



FEATURE ARTICLE

Acidification alters the composition of ammonia-oxidizing microbial assemblages in marine mesocosms

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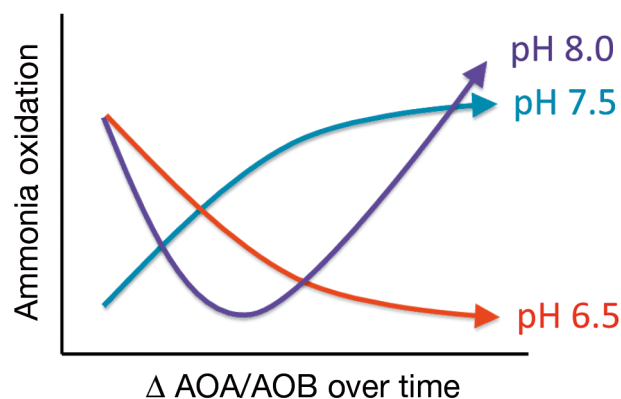
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ABSTRACT: Increasing atmospheric CO₂ concentrations are causing decreased pH over vast expanses of the ocean. This decreasing pH may alter biogeochemical cycling of carbon and nitrogen via the microbial process of nitrification, a key process that couples these cycles in the ocean, but which is often sensitive to acidic conditions. Recent reports have indicated a decrease in oceanic nitrification rates under experimentally lowered pH. How the composition and abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) assemblages respond to decreasing oceanic pH is unknown. We sampled microbes from 2 different acidification experiments and used a combination of qPCR and functional gene microarrays for the ammonia monooxygenase gene (*amoA*) to assess how acidification alters the structure of ammonia oxidizer assemblages. We show that despite widely different experimental conditions, acidification consistently altered the community composition of AOB by increasing the relative abundance of taxa related to the *Nitrosomonas ureae* clade. In one experiment, this increase was sufficient to cause an increase in the overall abundance of AOB. There were no systematic shifts in the community structure or abundance of AOA in either experiment. These different responses to acidification underscore the important role of microbial community structure in the resiliency of marine ecosystems.

KEY WORDS: Ocean acidification · Ammonia-oxidizing archaea · Ammonia-oxidizing bacteria · Nitrification

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Changes in microbial community structure under different pH regimes make it difficult to predict geochemical responses such as ammonia oxidation

Image: B. Ward

INTRODUCTION

Increasing atmospheric CO₂ has already led to decreased oceanic pH (Feely et al. 2004, Orr et al. 2005, Doney et al. 2009), but the effect that this decreasing pH will have on global carbon and nitrogen cycling is debated. Some have argued that, aside from calcification, pH change in the ocean will not fundamentally alter marine biogeochemistry (Joint et al. 2011). Nevertheless, significant reductions (8–38%) in ammonia oxidation rates were reported from short-term acidification experiments in the open ocean (Beman et al. 2011), and water column incubations from the English Channel showed a near complete

cessation of ammonia oxidation at pH 6.5 (Kitidis et al. 2011). In artificially acidified lakes, Rudd et al. (1988) also observed the complete shutdown of nitrification at pH below 5.7. Altered pH has also been shown to shift the overall structure of microbial communities (Krause et al. 2012). Ammonia oxidation, the first step in nitrification ($\text{NH}_3 + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+$), is thought to be pH sensitive (1) due to feedback inhibition through acidification of the medium and (2) because NH_3 , rather than NH_4^+ (the dominant form at pH < 9.3), is the substrate in the first step of the ammonia oxidation reaction. Given the importance of nitrification as both a source of oxidized nitrogen and a control on primary production in the oceans (Yool et al. 2007), understanding how ammonia-oxidizing communities respond to acidification is imperative.

MATERIALS AND METHODS

We opportunistically sampled from 2 very different experiments designed to test the effects of acidification on (1) diatom bloom development and (2) the temperate coral *Astrangia poculata* (Holcomb et al. 2012). In the diatom bloom experiment, 180 l of seawater collected from a depth of 70 m in Monterey Bay, California (USA), were inoculated with 20 l of surface seawater and incubated under ambient light for 6 d. One of the barrels was acidified using concentrated HCl (accompanied by bicarbonate addition to maintain alkalinity; Shi et al. 2009). An Oakton pH 11 meter (with an Oakton 35811-71 probe) was used to measure pH. The meter was inter-calibrated with spectrophotometric measurements using thymol blue (Zhang & Byrne 1996). pH of the ambient and acidified barrels evolved over the experiment, but pH in the acidified barrel was ~0.21 pH units lower than the ambient barrel through most of the experiment. A phytoplankton bloom dominated by diatoms developed in both barrels and the time courses of chlorophyll accumulation and nitrate drawdown were essentially identical (data not shown). Ammonia-oxidizing bacteria (AOB) and archaea (AOA) samples were collected after the initial acidification and 6 d later by filtering 2 l of water into capsule filters using a peristaltic pump. Ammonium concentrations were not measured, but previous reports indicate that the concentrations in Monterey Bay at this depth are low, averaging 0.25 μM (Kudela & Dugdale 2000). Initial ammonium concentrations in similar barrel experiments performed previously with Monterey Bay seawater were on the order of 1.0 μM

(Fawcett & Ward 2011). The fraction present as NH_3 (shown to be the substrate limiting ammonia oxidation, at least in AOB) is <5% and <3% of the total $\text{NH}_3 + \text{NH}_4^+$ at pH 7.89 and 7.69, respectively, assuming a pK of 9.23.

In the Vineyard Sound coral experiment, 1.9 l tanks (n = 4) containing live coral *Astrangia poculata* were incubated with flowing seawater from Vineyard Sound, Massachusetts (USA), maintained at 24°C. Ambient CO_2 tanks were maintained at a daytime pH of 8.00 ± 0.04 (night time pH in all tanks was up to 0.1 lower). Acidified tanks were maintained at a daytime pH of 7.8 ± 0.04 by bubbling incoming seawater with 800 ppm CO_2 . Both ambient and CO_2 -enriched experiments were performed with and without addition of inorganic nutrients. Nutrient-enriched tanks were supplied with seawater supplemented with NaNO_3 , K_2HPO_4 , and FeCl_2 solutions. NO_3^- was elevated by 5 μM , PO_4 by 0.3 μM , and Fe by 2 nM above ambient values. Ammonium concentrations ($\text{NH}_3 + \text{NH}_4^+$) ranged from undetectable to 1.1 μM , and there was no significant difference between control and acidified tanks, either initially or at the final sampling time. NH_3 comprises <6% and <4% of the total $\text{NH}_3 + \text{NH}_4^+$ at pH 8.0 and 7.8, respectively. Tanks were maintained for 5 mo under treatment conditions with regular removal of wall growth from the sides and bottoms of the tanks. Cleanings were suspended for approximately 3 wk before collecting biofilm material from the tank walls for genomic analysis. Additional details of the Vineyard Sound coral experiment have been published (Holcomb et al. 2012).

DNA was extracted from the Monterey Bay filters using the Puregene DNA kit (Gentra) and from the tank biofilm using the MoBio PowerSoil DNA extraction kit (MoBio Laboratories). AOA and AOB community composition was analyzed using functional gene microarrays containing archetype probes representing all known AOA and AOB *amoA* gene sequences (as of November 2009; Bouskill et al. 2011, 2012a). Target DNA was labeled from whole genomic DNA extracts using Klenow amplification (Ward 2008). Hybridization to the array, array scanning, and initial data quality assessment/control have been previously described (Bouskill et al. 2011, 2012a). The contribution of each archetype to the total community is indicated by its relative fluorescence ratio (RFR), the portion of the total fluorescence signal represented by that archetype (Ward et al. 2007). Correspondence analysis of the archetype RFR was performed using the 'cca' function contained in the R programming language package 'vegan' (Oksanen et al. 2011). Analysis of similarities

was performed using the anosim function in 'vegan' (Oksanen et al. 2011). Microarray data are archived at the Microarray Gene Expression Omnibus (accession number: GSE49956).

AOA and AOB gene abundances were quantified from extracted DNA using QuantIt™ PicoGreen® dsDNA reagent (Life Technologies) in triplicate reactions. Each sample was normalized to a concentration of 3 ng μl^{-1} prior to quantification of the *amoA* gene via quantitative PCR on an Agilent MX3005p qPCR system. Archaeal *amoA* genes were quantified in triplicate using 0.2 μM of primers Arch-amoAF (5'-STA ATG GTC TGG CTT AGA CG-'3) and Arch-amoAR (5'-GCG GCC ATC CAT CTG TAT GT-'3) (Francis et al. 2005), along with 10 μl of SYBR®Green Brilliant III Ultra-Fast master mix, 3 mM MgCl_2 (final concentration), and 300 $\mu\text{g ml}^{-1}$ BSA in a 20 μl reaction. Reaction conditions included an initial denaturation at 95°C followed by 42 cycles of 1 min at 94°C, 1.5 min at 59°C, and 1.5 min at 72°C followed by a final denaturation to generate a melt curve to test for amplification stringency. Bacterial *amoA* genes were quantified in triplicate using 0.1 μM of primers amoA-1F (5'-GGG GTT TCT ACT GGT GGT-'3) and amoA-2R (5'-CCC CTC KGS AAA GCC TTC TTC-'3) (Rotthauwe et al. 1997), along with 10 μl of SYBR®Green Brilliant III Ultra-Fast master mix, and 300 $\mu\text{g ml}^{-1}$ BSA in a 20 μl reaction. Reaction conditions included an initial denaturation at 95°C followed by 42 cycles of 1 min at 94°C, 1.5 min at 59°C, and 1.5 min at 72°C followed by a final denaturation to generate a melt curve to test for amplification stringency. All PCR products were visualized on a 1.5% agarose gel to assure proper fragment length. Amplification efficiencies varied from 78 to 85%.

RESULTS AND DISCUSSION

After only 6 d, changes were evident in the AOB assemblage in the Monterey Bay seawater incubations (Fig. 1). There was a 10% increase in the RFR of archetype B17 (*Nitrosomonas ureae* clade), from 47% of the fluorescence signal in the acidified barrel on Day 1 to 57% of the signal on Day 6. This increase, along with an increase in archetype B16, resulted in a shift to the upper left in the correspondence analysis of the acidified barrel AOB community structure (Fig. 1A). However, little change occurred in the community structure of the ambient barrel between Day 1 and Day 6 (Fig. 1). Although AOB are thought to turn over slowly, this somewhat rapid shift in community structure is not without precedent; Nicol et al.

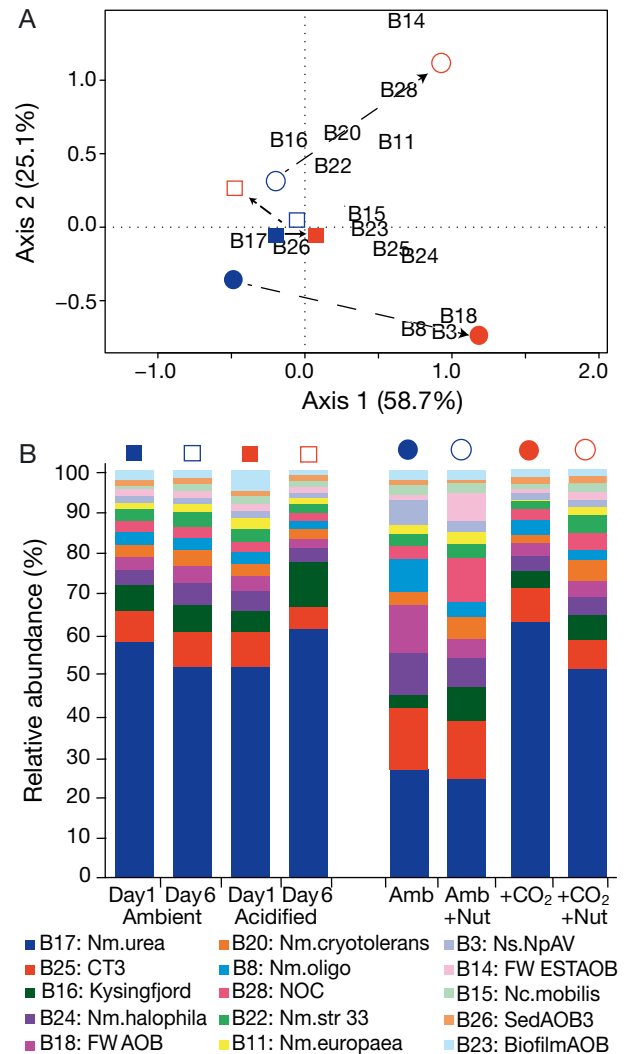


Fig. 1. Community composition of ammonia-oxidizing bacteria (AOB) in 2 different acidification experiments. (A) Correspondence analysis of community similarities in both experiments: Expt 1 (squares), Monterey Bay seawater experiment; Expt 2 (circles), Vineyard Sound coral experiment. Blue symbols indicate ambient CO_2 concentrations and red symbols indicate acidified samples. Arrows indicate the direction of change from the ambient to the acidified treatment. (B) The most abundant archetypes that explain the ordination patterns for both experiments. Symbols above each column correspond to symbols in the ordination probe list. See Appendix Table A1 for full archetype probe list (modified from Bouskill et al. 2011)

(2008) demonstrated a decrease in NH_4^+ concentrations in as little as 2 d and a shift in community structure within 2 wk in soil mesocosms of varying acidity.

In the 3 wk long Vineyard Sound coral experiment, the biofilm community demonstrated an even stronger response by the *Nitrosomonas ureae* clade (Fig. 1). Archetype B17 increased from approximately 23% of the AOB signal in the ambient tanks (Fig. 1), to over

54% in the acidified tanks (Fig. 1) and resulted in a clear separation of the 2 acidified samples along the primary axis (explaining 58.7% of the variance) in the correspondence analysis. The partitioning of the acidified samples along the secondary axis (explaining 25.1% of the variance) results from different AOB archetypes being abundant in the 2 ambient tanks (archetypes B18, B8, and B3 in the ambient tank, archetypes B14 and B28 in the ambient plus nutrients tank), but all being reduced to low levels in the acidified tanks. For both experiments, analysis of similarities indicates that the AOB display a significant difference in community composition between acidified and ambient treatments ($R = 0.631$, $p = 0.036$).

By contrast, the AOA indicated no such systematic shifts in community composition in either acidifica-

tion experiment (Fig. 2). In the Monterey Bay seawater experiment, AOA archetype A9 comprised a greater proportion of the community in the acidified barrels, whereas archetype A12 was more important in the ambient barrels (Fig. 2B), but this difference was due to different initial starting communities and did not change over the course of the experiment, thus the change cannot be attributed to a treatment effect. The differences in these 2 archetypes help to explain the separate partitioning of the acidified barrel, but the ambient barrel showed little change in the community over the course of the experiment. Thus, the remaining samples generally form 1 cluster of points with no clear segregation among the AOA as a result of the acidification (Fig. 2A) and no clear pattern in dominance of any one AOA archetype as a function of treatment (Fig. 2B). This result is further supported by a lack of difference with treatment as indicated by analysis of similarities ($R = 0.097$, $p = 0.233$).

We quantified the abundance of AOA and AOB in DNA extracted from both experiments via qPCR. We obtained widely different estimates of the relative importance of the 2 groups. The absolute gene abundances between the 2 experiments are not directly comparable, however. While both experiments are normalized to the total mass of DNA, the Monterey Bay seawater experiment is normalized to the mass of DNA extracted from 2 l of sea water and the Vineyard Sound coral experiment is normalized to 0.5 g wet weight biofilm material. Importantly, however, the relative abundances of AOA and AOB within each experiment are comparable, as are their changes as a result of acidification.

In the Monterey Bay seawater experiment (Fig. 3), both AOA and AOB *amoA* copies decreased from Day 1 to Day 6 of the experiment, suggesting that both communities were in decline. The declines, however, appeared to be slightly less severe in the acidified barrel, particularly for AOA where the gene abundances were already quite low. Surprisingly, AOB considerably outnumbered AOA in this Monterey Bay seawater experiment. Previous work by Mincer et al. (2007) reported AOB concentrations in Monterey Bay seawater that were largely undetectable through most of the upper 200 m of the surface ocean. Santoro et al. (2010), however, also reported AOA/AOB *amoA* quantities in the nearby Central California Current. While AOA were frequently more abundant than AOB, AOB sometimes outnumbered AOA at the depths sampled in this acidification experiment. Although the primers of Rotthauwe et al. (1997) do not detect the gamma-proteobacterial ammonia oxidizers, the microarray

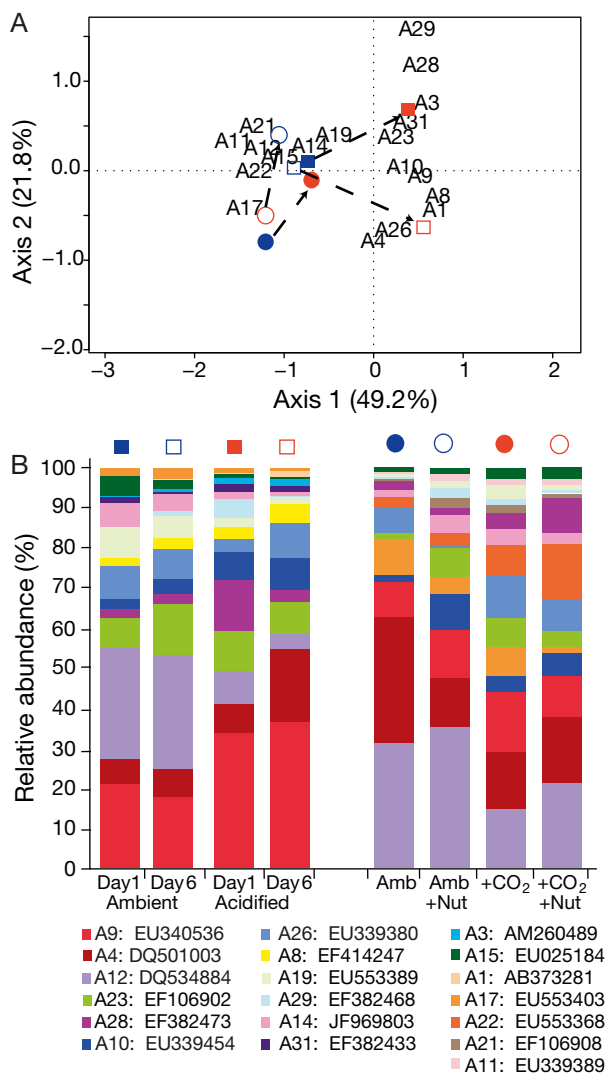


Fig. 2. As in Fig. 1, but for community composition of ammonia-oxidizing archaea (AOA). See Appendix Table A2 for full archetype probe list (modified from Bouskill et al. 2012a)

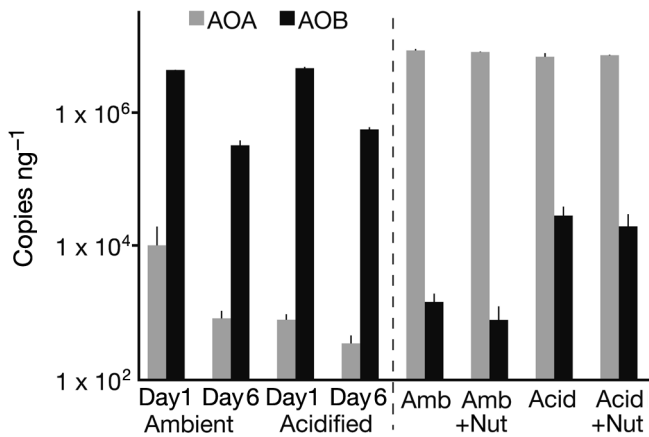


Fig. 3. Mean (+SD) *amoA* gene abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) from the Monterey Bay seawater experiment (left side) and the Vineyard Sound coral experiment (right side) as determined by quantitative PCR. Data are from triplicate qPCR reactions

probe that binds *Nitrosococcus* sequences accounted for only 2.0 to 2.6% of the relative fluorescence signal, suggesting that the abundance of this gamma-proteobacterial AOB were sufficiently low and uniform that their omission from the qPCR amplification does not change the observed trends.

By contrast, in the Vineyard Sound coral experiment, AOA were the dominant group and, consistent with the lack of community change in the microarray results, the AOA did not change in abundance as a result of acidification (Fig. 3). Contrary to our expectations, however, the AOB *amoA* gene abundance increased in both acidification treatments from an average of approximately 1200 gene copies per ng DNA in the ambient treatment to over 25 000 copies, on average, in the acidified treatments. This ~20-fold increase in gene abundance coincides with a dramatic increase in the relative fluorescence of the B17 probe on the microarray, suggesting that representatives of this clade were responsible for the increased gene abundances of AOB. As with the Monterey Bay experiment, the probe that binds *Nitrosococcus* sequences accounted for less than 10% of the AOB relative fluorescence signal for these samples, suggesting that if the gammaproteobacteria were included in the qPCR it would not alter the results that demonstrate a large increase in AOB abundance in the acidified samples.

The results from these widely different experiments illustrate a few key points regarding the potential response of ammonia oxidizers to ocean acidification. First, they suggest that the community structure of AOB may be more strongly affected by acidification than that of AOA. The shift in commu-

nity structure in both experiments was due to an increase in the relative abundance of one group, whether due to loss of other archetypes or growth of taxa represented by B17 (the probe for the *Nitrosomonas ureae* clade). In the Monterey Bay experiment, qPCR data indicate that the assemblages are in decline, and the increase in the clade represented by probe B17 may be a result of the decrease in abundance of other archetypes. In the Vineyard Sound experiment, however, the surprising increase in the bacterial *amoA* gene (Fig. 3) suggests that the increase in relative abundance of clade B17 that was detected on the microarray cannot be due solely to decreases in other clades.

At the time of the array design, probe B17 represented only 3 sequences, including *Nitrosomonas ureae* as the closest cultured representative. Since that time, with the addition of thousands of new *amoA* sequences, the probe now represents many more sequences, all of them from uncultured organisms, mostly derived from estuarine or lake sediments. It is not possible to know whether these sequences, which would hybridize with B17, represent organisms with the capacity for urea hydrolysis, but their association with the known organism *N. ureae* supports the possibility. Experiments with pure cultures indicate that the pH optimum for ureolysis by *N. ureae* is between 6 and 7 (Pommerening-Röser & Koops 2005), below the pH in our experiment but nonetheless evidence that these taxa are capable of utilizing urea as a substrate. Thus, one possible mechanism to explain the success of the *N. ureae* clade in our acidification experiments is that they are able to switch to growing on urea as a sole energy source (Pommerening-Röser & Koops 2005) to survive the decrease in NH_3 availability under acidified conditions. Although urea concentrations were not measured in this experiment, previous analysis of the nutrient concentrations in Vineyard Sound indicate that urea concentrations were as large or larger than the concentrations of NO_3^- and NH_4^+ (Glibert et al. 1985). Additional research will be needed to verify whether these AOB can switch to growth on urea in the face of declining NH_4^+ concentrations. Although ureases have also recently been detected in arctic *Thaumarchaeota* (Alonso-Sáez et al. 2012) and in the bathypelagic waters of the Tyrrhenian Sea (Yakimov et al. 2011), this ability is present in only 1 of the 3 complete genomes in the *Thaumarchaeota* (Hallam et al. 2006). The lack of response to acidification by AOA suggests that growth on urea may not be a mechanism employed by them in these experiments.

Second, the relative abundance of AOA and AOB differed between the 2 systems, underscoring the highly variable composition of the total ammonia-oxidizing assemblage. Many studies have reported the numerical dominance of AOA in coastal and marine waters (Mincer et al. 2007, Agogu e et al. 2008, Beman et al. 2008, 2012, Bouskill et al. 2012a). Studies from coastal and estuarine sediments, and the water columns of the freshwater reaches of estuaries, however, indicate a greater importance of AOB (Caffrey et al. 2007, Mosier & Francis 2008, Bouskill et al. 2012a, Cao et al. 2012). In the experiments presented here, the relative abundance of AOB versus AOA varied (Fig. 3). The average AOA:AOB ratio in the Monterey Bay seawater experiment was 0.0014 compared to 3853 in the biofilm from the Vineyard Sound coral experiment. Trait-based modeling of ammonia oxidizer dynamics suggests that as pH decreases, community diversity will decrease until AOA dominate, although this pattern was not directly observed in comparison with environmental data (Bouskill et al. 2012b). This modeling underscores the challenge in directly linking observable environmental processes, such as the measured reductions in ammonia oxidation (Beman et al. 2012), to patterns in community structure. The data we report here, however, demonstrate that the AOB component of the community is more likely to be altered by future acidification and that continued monitoring of the composition of AOA and AOB communities is needed to understand how ocean acidification could alter oceanic nitrogen cycling.

Determining the contribution of AOA and AOB to marine nitrification is critically important for understanding ecosystem resilience in the face of environmental change. Much more work is needed to assess the biogeographical patterns, and the underlying mechanisms controlling these patterns, for both AOA and AOB. Sequences closely related to *Nitrosomonas ureae* have been reported from San Francisco Bay (Mosier & Francis 2008), the Seine River Estuary (C eburon et al. 2003), the Chesapeake Bay (Bouskill et al. 2011), the South China Sea (Cao et al. 2012), and in wetland sediments in Australia (S. Domingos et al. unpubl., GenBank accession no. JF682366), suggesting that this is a widely distributed ammonia-oxidizer clade. If, as our data suggest, the AOB closely related to *N. ureae* play an increasingly important role in ammonia oxidation under acidified conditions, then much more information will be needed regarding the role that urea plays in our understanding of the nitrogen cycle.

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