N or P treatments (Figure 2).

Analysis of the *amoA* sequences from the clone libraries supports the result that N and P enrichment influences AOB composition in an interactive manner. Sequence similarity was significantly higher within treatments than across treatments (ANOSIM: $R = 0.027$, $P < 0.001$). In particular, sequence

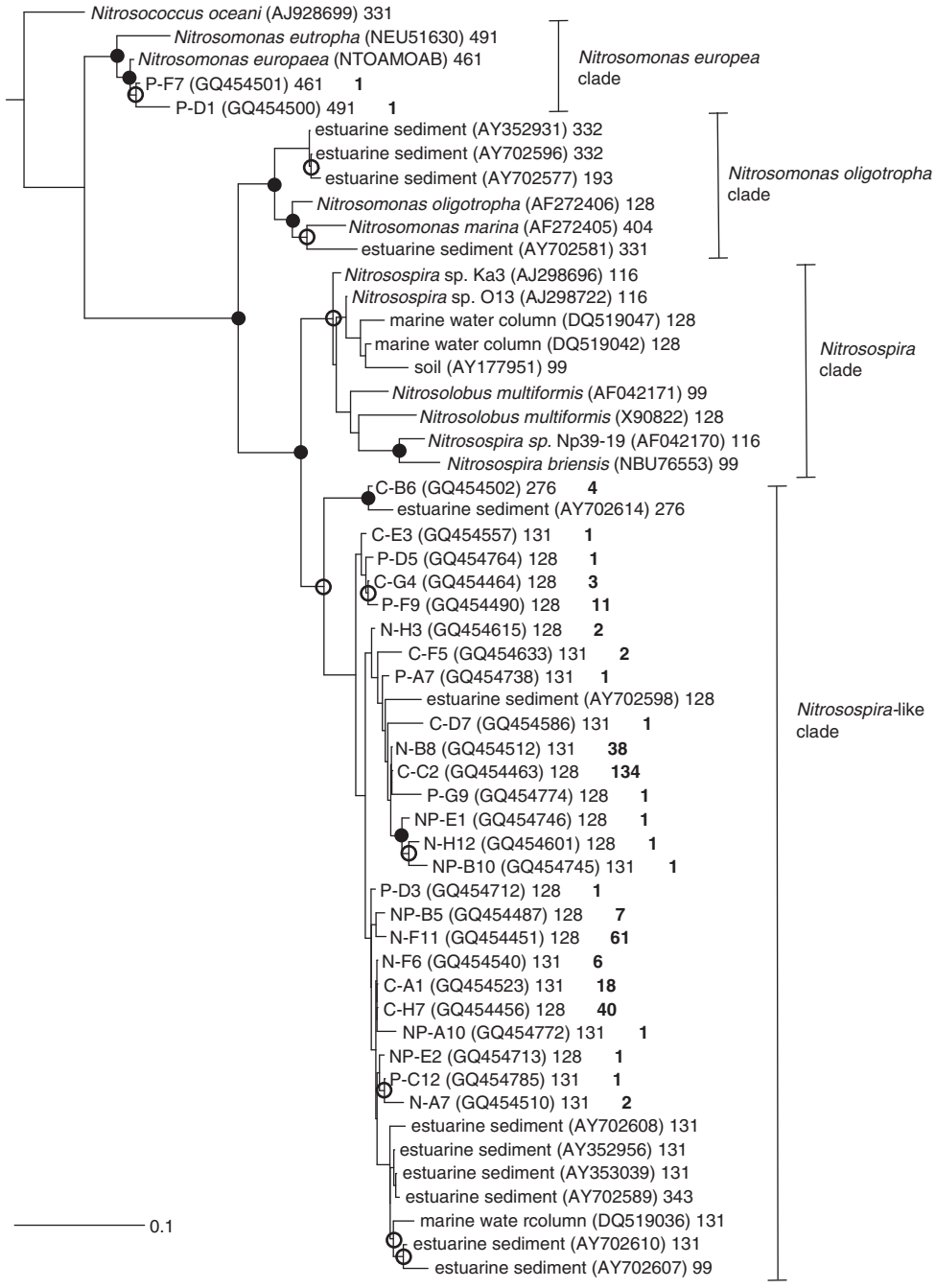

Figure 1 Phylogenetic relationships of the *amoA* sequences recovered from this study. The amino-acid neighbor-joining tree is based on 150 amino acids and includes one representative sequence from each OTU defined at ⩾99% DNA sequence similarity. *In silico* digestion terminal restriction fragment sizes (bp) are noted after the GenBank accession number. The bold number is the number of sequences within each OTU. Nodes supported by bootstrap values >50% are indicated by '○' and >80% by '●'.

composition in the N and P treatments differed from the control plots ($P=0.017$ and $P=0.029$, respectively), whereas those from the NP treatment did not ($P=0.598$). Sequence composition in the N plots was also significantly different from the P plots ($P=0.001$) and the NP plots ($P=0.007$), whereas there was not a significant difference in composition between the P and NP plots ($P=0.083$).

A phylogenetic analysis of all the sequences shows that the ANOSIM results are explained by fine-scale genetic differences within the *Nitrosospira*-like clade (Supplementary Figure 1). Sequences from the phosphorus and nitrogen treatments cluster in different parts of this clade, whereas sequences from the control and NP treatments are distributed more evenly throughout the tree.

Finally, the number of terminal restriction fragments observed did not differ among treatments ($F=1.247$, $df=3$, $P=0.3257$). However, N decreased AOB richness observed in the clone libraries.

Table 1 The percent variation explained (the relative eigenvalue) and significance (*P*) values for the effect of N and P (and the interactions between these treatments and over time) on AOB composition, as summarized by the top five principal coordinates (explaining 50.1% of the variation)

PCO	*Variation explained (%)*	*All between subjects*	*N*	*P*	*N × P*	*All within subjects*	*Time*	*Time × N*	*Time × P*	*Time × N × P*
1	21.4	0.7986	0.4096	0.7246	0.6891	**0.0272**	**<0.0001**	0.3254	0.3648	**0.0054**
2	9.3	**<0.0001**	**0.0001**	**0.0155**	**<0.0001**	**<0.0001**	0.1107	**0.0057**	0.1178	**<0.0001**
3	7.4	**0.0057**	0.1058	0.0987	**0.0029**	**0.0174**	**0.0102**	0.5169	0.0878	**0.0040**
4	7.0	0.2096	0.4842	0.3978	0.0691	0.5632	0.4637	0.5417	0.2629	0.5964
5	5.0	0.4957	0.6505	0.2032	0.4802	0.2091	**0.0501**	0.9493	0.3290	**0.0381**

Abbeviations: AOB, ammonia-oxidizing bacteria; N, nitrogen enrichment, NP, nitrogen and phosphorus enrichment; P, phosphorus enrichment; PCO, principal coordinate.
Values ⩽0.05 are shown in bold. 'All between subjects' represents the effect of all treatments on composition summed across the repeated measures. 'All within subject' represents all the compositional differences across the repeated measures (i.e., over time), including interactions between time and treatments.

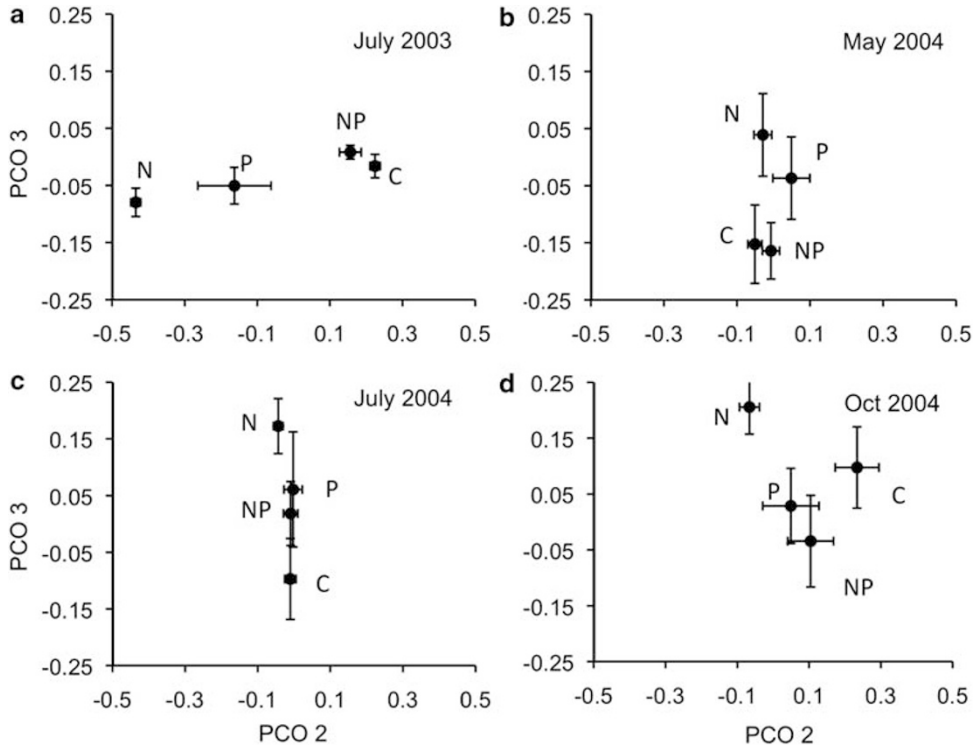

Figure 2 The average values of principal coordinates (PCO) 2 and 3 for all four nutrient treatments in (**a**) July 2003 and (**b**) May, (**c**) July and (**d**) October 2004. Error bars are ±1 s.e.

Rarefaction curves revealed that the number of OTUs observed was significantly lower in the N treatments and higher in the P treatments (Figure 3).

Temporal variation in AOB composition

Despite the effects of nutrient enrichment, most of the variation in AOB composition was related to temporal variability. Composition along PCO1, which accounted for 21.4% of the variability in the T-RFLP patterns, was not directly affected by nutrient treatment, but varied significantly by sampling time (Table 1).

To further examine seasonal and annual variability of AOB communities, we compared the composition in the control and NP treatment in three seasons over 2 years (the other treatment types were not sampled at all times). As in the previous analysis, enrichment with NP did not affect composition (defined by the first four PCOs, which comprise 52.5% of the variation in the C and NP plots; Table 2). Composition changed significantly over time, however (Table 2). Variation along PCO1 (33.1%) appears to reflect compositional differences between the 2 years, whereas variation along PCO2 (7.5%) is related to differences between sampling

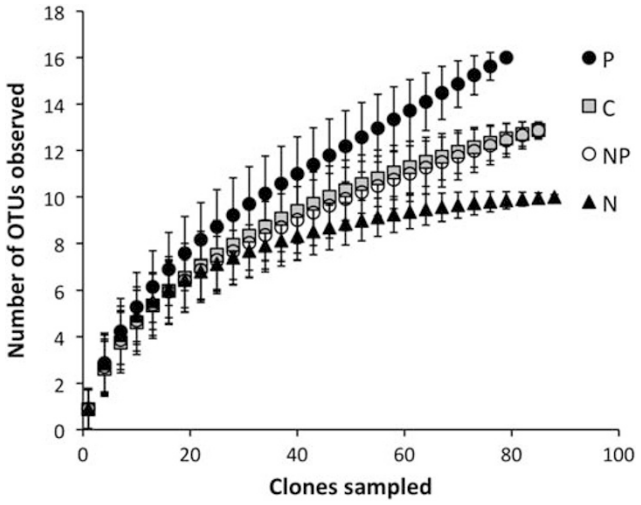

Figure 3 Rarefaction curves for *amoA* clone libraries from the four treatments: control (C), nitrogen alone (N), phosphorus alone (P) and a combination of nitrogen and phosphorus (NP). For clarity, only every third data point is plotted. Error bars are ±1 s.e.

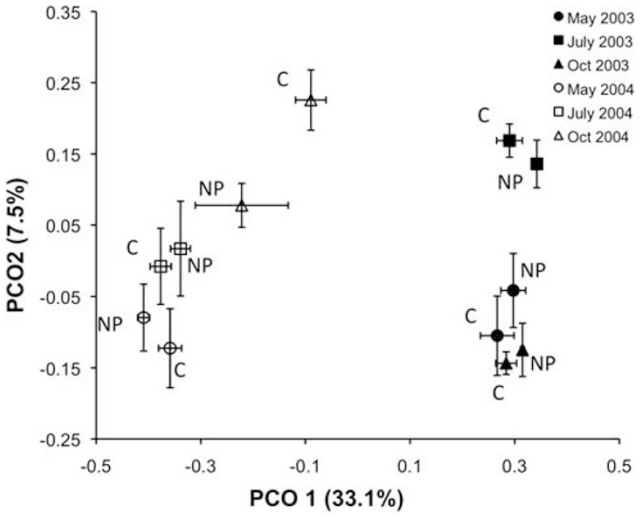

Figure 4 The average values of principal coordinates (PCO) 1 and 2 for the C and NP treatments in May, July and October of 2003 and 2004. Error bars are ±1 s.e.

Table 2 The percent variation explained (the relative eigenvalue) and significance (*P*) values for the effects of NP, time and their interaction on AOB composition, as summarized by the top four principal coordinates (explaining 52.4% of the variation)

PCO	*Variation explained (%)*	*NP*	*Time*	*Time × NP*
PC1	33.1	0.7969	**<0.0001**	0.5058
PC2	7.5	0.8645	**0.0022**	0.2287
PC3	6.5	0.8286	**0.0248**	0.9740
PC4	5.5	0.4586	**0.0260**	0.3604

Abbeviations: AOB, ammonia-oxidizing bacteria; N, nitrogen enrichment, NP, nitrogen and phosphorus enrichment; P, phosphorus enrichment; PCO, principal coordinate.
Values ⩽0.05 are shown in bold.

months (Figure 4). Thus, interannual and interseasonal variation in AOB composition was high in this salt marsh, even in the control communities.

In addition to this 'background' temporal variability, the effect of nutrient enrichment on AOB composition changed over time. Time and treatment interacted to affect four of the first five principal components (Table 1). For instance, in July 2003 the NP plots have a higher PCO2 value than both the N and P plots, whereas in May 2004 the NP plots have a PCO2 value intermediate between the N and P plots (Figure 2).

Similar to composition, the number of terminal restriction fragments differed significantly between sampling times ($F = 37.45$, $df = 3$, $P < 0.001$). The July 2003 samples produced an average of 20.5 fragments versus 8.8, 10.6 and 14.1 fragments in the May, July and October 2004 samples, respectively. Unlike composition, however, nutrient treatment and time did not interact to affect observed fragment richness (time × N: $F = 0.429$, $df = 3$, $P = 0.735$; time-P: $F = 1.627$, $P = 0.228$; time × N × P: $F = 2.036$, $P = 0.115$), suggesting that the number of fragments over time does not differ between treatments.

Effects of N and P on AOB abundance

We detected an average of $3.2 \times 10^7 \pm 2.8 \times 10^7$ copies of *amoA* g^{-1} wet sediment, with a range of 1.2×10^6–1.63×10^8 copies g^{-1} wet sediment. (Two of the 60 samples did not amplify and were removed from the analysis.) Neither nutrient treatment nor month sampled affected AOB abundance as estimated by quantitative PCR (repeated measures analysis of variance; $F = 0.061$, $df = 3$, $P = 0.8353$). In addition, there were no interactive effects of treatment and time on abundance. Within sample variability among the experimental replicates (three per sample) was low (average s.d. $= 5.2 \times 10^5$ copies g^{-1}) compared with across samples (s.d. $= 2.8 \times 10^7$). Thus, it appears that the vast majority of variability in the abundance estimates is due to actual variation among samples rather than measurement error.

Discussion

The majority of the AOB *amo*A sequences in our study fell within a clade of *Nitrosospira*-like sequences found in other salt marshes, estuarine sediments and the marine water column. This observation confirms previous studies that find that the dominant AOB in estuarine and marine environments appear to fall within divergent evolutionary clades (McCaig *et al.*, 1994; Freitag and Prosser, 2004; Ward *et al.*, 2007; Mosier and Francis, 2008). So far, there are no cultured isolates from this group, although Mosier and Francis, (2008) found sequences in an enrichment culture from the San Francisco Bay estuary. Most of the previous experiments on AOB communities are in soil; therefore,

this study provides important insights into these distinctive high-salinity communities.

Effects of nitrogen and phosphorus

Nitrogen and phosphorus enrichment significantly altered AOB composition, but not abundance, in this salt marsh. These composition changes appear to be driven by shifts in the relative abundance of taxa within the marine and estuarine *Nitrosospira*-like clade (Supplementary Figure S1), suggesting that there are fine-scale genetic differences with the AOB that correspond to differences in their ability to use nitrogen and phosphorus. Sequencing of genes that are more variable than *amoA* would be needed to resolve these taxa.

Previous experiments in soils also observed compositional shifts with nitrogen addition, whether in the form of nitrate, ammonium or both (Mendum and Hirsch, 2002; Horz *et al.*, 2004; Avrahami and Bohannan, 2009), and these results support evidence from enrichment cultures that AOB taxa may differ in their competitive abilities at different ammonium concentrations (Kowalchuk *et al.*, 2000; Koops and Pommerening-Röserm, 2001; Bollmann *et al.*, 2002). In contrast, studies from marine and estuarine environments have been observational and have revealed little or no relationship between nitrogen concentrations and AOB composition. For instance, ammonium concentrations in Chesapeake Bay sediments explained less than 3% of the variability in AOB composition (Francis *et al.*, 2003), and inorganic nitrogen was also not related to AOB composition in the Westerschelde estuary sediments (Sahan and Muyzer, 2008). The apparent discrepancy between the observational studies and our experiment is likely due to the range of the environmental variability sampled (O'Mullan and Ward, 2005). For instance, previous work documents that salinity is a primary determinant of AOB composition, such that if one samples along a salinity gradient, most of the variability in composition is explained by salinity (Bollmann and Laanbroek, 2002; Francis *et al.*, 2003; Sahan and Muyzer, 2008). Without experiments or sampling within a narrower range of salinity, the importance of secondary factors, such as nitrogen concentrations, may be obscured.

AOB composition in this salt marsh also responded to phosphorus enrichment. Little is known about the direct effects of phosphorus on β-AOB. In a different New England salt marsh, Horner-Devine *et al.*, (2004) observed a correlation between AOB composition and phosphate concentrations; pairwise samples with more similar phosphate concentrations contained more similar communities. In agricultural soils, Chu *et al.* (2007) found differences in DGGE banding patterns of the *amoA* gene between control and PK (phosphorus and potassium)-enriched plots, but did not find differences in *amoA* copy numbers (Chu *et al.*, 2008) or soil nitrification potentials.

Changes in AOB composition under P enrichment may reflect changes in interactions with other organisms that are P-limited. One possible interaction is with plants. Plants appear to compete for ammonium (Jackson *et al.*, 1989), whereas at the same time they can be positively affected by ammonia oxidizers because of a preference for nitrate (Austin *et al.*, 2006). During this study, *S. patens*, the dominant plant in our plots, responded primarily to N addition and was additionally stimulated by the NP addition, indicating secondary P limitation (Crain, 2007). P addition alone did not affect plant biomass, and plant tissue stoichiometry was not altered by P or NP addition. Thus, an interaction with plants does not entirely explain the effect of P addition on the AOB community.

Salt marsh AOB might also be interacting with sediment heterotrophs. In South Carolina, Sundareshwar and colleagues (2003) showed that P limited total microbial biomass in salt marsh sediments. The majority of this biomass is heterotrophic bacteria and fungi, and an increase in these heterotrophs could increase net mineralization rates and ammonium availability. In addition, AOB may directly compete with heterotrophs for ammonium or other limiting nutrients such that indirect changes in the heterotroph composition may favor some AOB taxa over others.

The fact that AOB abundance did not increase in response to the nutrient additions suggests that the community was not limited by nitrogen or phosphorus. Alternatively, the changes in abundance might have been too small to detect by our assay after only three years of fertilization. This seems unlikely as previous studies (albeit in agricultural soils) found striking changes in abundance within 6 weeks of fertilization (Mendum *et al.*, 1999; Okano *et al.*, 2004). We also cannot eliminate the possibility that abundance increased but was offset by a shift toward AOB taxa carrying fewer copies of the *amoA* gene (AOB appear to carry between 1 and 3 copies; Norton *et al.*, 2002).

There was no effect of nutrient enrichment on the number of terminal restriction fragments observed, but N addition appeared to decrease sequence diversity observed in the clone libraries (Figure 3). We hypothesize that these numbers do not reflect changes in AOB richness, as it seems unlikely that taxa are going extinct from (or invading) these plots during the course of this experiment. Rather, the result suggests that the addition of nitrogen, whether alone or in combination with phosphorus, disproportionately increases the relative abundance of a few taxa, thereby decreasing evenness. In agricultural soils, Bruns *et al.*, (1999) observed that *Nitrospira* cluster 3 dominated in N fertilization treatments. This response is similar to that of plants and fungi where taxa that are good N competitors increase relative to poor N competitors (Tilman, 1987; Dighton *et al.*, 2004; Suding *et al.*, 2005).

Finally, we observed an interactive effect of N and P addition on AOB communities such that composition

changed less when N and P were added simultaneously than when either nutrient was added alone (Tables 1 and 2; Figure 2). (Observed richness in the clone libraries was also similar in the control and NP treatments (Figure 3).) This surprising result suggests that the particular ratio of N and P applied in the NP treatment may have been similar to the control, such that there was no differential response among AOB taxa. Alternatively, perhaps in this ratio the plants were able to take up most of the nutrients before the AOB community could respond. In contrast, Chu *et al.* (2007) observed similar DGGE bands and *amoA* copy numbers in NPK, NP and NK treatments in agricultural soils. Experiments that apply different ratios of N and P would be necessary to tease apart the interactive effects of these nutrients.

Temporal variability and nutrient responses

Despite the influence of nutrient enrichment, most of the variability in AOB composition that we observed was unrelated to the experiment and due to unidentified, temporally varying environmental factors. Composition was highly variable over seasons and years, and compositional changes in response to the nutrient treatments were minor in comparison. High temporal variability in AOB communities (both abundance and composition) has been observed in both soils and the water column (Gray *et al.*, 2003; O'Mullan and Ward, 2005) and in estuarine sediments (Bernhard *et al.*, 2007; Ando *et al.*, 2009; Fortunato *et al.*, 2009). Given the importance of temperature on the spatial variability of AOB in soils (Fierer *et al.*, 2009), we speculate that temperature could be one important factor contributing to this temporal variability.

These results highlight the importance of considering the temporal variability of bacterial composition when performing experiments, lest the background variation overwhelm the treatment effects. Cordova-Kreylos *et al.* (2006) came to a similar conclusion in a study of pollutant effects on bacterial communities in Californian salt marshes. They showed that spatial and temporal variation in bacterial communities was greater than the variation attributable to pollutants. Once they accounted for this variability statistically, however, they could detect a correlation between contaminant concentrations and bacterial composition. Similarly, Gray *et al.* (2003) determined that soil amendments had a minor effect on eubacterial (and AOB) composition relative to natural, temporal and spatial variability.

Finally, the response to nitrogen and phosphorus additions varied over the course of the experiment. There are at least two non-exclusive reasons that could account for this result. First, composition could be related to the length of time since the start of nutrient application, for instance, if different AOB taxa respond at different rates to enrichment. Second, temporal variation of factors extrinsic to the experiment (for example, seasonal fluctuations in temperature) might influence the ability of particular taxa to respond to nutrient treatments.

Conclusions

AOB composition in salt marsh sediments appears to be highly variable over seasons and years, even without experimental disturbances. NP influences composition, but this influence changes over time. We conclude that closely related taxa within the marine/estuarine-specific *Nitrosospira*-like clade vary in their preference for nutrient concentrations, and this preference may depend on additional, temporally variable abiotic factors. In general, microbial community-process relationships likely depend on environmental conditions. For instance, in estuarine sediments, Bernhard *et al.* (2007) found that the strength of the correlation between AOB abundance and potential nitrification rates varies along a salinity gradient. Finally, AOB composition was highly variable within and across years, even in untreated plots. Further studies are needed to test which environmental variables are responsible for this aspect of compositional variability and its influence on nutrient cycling.

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