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Oxidation of urea-derived nitrogen by thaumarchaeota-dominated marine nitrifying communities

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Summary

Urea nitrogen has been proposed to contribute significantly to nitrification by marine thaumarchaeotes. These inferences are based on distributions of thaumarchaeote urease genes rather than activity measurements. We found that ammonia oxidation rates were always higher than oxidation rates of urea-derived N in samples from coastal Georgia, USA (means $\pm$ SEM: 382 $\pm$ 35 versus 73 $\pm$ 24 nmol L^{-1} d^{-1}, Mann-Whitney U-test $p < 0.0001$), and the South Atlantic Bight (20 $\pm$ 8.8 versus 2.2 $\pm$ 1.7 nmol L^{-1} d^{-1}, $p = 0.026$) but not the Gulf of Alaska (8.8 $\pm$ 4.0 versus 1.5 $\pm$ 0.6, $p > 0.05$). Urea-derived N was relatively more important in samples from Antarctic continental shelf waters, though the difference was not statistically significant (19.4 $\pm$ 4.8 versus 12.0 $\pm$ 2.7 nmol L^{-1} d^{-1}, $p > 0.05$). We found only weak correlations between oxidation rates of urea-derived N and the abundance or transcription of putative Thaumarchaeota *ureC* genes. Dependence on urea-derived N does not appear to be directly related to pH or ammonium concentrations. Competition experiments and release of $^{15}NH_3$ suggest that urea is hydrolyzed to ammonia intracellularly, then a portion is lost to the dissolved pool. The contribution of urea-derived N to nitrification appears to be minor in temperate coastal waters, but may represent a significant portion of the nitrification flux in Antarctic coastal waters.

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Introduction

Thaumarchaeota play an important role in the marine nitrogen cycle by oxidizing ammonia to nitrite (Könneke *et al.*, 2005; Capone *et al.*, 2008; Prosser and Nicol, 2008; Ward, 2011). These Ammonia-Oxidizing Archaea (AOA) are the most abundant Ammonia-Oxidizing Organisms (AOO) in most pelagic marine systems, with concentrations of the gene encoding the α subunit of ammonia monooxygenase (*amoA*) up to 10^8 copies L^{-1} (reviewed in Ward, 2011). Members of the Beta- and Gammaproteobacteria can also oxidize ammonia (known collectively as Ammonia-Oxidizing Bacteria, or AOB), though they are now thought to play a relatively minor role in most pelagic marine environments (e.g., Beman *et al.*, 2008; Prosser and Nicol, 2008; Ward, 2011; Beman *et al.*, 2012; Bouskill *et al.*, 2012).

Ammonia (NH_3), versus ammonium (NH_4^+), has been shown to be the substrate for ammonia oxidation by AOB (Suzuki *et al.*, 1974; Burton and Prosser, 2001) and is assumed to be the substrate for AOA (Martens-Habbena *et al.*, 2009; Beman *et al.*, 2011; for clarity we will use the term "ammonia" in the context of the process of "ammonia oxidation" and use "ammonium" to refer to the combined pool of the species that are at equilibrium in seawater: NH_3 and NH_4^+). The ability of many AOB to oxidize nitrogen supplied as urea has been known for some time and is thought to be particularly important in acidic soils where the availability of NH_3 is limited by protonation (e.g., Burton and Prosser, 2001; Nicol *et al.*, 2008; Gubry-Rangin *et al.*, 2010). Identification of genes for putative ureases and urea transporters in Thaumarchaeota genomes (Hallam *et al.*, 2006; Tourna *et al.*, 2011) suggested that AOA might also be able to oxidize N supplied as urea, though the first Thaumarchaeota isolate "*Candidatus* Nitrosopumilus maritimus" strain SCM1 was not able to grow on urea (Walker *et al.*, 2010). Subsequently, Qin *et al.*, (2014) compared the capacity for growth on urea by "*Ca* N. maritimus" strain SCM1 with two new Thaumarchaeota isolates from coastal waters of Washington, USA. One strain (PS0) showed an almost stoichiometric conversion of urea-derived N to nitrite, while the other two strains did not oxidize N supplied as urea. More recently Bayer *et al.*, (2016) found that one of two new Nitrosopumilus isolates from the northern

Adriatic Sea (D3C) was able to oxidize urea-derived N while the other was not. Similarly, a thaumarchaeote isolated from soil, *Nitrososphaera vienennsis* (Tourna *et al.*, 2011), is able to use urea, while an acidophilic soil thaumarchaeote, "*Ca.* Nitrosotalea devanaterra" cannot (Lehtovirta-Morley *et al.*, 2011). These studies demonstrate that the ability to use N supplied as urea is not a universal attribute of Thaumarchaeota, even among closely related isolates from the same environment.

Here we compare the oxidation of N supplied as ammonium or urea by nitrifying communities from a variety of pelagic marine environments to assess the relative contribution of ammonia versus urea N to nitrification in the ocean. We measured the production of ^{15}N-labeled nitrite plus nitrate ($^{15}NO_x$) from ^{15}N-labeled ammonium and urea in samples from near-shore and coastal environments of temperate and polar oceans (Fig. S1). Our goal was to test the hypothesis, based primarily on *ureC* distributions (Alonso-Sáez *et al.*, 2012), that urea-derived N contributes significantly to nitrification. Further, since the work that led to this hypothesis was performed with samples from polar waters, we wanted to determine if this generalization applied equally to nitrification in temperate waters. Because many organisms hydrolyze urea and release ammonium to the environment (Mobley *et al.*, 1995; Burton and Prosser, 2001; Solomon *et al.*, 2010), we performed experiments to determine whether N supplied as urea was released into the extracellular ammonium pool where it would be available to other organism, and we conducted competition experiments to determine whether ammonium affected oxidation of urea N and vice versa. Finally, we extracted DNA and RNA from these samples to investigate relationships between *amoA* and *ureC* gene abundance and transcription and oxidation rates of ammonia and urea-derived N.

Results and Discussion

Thaumarchaeota were the numerically dominant ammonia oxidizers (>50% of *amoA* genes) in 41 of the 44 samples we examined (Table S1). AOB *amoA* was more abundant than AOA *amoA* in surface waters at three stations in the South Atlantic Bight (SAB, Fig. S1) and SAB stations generally had higher AOB/AOA ratios than the rest of the dataset. However, the abundance of both groups of AOO and rates of urea and ammonia oxidation were near the limit of detection at most of these stations (Table S1).

Oxidation rates of ^{15}N-labeled ammonium differed among the 4 sites sampled (Fig. 1, Table 1; Kruskal-Wallis H test, $p < 0.0001$). Rates in nearshore samples from coastal Georgia (Fig. S1) in mid-summer were higher (mean ± SEM: 382 ± 35 nmol L^{-1} d^{-1}) than rates measured in SAB samples taken further offshore in April and

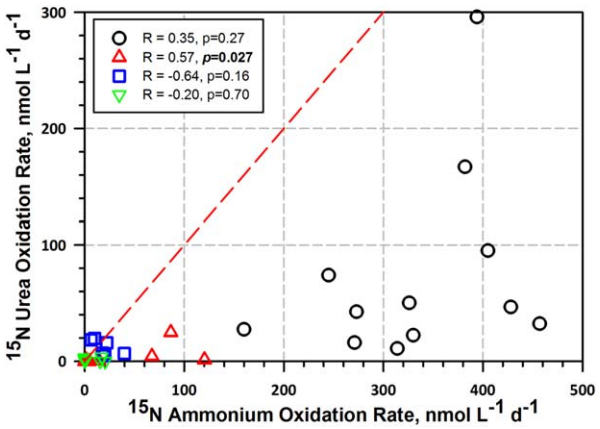

Fig. 1. Oxidation rates of ^{15}N-labeled ammonia and urea in samples taken from coastal Georgia (black circles), the South Atlantic Bight (red upward triangles), continental shelf waters west of the Antarctic Peninsula (blue squares) and the Gulf of Alaska (green downward triangles). Pearson Product moment correlation coefficients (R) for each sample set are given in the inset, statistically significant correlations ($p<0.05$) are indicated in **bold**. Rates that were at the Limit of Detection (LD; Table S1) are plotted as 0. The dashed red line has a slope of 1.

October (20.0 ± 8.8 nmol L^{-1} d^{-1}; Mann-Whitney U test $p < 0.001$; Fig. 1, Table 1, Table S1). Rates of ^{15}N-ammonia oxidation (19.4 ± 4.8 nmol L^{-1} d^{-1}) in samples from continental shelf and slope waters collected west of the Antarctic Peninsula (Fig. S1) during summer (January) were not different from those measured in the SAB (Mann-Whitney U test $p = 0.056$) or from the Gulf of Alaska (Mann-Whitney U test $p = 0.13$). The rates measured in Antarctic samples are comparable to those in a larger data set reported in Tolar *et al.*, (2016). Rates for the Gulf of Alaska were the lowest we observed, but were comparable to rates for the same region reported in Shiozaki *et al.*, (2016). When normalized to the abundance of Thaumarchaeota in these samples, ammonium oxidation rates in populations from coastal Georgia and the SAB were similar (rates of 8.6 ± 2.0 and 9.2 ± 3.4 fmol $cell^{-1}$ d^{-1}, respectively; Mann-Whitney U test $p = 0.58$; Table 1, Table S1) and in the range of maximum ammonium oxidation rates for Thaumarchaeota isolates reported in Qin *et al.*, (2014): 2.9-12.7 fmol $cell^{-1}$ d^{-1}. Mean rates for Antarctic and Gulf of Alaska samples were lower: 0.7 ± 0.3 and 1.5 ± 1.0 fmol $cell^{-1}$ d^{-1} (Table 1, Table S1).

We found that nitrogen supplied as urea was oxidized in most samples (i.e., $^{15}NO_x$ was produced from ^{15}N-labeled urea); however, we do not have measurements of urea concentrations for Antarctic samples or for some of the samples from Georgia coastal waters taken in 2012. We used average urea concentrations measured in other samples from the same water masses at these locations to calculate oxidation rates (discussed further in Methods). Further, urea and ammonia concentrations were so low in

Table 1. Summary of ^{15}N-labeled ammonia and urea oxidation rates.

				$^{15}NH_4^+$ Rate		^{15}N Urea Rate		
Location	Date	NH_4^+, μM	Urea[a], μM	nmol L^{-1} d^{-1}	fmol cel L^{-1} d^{-1}	nmol L^{-1} d^{-1}	fmol cel L^{-1} d^{-1}	Rate Ratios: Urea /NH_4^+
Coastal Georgia	8/2011	0.70 ± 0.13	0.81 ± 0.13	318 ± 34	12 ± 4.1	103 ± 38	3.6 ± 1.3	0.30 ± 0.09
	8/2012	1.96 ± 0.25	*0.37*	444 ± 55	6.9 ± 2.0	*29 ± 6.7*	*0.3 ± 0.1*	*0.08 ± .01*
	All samples	1.41 ± 0.22	0.56 ± 0.08	389 ± 37	9.1 ± 2.1	76 ± 26	2.4 ± 0.9	0.2 ± 0.06
South Atlantic Bight	4/2011	1.03 ± 0.39	0.16 ± 0.04	11 ± 9.5	11 ± 7.3	LD[b]	LD[b]	LD[b]
	10/2011	0.72 ± 0.20	0.62 ± 0.18	28 ± 17	7.7 ± 3.6	3.5 ± 3.0	5.6 ± 4.8	0.3 ± 0.1
	All Samples	0.86 ± 0.20	0.42 ± 0.12	20 ± 10	9.2 ± 3.8	2.2 ± 1.6	3.0 ± 2.6	0.2 ± 0.1
Antarctic	1/2011	1.16 ± 0.34	*0.38*	19 ± 4.8	0.7 ± 0.3	*12 ± 2.7*	*4.8 ± 1.6*	*1.1 ± 0.5*
Gulf of Alaska	8/2013	0.11 ± 0.06	0.13 ± 0.02	13.2 ± 4.5	2.3 ± 1.5	1.8 ± 0.69	0.11 ± 0.04	0.7 ± 0.6

Values are means ± SEM of the data for a given set of samples. See Table S1 for individual measurements.
[a]Urea concentrations in italics (and rates based on them) were estimated from concentrations measured in other samples taken from the same water masses (see Table S3).
[b]Most of the oxidation rates of ^{15}N-labeled urea were at the limit of detection on the April 2011 South Atlantic Bight cruise (see Table S1).

some samples that some of the amendments (50 nM of ^{15}N-labeled substrate) were not at tracer level. With these caveats, oxidation of ^{15}N supplied as urea was generally lower than ammonia oxidation rates in the same sample (Fig. 1, Table 1), and rates were below the limit of detection in many samples (Table S1). Oxidation of ^{15}N supplied as urea also differed among the 4 sites sampled (Fig. 1, Table 1; Kruskal-Wallis H test, $p < 0.0001$). Oxidation rates of ^{15}N-labeled urea were much higher in samples of Georgia coastal waters (73 ± 24 nmol L^{-1} d^{-1}) than measured in samples from the SAB (2.2 ± 1.7 nmol L^{-1} d^{-1}; Mann-Whitney U test $p < 0.0001$; Table 1, Table S1); however, rates for many of the surface water samples from the SAB were at the limit of detection. Oxidation of ^{15}N supplied as urea was faster in Antarctic samples (12 ± 2.7 nmol L^{-1} d^{-1}) than in samples from the SAB or the Gulf of Alaska (1.5 ± 0.6 nmol L^{-1} d^{-1}; Mann-Whitney U test $p = 0.0051$). Cell-specific rates of ^{15}N-labeled urea oxidation also differed significantly among sampling sites (Table 1; Kruskal-Wallis H test, $p = 0.0004$) with rates of 2.6 ± 0.9, 3.0 ± 2.3, 4.8 ± 1.6 and 0.09 ± 0.04 fmol urea-N oxidized $cell^{-1}$ d^{-1} in samples from coastal Georgia, the SAB, Antarctic continental shelf waters and the Gulf of Alaska, respectively.

Cell-specific rates of ^{15}N-labeled urea oxidation in Antarctic samples were higher on average in Winter Water (WW, 50-100 m; 8.2 ± 1.2 fmol $cell^{-1}$ d^{-1}) versus the Circumpolar Deep Water (CDW, 150-400 m; 1.4 ± 0.5 fmol $cell^{-1}$ d^{-1}; $p = 0.007$, Student's t-test; Table S1), while there was no significant difference in cell-specific ammonia oxidation rates between these water masses (0.7 ± 0.6 vs. 0.6 ± 0.3 fmol $cell^{-1}$ d^{-1}, respectively; t-test, $p = 0.84$; Table S1). This difference in cell-specific activities coincides with a shift in the phylogenetic composition of Thaumarchaeota populations in WW versus CDW shown in Figure 5 of Tolar *et al.*, (2016), similar to the distributions for Antarctic coastal waters shown in Figures 1 and 2 of Kalanetra *et al.*, (2009) and Figure 4 of Alonso-Sáez *et al.*, (2011). A single thaumarchaeote phylotype dominated (~98% of sequences) the WW communities sampled on these cruises, while CDW communities were more diverse. This suggests that the dominant WW phylotype oxidizes N from both urea and ammonia, while phylotypes found in CDW populations (which include a significant portion of the dominant WW phylotype), may preferentially oxidize ammonia.

Ratios of the rate of ^{15}N-labeled urea oxidation versus ^{15}N-labeled ammonium oxidation differed among the 4 sites sampled (Fig. 1, Table 1; Kruskal-Wallis H test, $p = 0.0026$). Ratios were similar for Georgia coastal and SAB stations (0.2 ± 0.06 and 0.1 ± 0.06, respectively; however, urea oxidation rates were at the limit of detection for many SAB samples. Ratios for samples from Antarctic continental shelf waters were 1.1 ± 0.5 and were significantly greater than for samples from coastal Georgia and the SAB combined (0.2 ± 0.05; Mann-Whitney U test, $p = 0.0027$) or the Gulf of Alaska (0.35 ± 0.30, Mann-Whitney U test $p = 0.046$).

We determined the correlations between oxidation rates of ^{15}N supplied as ammonium or urea and *in situ* concentrations of ammonia and urea. We used a reduced data set for this analysis that excluded urea concentrations estimated from regional averages and that assigned values of 0 to analyses that were at the limit of detection. We found that ammonia oxidation rates correlated with both ammonium and urea concentrations when all data were considered together (ammonia: Fig. 2a, Pearson's $R = 0.48$, $n = 44$, $p = 0.00096$; urea: Fig. 2b, $R = 0.50$, $n = 29$, $p = 0.0057$); however, the relationship was not always statistically significant when subsets by location were considered separately (Figs. 2a,b). Oxidation of ^{15}N supplied as urea was not correlated with

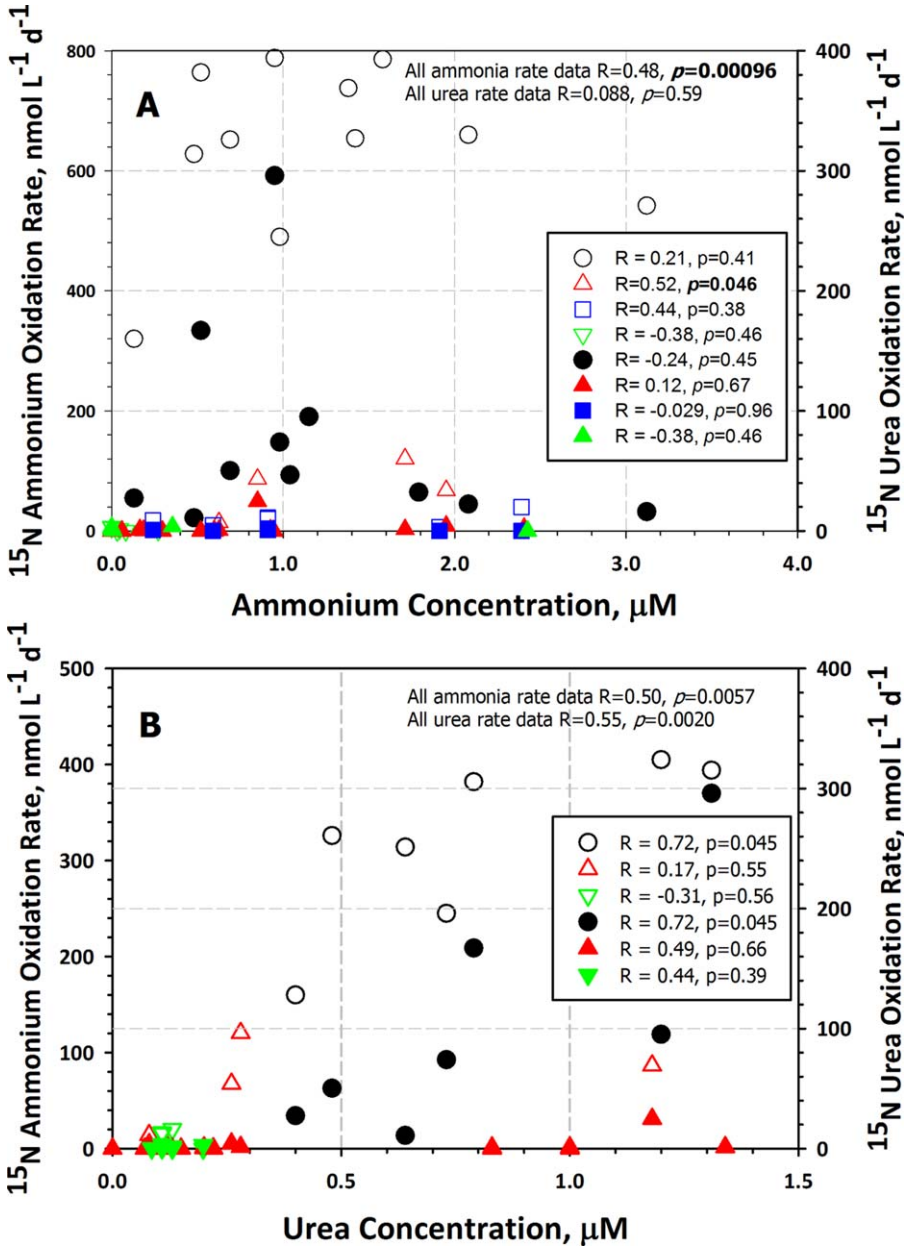

Fig. 2. A) Oxidation rates of ^{15}N-labeled ammonium (open symbols) or urea (filled symbols) versus ammonium concentration in the same sample. B) Oxidation rate rates of ^{15}N supplied as ammonium or urea versus urea concentration in the same sample. Symbols are as in Figure 1. Rates and concentrations that were at the limit of detection (Table S1) are plotted as 0. Insets give Pearson's R for each sample subset, statistically significant correlations ($p<0.05$) are indicated in **bold**. Values derived from experiments where urea concentrations were estimated from averages are shown on plot B), but were omitted from the statistical analysis.

ammonium concentration (Fig. 2a; $R = 0.088$, $n = 34$, $p = 0.59$) when all data were considered together; however, the correlation with urea concentration was significant (Fig. 2b; $R = 0.55$, $n = 29$, $p = 0.0020$). The correlation was only significant ($p = 0.045$) for the subset of samples from coastal Georgia (Fig. 2b). In particular, oxidation rates of ^{15}N supplied as urea were not higher in samples with low ammonium concentrations (Fig. 3; $R = -0.012$, $p = 0.45$), though none of the environments we sampled are likely to experience prolonged ammonium depletion. There was no clear trend in the oxidation of ^{15}N supplied as ammonia or urea with tide or time of day during diurnal studies of Georgia coastal waters in any of the years sampled, and rates were of the same order of magnitude in all years (Table S1).

Ratios of the abundances of Thaumarchaeota *ureC* to Thaumarchaeota *amoA* and of Thaumarchaeota *ureC* to Thaumarchaeota 16S rRNA (*rrs*) genes differed significantly among sets of samples (Fig. 4, S1; Kruskal-Wallis H test, $p < 0.0001$ and $p = 0.0011$, respectively). *ureC* genes are not abundant in Thaumarchaeota populations from Georgia coastal waters, (ratios of *ureC* to *amoA* or *rrs* genes averaged 0.16 ± 0.03 and 0.23 ± 0.04, respectively), in contrast to larger ratios we found in populations from Antarctic samples (0.8 ± 0.28 and 1.41 ± 0.39, respectively; Fig. 4, Table S1). Three SAB samples taken at depth at

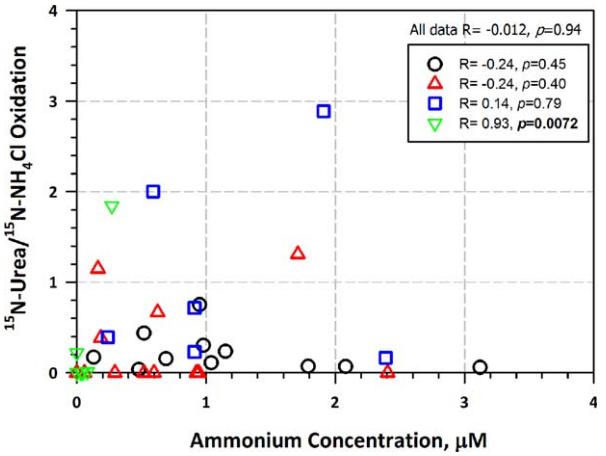

Fig. 3. Ratio of the oxidation rates of ^{15}N-labeled urea to ^{15}NH4Cl versus ammonia. Symbols as in Figure 1. Insets give Pearson's R for each subset of samples, statistically significant correlations ($p<0.05$) are indicated in **bold**.

shelf-break stations had high ratios (Fig. 4, Table S1). The phylogenetic composition of the Thaumarchaeota population in these samples was distinct from that of the population found in other SAB samples or in Georgia coastal waters (Liu *et al.*, unpublished data). The mean ratios of Thaumarchaeota *ureC* /*amoA* and *ureC* /*rrs* genes from Antarctic samples reported here were not significantly different from the mean of a larger data set from the same cruise reported in Tolar *et al.*, (2016) ($n = 71$; 1.99 ± 0.30 and 1.25 ± 0.19, respectively; Mann-Whitney U test, $p = 0.17$ and $p = 0.26$). The ratios for *ureC* /*amoA* and *ureC* /*rrs* in the larger data set are both significantly greater than the means obtained for the coastal Georgia samples reported here (Mann-Whitney U tests, $p = 0.0036$ and $p < 0.0001$, respectively).

The differences in the ratios of Thaumarchaeota *ureC* to *amoA* and *ureC* to *rrs* between coastal Georgia and Antarctic samples were statistically significant (Mann-Whitney U tests, $p = 0.028$ and $p = 0.0036$, respectively). We were concerned that our results from coastal Georgia might be influenced by using primers that were developed by Alonso-Sáez *et al.*, (2012) for use with polar populations (see their Supporting Information Fig. S8); however, metatranscriptomes from Georgia coastal samples (Gifford *et al.*, 2011; Gifford *et al.*, 2013) also indicated low transcription of *ureC* and other Thaumarchaeota genes related to urea utilization (transporters, urease subunits and accessory proteins; Hollibaugh *et al.*, 2011; Hollibaugh *et al.*, 2014). Our initial analysis used BLASTx of metatranscriptome sequences against the Genbank RefSeq protein database to assign reads to genes. At the time we performed this analysis, the only Thaumarchaeota genomes included in RefSeq were from Cenarchaeum symbiosum A, "*Ca.* N. maritimus" and "*Ca.* Nitrosoarchaeum limnia". Of these, only C. symbiosum A contains genes for urease. We performed a limited re-analysis of these metatranscriptomes using custom databases (Table S2) containing Thaumarchaeota *ureC* and *amoA* sequences that were not available when the original analysis was performed.

The re-analysis recruited many putative Thaumarchaeota *ureC* sequences that were subsequently discarded as not being from Archaea (7.6% retained, Table 2); however, this was not a problem with putative Thaumarchaeota *amoA* reads (99.9% retained, Table 2). Most of the reads that were retained recruited to Nitrosopumilus strain AR2, to a Thaumarchaeota single cell genome and to Global Ocean Survey metagenomic sequences (Table S3). It is conceivable that there are divergent Thaumarchaeota urease genes in our samples that were not detected by qPCR using the Alonso-Sáez *et al.*, (2012) primer set; however, it seems unlikely that transcripts of these genes would not be found in the metatranscriptomes, which were generated using random hexamer primers. The results of the reanalysis support the conclusion reached in our initial analysis of the metatranscriptomes that Thaumarchaeota *ureC* transcripts occurred infrequently relative to *amoA* transcripts (Table 2). We conclude that the differences between sites in the ratio of Thaumarchaeota *ureC* /*amoA* genes we detected by qPCR was not due to primer bias, but rather reflects a lower frequency of Thaumarchaeota *ureC* genes in the coastal Georgia population.

Mean ratios of both *ureC* /*amoA* and *ureC* /*rrs* genes found in SAB and Gulf of Alaska samples (Table S1) were greater than those from the other sites. The SAB ratios were driven by extreme values in samples where very few Thaumarchaeota were detected. These ratios are unreasonably high (7.2 ± 3.8, range 0.12-58 and 9.6 ± 3.6 range 0.5-46 for the SAB; 8.8 ± 1.7, range 4.3-14.1 and 3.5 ± 0.9, range 0.6-5.9 for the Gulf of Alaska), given the *ureC* copy number expected for Thaumarchaeota genomes (1-2). We speculate that they are due to nonspecific amplification of urease genes from organisms other than Thaumarchaeota, combined with low abundance of Thaumarchaeota *amoA* and *rrs* genes in these samples. Melting curves of *ureC* qPCR amplicons gave no indication of multiple products; however, *in silico* tests we performed indicated that the main non-Thaumarchaeota sequences that might have yielded PCR products with the Alonso-Sáez *et al.*, (2012) primers are urease genes from marine group II Euryarchaeota, which are abundant in the Arctic Ocean (Galand *et al.*, 2009) and can be relatively abundant in continental shelf waters of the SAB (Liu *et al.*, unpublished data), but were not found in the initial analyses of metatranscriptomes from Marsh Landing (Gifford *et al.*, 2011; Gifford *et al.*, 2013). Seven of the 108 *ureC* sequences retrieved during our re-analysis of these

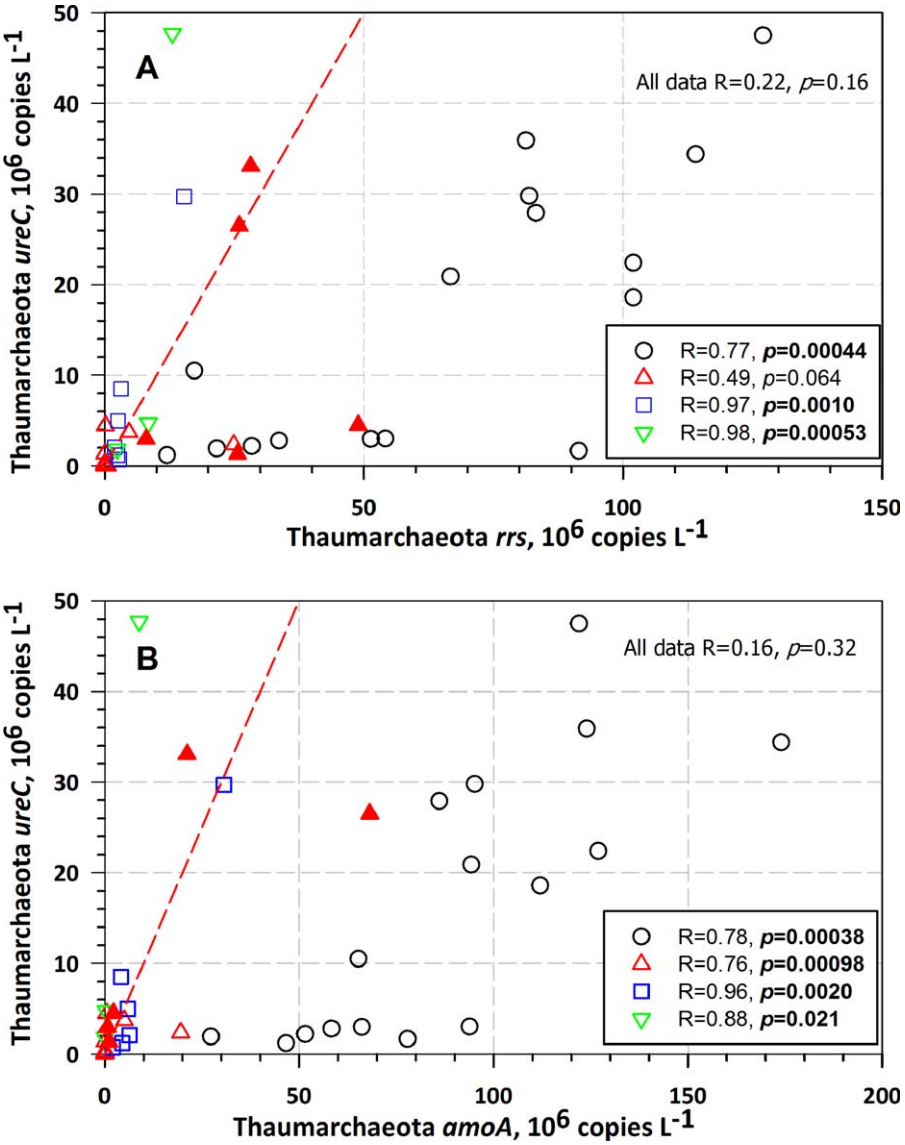

Fig. 4. Relationship between the abundance of Thaumarchaeota *ureC* genes and the abundance of A) Thaumarchaeota 16S rRNA genes; and B) Thaumarchaeota *amoA* genes. Symbols as in Figure 1; insets give Pearson's R for each subset of samples, statistically significant correlations ($p<0.05$) are indicated in **bold**. The dashed red lines have slopes of 1.

Table 2. Abundance of sequences identified as Thaumarchaeota *ureC* and *amoA* in metatranscriptomes from Georgia coastal waters.

Library[a]	Sample Date	Number of Reads Queried	Number of verified Thaum *ureC* Hits[b]	Number of verified Thaum *amoA* Hits[c]	Ratio, *ureC* /*amoA* hits[d]
FN56_FN57	8/6/2008	1,080,736	4	859	0.0047
FN66	8/7/2008	3,174,161	9	5,466	0.0016
FN71	8/7/2008	6,113,442	25	16,028	0.0016
FN140	8/12/2009	2,513,708	16	637	0.0251
FN146A	8/13/2009	2,270,525	11	691	0.0159
FN146B	8/13/2009	2,366,038	13	785	0.0166
FN151	8/14/2009	3,025,727	17	428	0.0400
FN158	8/14/2009	3,899,029	13	1,301	0.0100

[a]Libraries described in Gifford *et al.* (2011; 2013) and Hollibaugh *et al.* (2011, 2014). Libraries FN56 and FN57 were sequenced by GS-FLX 454 pyrosequencing and were combined for this analysis, the remainder were sequenced by Illumina HiSeq.
[b]Metatranscriptomic reads were identified as *ureC* by BLASTn against a custom database (Table S2) and retained (7.6%) after verifying hit identity (regardless of taxonomic affiliation) by BLASTx against the RefSeq protein database.
[c]As above for Archaea *amoA*, 99.9% of reads were retained.
[d]Ratio of verified hits.

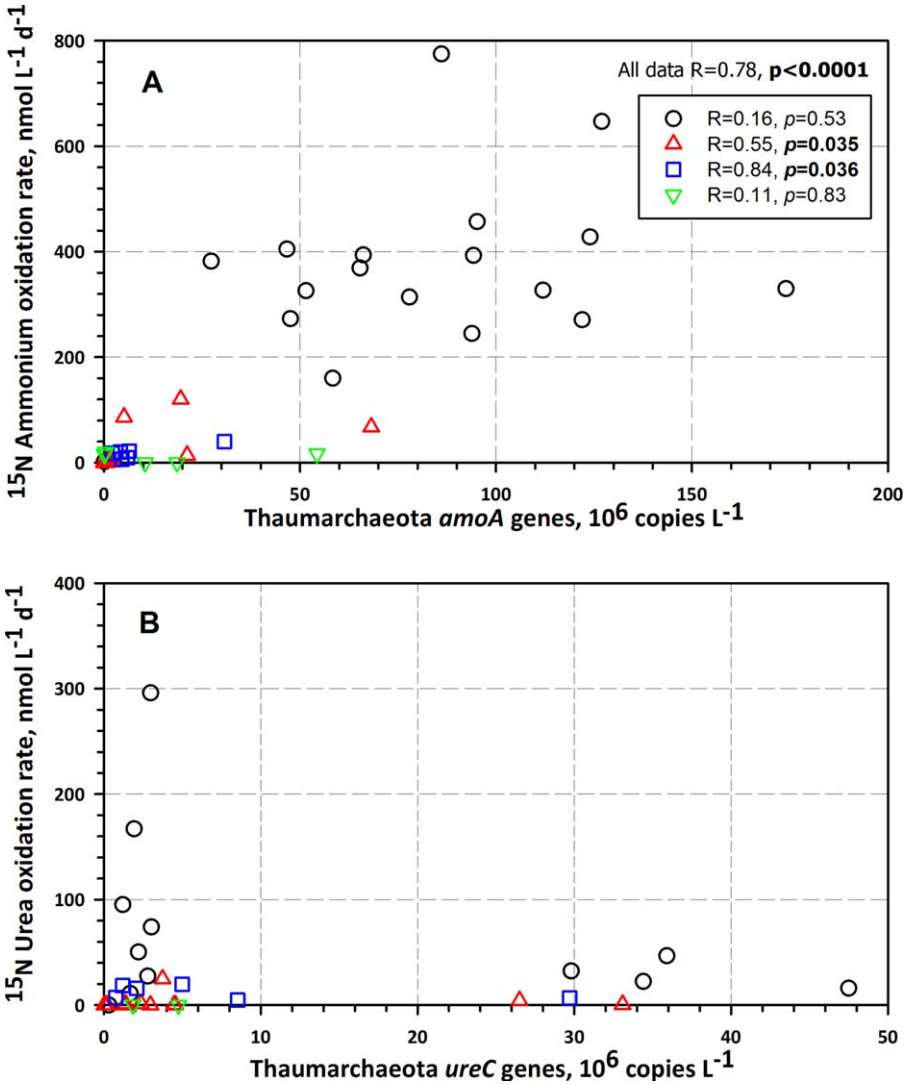

Fig. 5. Relationship between A) oxidation of ^{15}N supplied as ammonium and Thaumarchaeota *amoA* gene abundance; and B) oxidation of ^{15}N supplied as urea and Thaumarchaeota *ureC* gene abundance. Symbols as in Figure 1, inset gives Pearson's R for each subset of samples. Statistically significant correlations ($p<0.05$) are indicated in **bold** (not shown for panel B because none of the correlations were significant). Urea oxidation rates that were at the Limit of Detection (LD; Table S1) are plotted as 0.

metatranscriptomes were most closely affiliated with *ureC* genes from two marine group II Euryarchaeota single-cell genomes (Table S3).

We found no significant correlations between ammonia oxidation rate and *amoA* gene abundance, or between oxidation rates of ^{15}N supplied as urea and *ureC* gene abundance (Fig. 5a-b). We found no correlation between oxidation rates of ^{15}N supplied as ammonium or urea and *amoA* or *ureC* transcript abundance or transcript:-gene ratios, or between cell-specific oxidation rates and transcript:gene ratios (Fig. S2a-f). The lack of statistically significant correlations could result from errors in the measurements, including specificity of the Alonso-Sáez *et al.*, (2012) *ureC* primer set, or to inherently poor coupling between gene transcription and activity.

The distribution by sampling site of relative rates of urea versus ammonia oxidation we measured indicates that the contribution of urea to nitrification may be important in polar oceans, as suggested by Alonso-Sáez *et al.*, (2012). In contrast, urea nitrogen does not appear to contribute significantly to nitrification in the subtropical environments we studied. The basis for this difference in the ecophysiology of Thaumarchaeota is not clear, but our data suggest that it is not related to ammonia availability (Fig. 2a). pH, which has been shown to affect urea use by soil ammonia-oxidizers through its influence on the availability of free ammonia (Burton and Prosser, 2001; Pommerening-Röser and Koops, 2005; Nicol *et al.*, 2008; Gubry-Rangin *et al.*, 2010; Lehtovirta-Morley *et al.*, 2011) seems unlikely to explain the difference, as pH is lower (7.2-8.0; Hollibaugh *et al.*, 2014; Sapelo Island National Estuarine Research Reserve data archived at http://cdmo.baruch.sc.edu/get/export.cfm) in strongly heterotrophic Georgia coastal waters (Wang and Cai, 2004; Wang *et al.*, 2005; Cai *et al.*, 2011) than in polar oceans (Antarctic: 8.05-8.16, McNeil and Matear, 2008; Arctic: 8.03-8.22, Robbins *et al.*, 2013). Field data indicate growth (Gubry-Rangin *et al.*, 2010) and urea oxidation (Lu *et al.*, 2012) by thaumarchaeotes in

Table 3. Effect of unlabeled ammonium or urea amendments on the oxidation of ^{15}N-labeled ammonium or urea in samples from coastal Georgia

	Competitor		
Substrate	Ammonia, 6 μM	Urea, 12 μM	No Addition
^{15}N-labeled ammonium, 50 nM	8,428 ± 214	1,778 ± 26	3,640 ± 442
^{15}N-labeled urea, 50 nM	245 ± 83	483 ± 38	277 ± 20

Data are mean ± SEM (triplicate incubations) of the oxidation rates of the total ammonium or urea pools, reported as nmol L^{-1} d^{-1}. Ammonium and urea concentrations in the "No Additions" controls were 2.90 and 1.14 μM, respectively.

acidic soils and Qin *et al*., (2014) and Lehtovirta-Morley *et al*., (2011) have shown that cultures of some ammonia-oxidizing thaumarchaeotes sustain maximal growth rates on ammonium at pH ≤7. Ammonium concentrations in our data set were similar between Georgia coastal waters and the Antarctic (Mann-Whitney U test $p = 0.38$); however, the lower pH of Georgia coastal waters should make ammonia less available there than in the Antarctic, in contrast to the distribution of *ureC* genes and ratios of oxidation rates. Ammonium concentrations at both locations are likely greater than those that limit thaumarchaeote growth (Martens-Habbena *et al*., 2009), and ammonium concentrations are likely variable at all of the sites we sampled. There appears to be a cost to Thaumarchaeota for growth on urea as cell yields are lower than for growth on ammonia (Table S4), possibly due to the additional metabolic machinery required to use urea and/or to loss of a significant fraction of the ammonia produced by urea hydrolysis to the environment (see below), suggesting that the ability to grow on urea is only advantageous under conditions of chronically low ammonia concentrations. It is also possible that these putative ureases serve another, or additional, function, as ureases (urea amidohydrolase; EC 3.5.1.5) are members of the amidohydrolase superfamily that has broad hydrolytic function (Seibert and Raushel, 2005) and Thaumarchaeota sequences diverge from more fully characterized Bacteria ureases (Yakimov *et al*., 2011).

Urea hydrolysis by AOB is thought to occur intracellularly, though Burton and Prosser, (2001) have shown that ammonia "leaked" from cells during growth of an AOB on urea at low pH. We examined the contribution of urea hydrolysis to the ammonium pool by measuring production of ^{15}N-ammonium in samples amended with ^{15}N-labeled urea. We recovered 19% and 21% of the ^{15}N-label from urea as ^{15}N-labeled ammonium in experiments conducted with samples from coastal Georgia (sample ML-13 2F) and the Gulf of Alaska (200 m at Station 033, Table S1), respectively. We explored the mechanism of urea utilization further by comparing oxidation of ^{15}N supplied as ammonium or urea in treatments amended with unlabeled ammonium or urea (Table 3). We found that an ammonium amendment had no effect on the oxidation of ^{15}N-labeled urea relative to an unamended control (two-tailed t-test, $p = 0.70$), while an amendment of unlabeled urea reduced the rate of ^{15}N-labeled ammonia oxidation 51% (two-tailed t-test, $p = 0.014$). Oxidation rates of ^{15}N-labeled ammonium and urea increased by 2.3- and 1.7-fold in response to the addition of unlabeled ammonium and urea, respectively (Table 3). This pattern suggests that both ammonia and urea-derived N are oxidized intracellularly by Thaumarchaeota, as is the case for AOB (Burton and Prosser, 2001).

Ureases are produced by many organisms (Mobley and Hausinger, 1989; Solomon *et al*., 2010), so if ammonia is released to the environment, urease activity need not be specifically from Thaumarchaeota in order to supply the ammonia they need for energy generation. Recently, *Nitrospira moscoviensis*, a representative of a ubiquitous group of nitrite-oxidizing bacteria, has been shown to produce urease and Koch *et al*., (2015) have proposed a reciprocal feeding model wherein urease-positive *Nitrospira* hydrolyze urea to produce ammonium that is subsequently oxidized by AOO to supply the nitrite oxidized by *Nitrospira*. *Nitrospina* is an important nitrite-oxidizing bacteria in the sea (Mincer *et al*., 2007) and single cell genome sequences from 3 marine *Nitrospina* sp. (*Nitrospina* sp. SCGC AAA288-L16, *Nitrospina* sp. AB-629-B06, *Nitrospina* sp. AB-629-B18) also contain genes annotated for urease production. Mincer *et al*., (2007) report that the abundance of *Nitrospina rrs* paralleled that of Thaumarchaeota *rrs* and *amoA* genes in samples from Monterey Bay and the North Pacific Subtropical Gyre and that *Nitrospina* and Thaumarchaeota were of comparable abundance. Santoro *et al*., (2010) reported that the abundance of *Nitrospina rrs* genes in the central California current correlated with AOA *amoA* and Thaumarchaeota *rrs* genes ($R^2 = 0.65$ and 0.69, respectively) and average 25% of their abundance. We have measured the abundance of *Nitrospina* in samples from the Georgia coast ($n = 166$, Hollibaugh unpublished data) and the Antarctic ($n = 69$; Tolar *et al*., 2016). The relative abundance of *Nitrospina rrs* compared to Thaumarchaeota *rrs* was 0.42 ± 0.020 in samples from coastal Georgia versus 0.18 ± 0.056 in our Antarctic samples. The difference was statistically significant (Student's t-test, $p = 0.0058$). Although many factors can contribute to the dynamics of nitrifier communities, the difference in relative abundance of *Nitrospina* between these sites and

lower thaumarchaeote *ureC* /*amoA* or *ureC* /*rrs* ratios is consistent with potentially greater reliance by AOO on ureases supplied by other organisms in Georgia coastal waters than in the Antarctic.

In summary, we have shown that nitrogen supplied as urea is oxidized in populations of pelagic marine nitrifiers dominated by Thaumarchaeota (Fig. 1). However, the contribution of urea to nitrification was generally less than that of ammonium (Fig. 1, Table 1). We found no significant correlation between urea oxidation rates and ammonium concentration (Figs. 2a, Fig. 3), suggesting that selection for the ability to oxidize urea is independent of ammonia availability, at least at the ammonium concentrations we encountered. We found weak relationships, at best, between oxidation of urea N and *ureC* gene or transcript abundance, or between rates and ratios of transcripts: gene. The ability of Thaumarchaeota to use urea and their preference for it as an alternate substrate appears variable, highly dependent on sample location and is likely determined by the ecotypes present. The exact mechanism by which urea is used by Thaumarchaeota is unclear, but the appearance of labeled ammonium during incubations and the lack of an inhibitory effect of unlabeled ammonium on oxidation of urea-derived ^{15}N (Table 3) suggests that urea is hydrolyzed and oxidized intracellularly. Some of the ammonium derived from urea is lost during processing and appears in the medium where it is presumably taken up by other organisms or oxidized as part of the extracellular ammonium pool. Urea-derived ammonium that is lost to the environment thus contributes to ammonium regeneration and indirectly to nitrification. Depending on the portion of intracellular ammonium that is lost to the extracellular pool, ammonium regenerated by urea hydrolysis may be a source of significant isotope dilution in ^{15}N-ammonia oxidation rate measurements, particularly in regions of active urea cycling.

Methods

Sample collection

We sampled a variety of marine environments to compare rates of ammonia and urea oxidation (Fig. S1, Table S1). Surface water (~0.2 m) samples were collected into opaque plastic bottles (Nalge) at Marsh Landing on Sapelo Island, Georgia, USA from the Duplin River—a tidal creek dominated by salt marshes ("coastal Georgia," Fig. S1)—in August 2011 and 2012, and September 2013, during the annual peaks of Thaumarchaeota abundance (Hollibaugh *et al*., 2014). The competition experiment was performed with a sample of surface water collected from a dock on Lighthouse Creek, another tidal creek adjacent to the University of Georgia Marine Institute on Sapelo Island, Georgia (31.396316°, −81.280044°) on 25 July 2015. The South Atlantic Bight ("SAB," Fig. S1) was sampled using Niskin bottles from the *R/V Savannah* in April and October of 2011. Antarctic experiments were performed with samples from the Winter Water (WW, 70-100 m) and Circumpolar Deep Water (CDW, 350-400 m) water masses of the Southern Ocean west of the Antarctic Peninsula (Fig. S1) collected using Niskin bottles from the *ARSV Laurence M. Gould* on cruise LMG 11-01 in January 2011 (Tolar *et al*., 2016; Table S1). Finally, we sampled the Gulf of Alaska (Fig. S1, Table S1) from the *R/V Melville* cruise MV1310 in August, 2013.

Seawater from each site was filtered onto 0.22 μm pore size, 47 mm diameter GVWP Durapore® filters (EMD Millipore) and frozen at −80°C for subsequent DNA and RNA extraction. Fifty or 100 mL of the filtrate was frozen at −80°C in new 50 mL polypropylene tubes (VWR) for subsequent nutrient analysis.

DNA and RNA extraction and quantification

DNA was extracted from frozen Durapore filters using lysozyme, proteinase K and SDS, followed by purification as described previously (Ferrari and Hollibaugh, 1999; Bano and Hollibaugh, 2000). Marine group I Archaea (Thaumarchaeota) 16S rRNA (*rrs*), *Nitrospina rrs*, betaproteobacterial *amoA* (AOB) and archaeal *amoA* (AOA) genes were quantified using primers (Table S5) and protocols described previously (Mincer *et al*., 2007; Kalanetra *et al*., 2009; Tolar *et al*., 2013) with an iCycler iQ™ Real-Time qPCR detection system (BioRad). Archaeal *ureC* genes were quantified under the same conditions as *amoA* with an annealing temperature of 53°C (Alonso-Sáez *et al*., 2012). RNA was extracted from filters as described previously (Gifford *et al*., 2011) using 200 μm zirconium beads (OPS Diagnostics) for the initial bead-beating step. We used the TURBO DNase-Free Kit® (Ambion) to remove DNA after extraction as per the manufacturer's instructions, except that we added a second enzyme treatment at 2X concentration. Thaumarchaeota *amoA* and *ureC* transcripts were quantified with the iScript™ One-Step RT-qPCR Kit® with SYBR Green (BioRad). RT-qPCR assay for transcripts used the same cycling conditions as the qPCR assay for the respective gene, with the addition of a 10 minute reverse transcription step at 50°C before the initial denaturation step.

Re-analysis of marsh landing metatranscriptomes

The Marsh Landing metatranscriptomes that we re-analyzed here are described in detail in Gifford *et al*., (2011; 2013) and Hollibaugh *et al*., (2011; 2014). Our re-analysis involved searching the metatranscriptomes for homologues of *ureC* and *amoA* gene sequences from Thaumarchaeota and marine group II Euryarchaeota using target sequences from isolates, from genomes assembled by amplification of DNA from single cells, or from large insert clone libraries. These target sequences are given in Table S2. Sequences of verified transcripts from the Marsh Landing metatranscriptomes (i.e., no rRNA or internal standards; library information is given in Table 2 and Table S3) were compared to the sequences in our custom database using BLASTn. Hits with bit scores >40 were retained and compared to sequences in the Genbank RefSeq protein sequence database using BLASTx. Sequences with hits to *amoA* or *ureC* (regardless of taxonomic affiliation) with bit scores >40 were accepted as verified

transcripts of archaeal *amoA* (99.9% of the putative archaeal *amoA* transcripts identified in the initial BLASTn search) or *ureC* (7.6% of the putative archaeal *ureC* transcripts). Libraries FN56 and FN57 were combined for these analyses because they were small libraries from the same sampling period (August, 2008) and were obtained using a GS-FLX 454 pyrosequencer, while the others were run on an Illumina HiSeq sequencer. Table 2 presents a summary of these results with details of hit recruitment given in Table S3.

Nutrient analysis

Concentrations of dissolved inorganic nitrogen and urea (Table S1) were measured using standard methods (ammonia: Solórzano, 1969, Holmes *et al.*, 1999; nitrite and nitrite + nitrate: Strickland and Parsons, 1972, Jones, 1984; and urea: Rahmatullah and Boyde, 1980, Mulvenna and Savidge, 1992). Urea concentrations were not available for samples used in experiments from Antarctic continental shelf waters or for the August 2012 coastal Georgia data set. We used an average of values for samples collected from the same water masses at other stations on the Antarctic continental shelf and an average of the urea concentrations measured approximately weekly from 5/2013 to 9/2015 at Marsh Landing (Table S6) in the calculations for these experiments (values shown in italics in Table S1).

Rate measurements

Samples were amended with 50 nM ^{15}N-labeled ammonium chloride ($^{15}NH_4Cl$; >99 at-% ^{15}N, Cambridge Isotope Laboratories) or urea ($^{13}CO(^{15}NH_2)_2$; >98 at-% ^{15}N, Cambridge Isotope Laboratories). Duplicate or triplicate samples were incubated in 50 mL screw-cap, conical polypropylene tubes or 250 mL polycarbonate bottles. Samples were incubated at *in situ* temperatures in the dark for 24 hours (coastal Georgia, SAB) 124-139 hours (Antarctic), or 48 hr (Gulf of Alaska); (Table S1). Incubations were terminated by freezing at −80°C and samples were kept frozen until analysis. Controls consisted of samples frozen immediately after adding ^{15}N-labeled substrates or sample water that had been filtered through 0.22 μm pore size GVWP filters (EMD Millipore®) prior to substrate addition.

The amount of ^{15}N oxidized to nitrite + nitrate (NO_x) was measured by the denitrifier method (Sigman *et al.*, 2001) as described previously (Popp *et al.*, 1995; Dore *et al.*, 1998; Beman *et al.*, 2011) using a Finnigan MAT-252 isotope ratio mass spectrometer. Ammonia or urea oxidation rates were calculated from δ^{15}N values as described previously (Christman *et al.*, 2011; Beman *et al.*, 2012). *In situ* urea and ammonium concentrations that were at the limit of detection (~50 nM) were set to 0 nM for these calculations and urea concentrations were multiplied by 2 moles of ^{15}N added per mole of urea. Formation of $^{15}NO_x$ was negligible in filtered controls or in samples taken immediately after substrate addition, and no $^{15}N_2O$ was formed when ^{15}N-labeled substrates were added directly to denitrifier cultures of *Pseudomonas aureofaciens*. Cell specific rates were calculated by dividing measured rates by Thaumarchaeota abundance, calculated from 16S rRNA gene abundance assuming one 16S rRNA gene copy per Thaumarchaeota genome as described previously (Kalanetra *et al.*, 2009; Tolar *et al.*, 2013).

We performed an analysis of the sensitivity of ^{15}N oxidation rate calculations to *in situ* urea concentrations. We calculated ^{15}N-labeled urea oxidation rates from $^{15}NO_x$ production rates using *in situ* urea concentrations set to 0 μM (thus a 50 nM addition of ^{15}N-labeled urea yields a potential rate) or 1.0 μM, then compared these to rates calculated using our estimates of *in situ* concentrations (Table S7). We also calculated the ratio of ^{15}N-urea oxidation to ^{15}N-NH_4^+ oxidation in these 3 sets of samples to see how our assumption affected conclusions about the relative importance of urea versus ammonium as N sources for AOO in Georgia coastal waters versus Antarctic continental shelf waters.

Oxidation rates of ^{15}N supplied as urea and calculated assuming that *in situ* concentrations of urea were 0 were lower than rates calculated using mean urea concentrations from each environment; however, rates in the subset of samples from Georgia coastal waters were still greater than for samples from the Antarctic continental shelf (two-tailed t-test, $p = 0.033$). The ratio of oxidation rates of ^{15}N-labeled urea versus $^{15}NH_4Cl$ was not significantly different between these two data sets (two-tailed t-test, $p = 0.095$). When ^{15}N oxidation rates were calculated assuming 1.0 μM of exogenous urea, rates in the subset from Georgia coastal waters were still greater than those from the Antarctic continental shelf (two-tailed t-test $p = 0.035$), and the mean ratios of oxidation rates were not significantly different (two-tailed t-test $p = 0.095$). These comparisons were made with small subsets of the samples from Georgia coastal waters. It is unlikely that there is no urea in either of these environments (Table S6); however, uncertainty in the exact concentration of urea in the samples used for rate measurements constrains interpretation of these data. We have retained them in this comparison as the Antarctic is a difficult environment to access and, as far as we know, these are the only measurements of urea oxidation to have been made there.

Contribution of urea hydrolysis to ammonium pools

We used a modified version of the ammonia diffusion method (Holmes *et al.*, 1998) to measure ^{15}N-NH_3 in treatments amended with ^{15}N-labeled urea. We have performed this experiment several times with similar results, the experiments reported here were conducted with surface water samples from Marsh Landing taken on September 1, 2013 and from 200 m on August 22, 2013 at Station 033 off Vancouver Island on *R/V Melville* cruise MV1310 (Figure S1). We used 200 mL samples amended as necessary with carrier NH_4Cl of known isotopic composition. An ammonia trap (filter pack containing combusted, acidified 1 cm Whatman GF/D filters sealed between two 2.5 cm, 10 μm pore size Teflon membranes) was added to each sample, followed by addition of 1.5 g MgO to stop microbial activity and to convert all NH_4^+ to NH_3. Samples were incubated for 4 weeks with shaking at 40°C to allow NH_3 to diffuse into the filter pack. Each filter was then dried and the δ^{15}N of each sample was obtained using an isotope ratio mass spectrometer operated by T. Maddox, UGA Center for Applied Isotope Studies.

Competition experiment

We examined the interaction between extracellular ammonium and urea in a competition experiment. Competitor (final concentrations = 6 μM ammonium or 11 μM urea) and substrate (50 nM of ^{15}N-labeled ammonium or urea) were mixed together in sets of 4 tubes per treatment, then 50 mL of sample was added to each tube. One tube from each treatment was immediately frozen at −80°C as a control. The remaining tubes (triplicates per treatment) were incubated for 13.5 hours at the *in situ* temperature (30°C) before being frozen at −80°C. Concentrations of ammonium and urea in unamended controls were 2.99 and 3.02 μM, respectively, at the beginning of the experiment.

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Author contributions

B.B.T. and J.T.H. planned and performed experiments, analyzed the data and wrote the paper. N.J.W. and B.N.P. provided training and equipment for rate measurements, performed ^{15}N analyses (with B.B.T.), and assisted with isotopic calculations.

Conflict of interest statement

The authors declare no competing financial interests.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table S1. Gene abundance, transcript abundance, nutrient and ^{15}N-labeled ammonium and urea oxidation rate data from samples collected for this study. See file "Supplemental Table S1.xlxs".

Table S2. Sources of *ureC* and *amoA* sequences used to create the custom databases used for re-analysis of Marsh Landing metatranscriptomes. Sequences were retrieved from JGI-IMG by keyword searches of annotations and BLASTn searches of Archaea genomes. BLASTn searches of IMG for Thaumarchaeota ureases used sequences from Cenarchaeum symbiosum (Hallam *et al.*, 2006), "*Ca.* Nitrosopelagicus brevis" strain CN25 (supplied by A. Santoro), as well as putative urease sequences from the environment (Yakimov *et al.*, 2011; Alonso-Sáez *et al.*, 2012). SAG sequences AAA282 and AAA287 are from the Hawaii Ocean Time Series station and were collected by E. Delong (see Swan *et al.*, 2011). SAG generation was sponsored by NSF OCE-1232982 to R. Stepanauskas. SAG AAA633 is from a sample collected by G. Herndl who sponsored its production. See file "Supplemental Table S2.txt".

Table S3. Archaea *ureC* transcripts found in metatranscriptomes from the Duplin River at Marsh Landing, Sapelo Island in August 2008 and 2009. Putative *ureC* transcripts were assigned to target sequences in a custom database of Thaumarchaeota and Marine Group II Euryarchaeota *ureC* gene sequences (see Table S2). Libraries were first screened against the custom *ureC* database using BLASTn. Reads from BLASTn hits with bit scores $>$40 were then run against the Genbank RefSeq protein database using BLASTx and retained if the top hit with a bit score $>$40 was to a *ureC* gene, regardless of the taxonomic affiliation of the sequence. Production and initial analysis of the metatranscriptomes are described in detail in Gifford *et al.* (2011; 2013) and Hollibaugh *et al.* (2011; 2014). See file "Supplemental Table S3.xlxs".

Table S4. Cell yields for Thaumarchaeota cultures grown on ammonium versus urea. Data used to calculate yields were taken from growth curves presented in the references cited.

Table S5. List of qPCR primers and probes used in this study.

Table S6. Urea data used to estimate concentrations for rate calculations. Values enclosed in parentheses were ommitted from the calculation of mean concentrations.

Table S7. Effect of varying concentrations of exogenous urea on calculation of ^{15}N-labeled urea oxidation rate from $^{15}NO_x$ production rate.

Figure S1. Locations sampled for experiments, including **A)** Marsh Landing ("ML") on the Duplin River adjacent to Sapelo Island, Georgia and the South Atlantic Bight, **B)** the continental shelf west of the Antarctic Peninsula; and **C)** North Pacific Ocean and Gulf of Alaska.

Figure S2. Oxidation rates plotted against transcript abundance or transcript:gene ratios. **A)** Ammonia oxidation rates plotted against *amoA* transcript abundances; **B)** urea oxidation rates versus *ureC* transcript abundances; **C)** ammonia oxidation rate versus *amoA* transcript:gene ratios; **D)** urea oxidation rates versus *ureC* transcript:gene ratios; **E)** cell-specific ammonia oxidation rates versus *amoA* transcript:-gene ratios; and **F)** cell-specific urea oxidation rates versus *ureC* transcript:gene ratios. Transcript:gene ratios have been multiplied by 1,000 to simplify plots.