



# Ammonia Oxidation in the Ocean Can Be Inhibited by Nanomolar Concentrations of Hydrogen Peroxide

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Marine Thaumarchaeota were discovered over 20 years ago and although a few isolates from this group are now available for study, we do not yet understand the environmental controls on their growth and distribution. Thaumarchaeotes oxidize ammonia to nitrite, mediating a key step in the global nitrogen cycle, and it is estimated that about 20% of all prokaryotic cells in the ocean belong to this phylum. Despite their almost ubiquitous distribution, marine Thaumarchaeota are rarely abundant in open-ocean surface (<100 m) waters. We tested the hypothesis that this vertical distribution is driven by reactive oxygen species (ROS), specifically H<sub>2</sub>O<sub>2</sub>, generated by photochemical and biological processes—"indirect photoinhibition" rather than light inhibition as previously postulated for ammonia-oxidizing Archaea. Here we show that H<sub>2</sub>O<sub>2</sub> can be surprisingly toxic to Thaumarchaeota from the Southern Ocean, with ammonia oxidation inhibited by additions of as little as 10 nM H<sub>2</sub>O<sub>2</sub>, while temperate Thaumarchaeota ecotypes were more tolerant. This sensitivity could explain the seasonal disappearance of Thaumarchaeota from polar surface waters and the increase in ammonia oxidation rates with depth commonly observed in marine environments. Our results highlight the need for further physiological studies of Thaumarchaeota, and indicate that ROS sensitivity could be used as a characteristic for dividing the group into meaningful ecotypes.

**Keywords:** Thaumarchaeota, ammonia oxidation, nitrification, nitrogen, ammonia, reactive oxygen species, hydrogen peroxide

## INTRODUCTION

Photoinhibition has been hypothesized to reduce ammonia oxidation (AO), the first step of nitrification, in both ammonia-oxidizing Bacteria (AOB; Hooper and Terry, 1973; Olson, 1981; Ward, 1985; Horrigan and Springer, 1990) and Archaea (AOA; Murray et al., 1998, 1999a; Church et al., 2003; Qin et al., 2014). Nitrification links key processes within the nitrogen cycle (e.g., nutrient regeneration, assimilation, respiration) by converting the most reduced form of inorganic N (ammonium) into the most oxidized (nitrate), which can be denitrified and removed from the pool of fixed N (Ward, 2011). Early measurements of nitrification revealed reduced or undetectable rates in surface waters (Alleman et al., 1987), which were attributed to light sensitivity of AOB. AOA are now generally regarded as the dominant ammonia-oxidizing organisms (AOO) in the

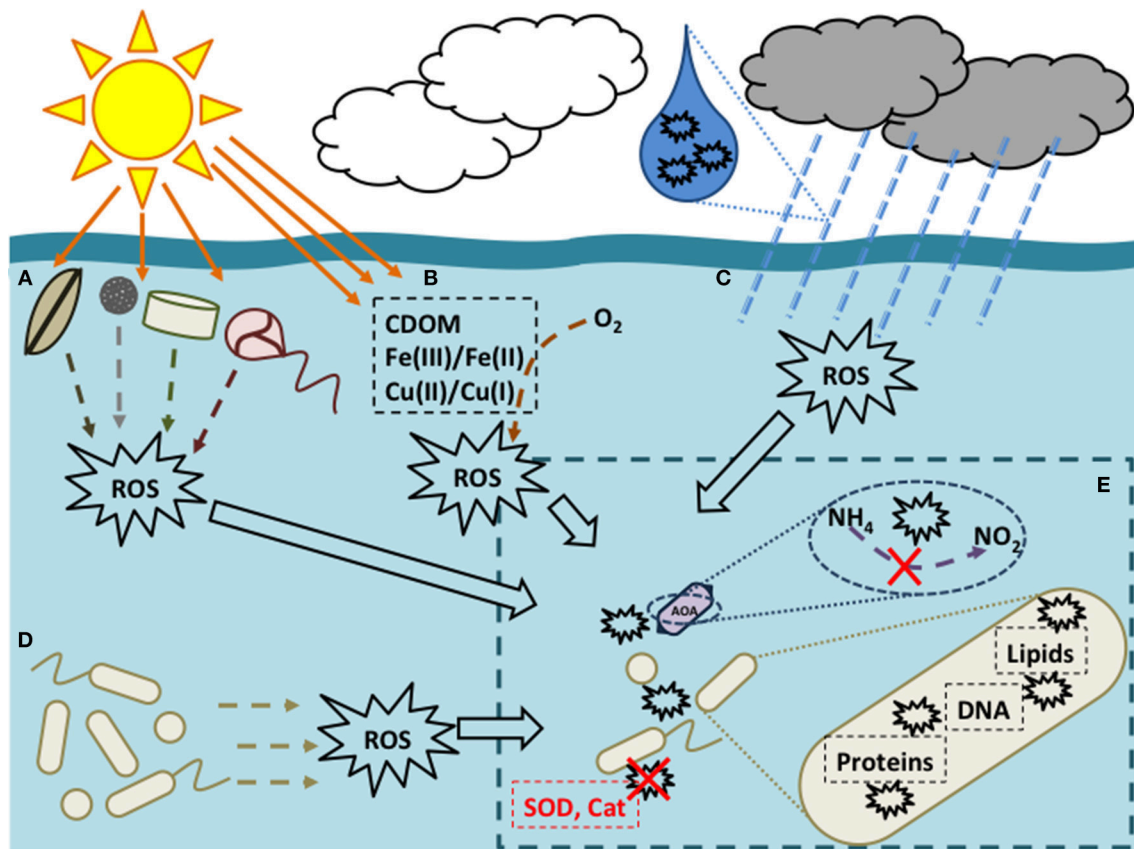
ocean (e.g., Wuchter et al., 2006; Francis et al., 2007; Mincer et al., 2007; Prosser and Nicol, 2008; Santoro et al., 2010; Ward, 2011; Tolar et al., 2013) and belong to the Marine Group I Archaea, now classified as Thaumarchaeota (Spang et al., 2010). However, Thaumarchaeota do not typically achieve high abundances in the surface ocean (Karner et al., 2001; Mincer et al., 2007), with notable exceptions during winter at higher latitudes and in polar regions (Murray et al., 1998, 1999a; Church et al., 2003; Wuchter et al., 2006). Attempts to explain these patterns have focused primarily on competition with Bacteria or phytoplankton and on light inhibition (Murray et al., 1999a; Church et al., 2003; Wells and Deming, 2003; Merbt et al., 2012; Smith et al., 2014b). However, most of these theories are derived from negative correlations between environmental variables and abundance. In the Southern Ocean, Thaumarchaeota populations decrease dramatically—especially in surface waters (Church et al., 2003)—as irradiance increases during the transition from winter to summer (Murray et al., 1998). This could indicate that photoinhibition or a product of increased irradiance (such as reactive oxygen species, or ROS) affects Thaumarchaeota and, perhaps, nitrification in general, as AOB have also been shown to be sensitive to light (Hooper and Terry, 1973; Olson, 1981; Ward, 1985; Horrigan and Springer, 1990).

The photochemical production of ROS [e.g., H<sub>2</sub>O<sub>2</sub>, superoxide, singlet oxygen, and hydroxyl radical; (Imlay, 2008)] proceeds at significant rates in sunlit surface waters (Figures 1A–D), creating a daily, ubiquitous increase in ROS concentration of 1–2 orders of magnitude. Of these ROS species, H<sub>2</sub>O<sub>2</sub> has the longest half-life (Kieber et al., 2003, and references therein; Powers and Miller, 2014). H<sub>2</sub>O<sub>2</sub> is primarily introduced by photochemical reactions with colored dissolved organic matter (CDOM; Figure 1B; Cooper et al., 1994; Blough and Zepp, 1995); rates of this production vary with solar irradiance, temperature, and [CDOM], but generally range from 9 to 134 nM h<sup>-1</sup> in marine systems (reviewed in Kieber et al., 2003). Superoxide (O<sub>2</sub><sup>-</sup>) also reacts with trace metals (Figure 1B) to produce H<sub>2</sub>O<sub>2</sub>; reactions with Cu(I) can proceed rapidly with rate constants (k) on the order of 2 × 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup> (Zafiriou et al., 1998), although [Cu(I)] is relatively low (~0.1 nM; Moffett and Zika, 1988). Other sources include atmospheric input through fluxes at the air-sea interface (0.09–4.0 nM h<sup>-1</sup>; Thompson and Zafiriou, 1983) and rain (Figure 1C), which can rapidly increase surface water [H<sub>2</sub>O<sub>2</sub>] due to its high concentration (8.4–82 μM; Zika et al., 1982; Cooper et al., 1987) relative to seawater (<10 nM–4.5 μM; Kieber et al., 2003). There are also biological sources of ROS; phytoplankton produce H<sub>2</sub>O<sub>2</sub> as part of their metabolism during photosynthesis and respiration (Figure 1A; Palenik et al., 1987; Zepp et al., 1987; Palenik and Morel, 1988) up to 0.46 nmol h<sup>-1</sup> cell<sup>-1</sup> (Twiner and Trick, 2000), though some pathways are not directly linked to photosynthesis. Extracellular biological ROS production (O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>, specifically; Figure 1D) in the absence of light has also been observed, with rates 0.01–11 nM h<sup>-1</sup> (Palenik and Morel, 1988; Moffett and Zafiriou, 1990; Roe et al., 2016) that have been attributed to microorganisms such as heterotrophic bacteria and

phytoplankton (Palenik et al., 1987; Rose et al., 2008; Diaz et al., 2013).

Since ROS can also be formed internally as by-products of biochemical reactions including photosynthesis and respiration (e.g., Marshall et al., 2005; Diaz et al., 2013), they must be removed to prevent cell damage. Prokaryotes possess a number of mechanisms to protect against ROS damage, including detoxifying enzymes, and DNA repair systems (Imlay, 2008; Figure 1E). A survey of the Integrated Microbial Genomes (IMG) database (<http://img.jgi.doe.gov/>) indicated a potential deficiency of genes from ROS-protection pathways in Thaumarchaeota relative to Bacteria or Euryarchaeota (Supplementary Table 1). All Thaumarchaeota genomes sequenced to date possess at least one copy of superoxide dismutase, which converts superoxide into H<sub>2</sub>O<sub>2</sub>; however, only *Nitrososphaera gargensis* (Hatzenpichler et al., 2008) and *N. evergladensis* (Zhalnina et al., 2014) have an annotated catalase (Supplementary Table 1)—the most effective enzyme known for detoxifying H<sub>2</sub>O<sub>2</sub> (Imlay, 2008). These particular catalases have manganese cofactors instead of the more typical heme cofactor, and are less efficient (Mishra and Imlay, 2012). A putative catalase gene was also identified from two Thaumarchaeota single-cell amplified genomes (Luo et al., 2014); a homolog of deoxyribodipyrimidine photolyase, a key enzyme involved in the repair of UV-damaged DNA, was also found in one genome in the same study. Thaumarchaeota have genes annotated as peroxiredoxin; however, peroxiredoxin is less efficient than catalase (Parsonage et al., 2008) and has been shown to remove H<sub>2</sub>O<sub>2</sub> effectively only at low intracellular [H<sub>2</sub>O<sub>2</sub>] (<0.1 μM; Imlay, 2008). The other peroxidases encoded in Thaumarchaeota genomes may or may not directly detoxify H<sub>2</sub>O<sub>2</sub> (Mishra and Imlay, 2012). It is therefore possible that the distribution of Thaumarchaeota could be explained by the lack of ROS detoxifying enzymes rather than direct photoinhibition or “competition” with Bacteria or phytoplankton for ammonia.

Here we test the hypothesis that this “missing” ROS protection makes Thaumarchaeota highly sensitive to H<sub>2</sub>O<sub>2</sub> generated external to the cell. We examined the sensitivity of AO to the ROS species H<sub>2</sub>O<sub>2</sub> in AOO assemblages dominated by Thaumarchaeota (Supplementary Tables 2, 3) from a wide range of marine environments, including the Southern Ocean near Palmer Station (Antarctica), the Gulf of Mexico, the Gulf of Alaska, and coastal waters at Marsh Landing, Georgia, USA (See Materials and Methods for details; Figure 2). Previous work supports the notion that Thaumarchaeota are the abundant ammonia oxidizer at these sites (Hollibaugh et al., 2014; Tolar et al., 2016a,b). Whole-seawater incubations were performed at *in situ* temperatures with additions of <sup>15</sup>N-labeled ammonium to determine rates of AO in the presence of H<sub>2</sub>O<sub>2</sub> added at environmentally-relevant (nM) concentrations. We also quantified the effect of H<sub>2</sub>O<sub>2</sub> additions on the abundance of transcripts of *amoA*, a highly-transcribed Thaumarchaeota gene that encodes the α-subunit of the ammonia monooxygenase enzyme as an index of another, more general (transcription broadly) Thaumarchaeota-specific process. Finally, we measured overall microbial activity using



**FIGURE 1 | Sources (A–D) and effects (E) of reactive oxygen species (ROS) in marine environments.** ROS (here, superoxide and hydrogen peroxide) can be introduced in surface waters (Kieber et al., 2003) via (A) phytoplankton as by-products of photosynthesis, (B) photochemical reactions of colored dissolved organic matter (CDOM) and metals, (C) atmosphere exchange and rain, and (D) production by heterotrophic bacteria (beige). Inset (E) gives a cellular-level view of ROS toxicity: when prokaryotic cells come into contact with ROS, molecules such as DNA, protein, and lipids can become damaged unless enzymes like superoxide dismutase (SOD) or catalase (Cat) are produced to detoxify ROS (Imlay, 2008; detoxification indicated by a red X). As shown in the text, ammonia oxidation by marine Thaumarchaeota (AOA) can be sensitive to ROS.

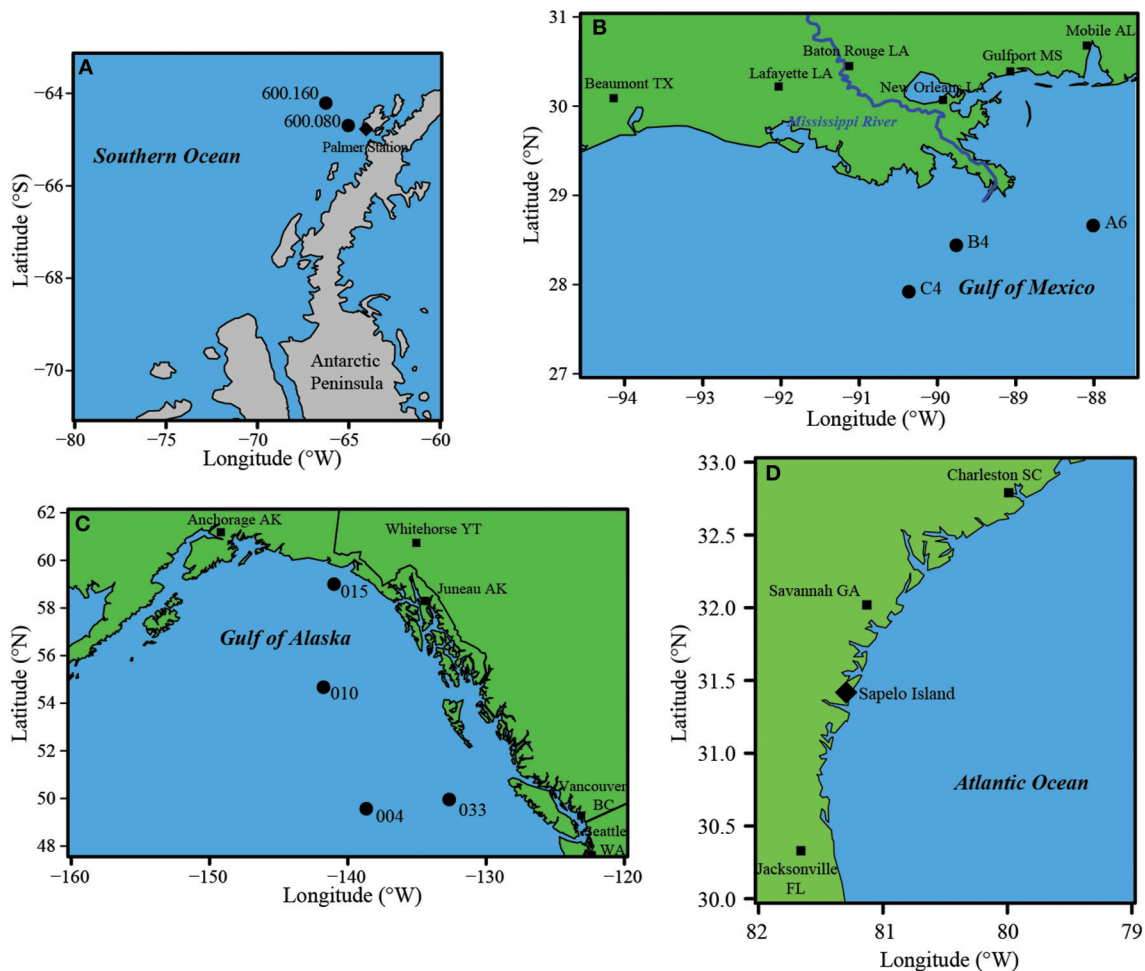
leucine incorporation to determine the effects of H<sub>2</sub>O<sub>2</sub> on general cellular function (protein synthesis) for comparison.

## MATERIALS AND METHODS

### Sample Collection

Seawater samples were collected (Figure 2, Supplementary Table 2) from Niskin bottles attached to a CTD rosette along the Palmer LTER 600 line in the Southern Ocean (*ARSV Laurence M. Gould*, September 2010; Station 600.160—64° 12.657' S, 66° 15.600' W; and Station 600.080—64° 41.682' S, 65° 01.849' W), in the Gulf of Mexico (*GoM—R/V Pelican*, May 2012; Station C4—27° 55.169' N, 90° 22.072' W; Station B4—28° 26.551' N, 89° 45.577' W; Station A6—28° 39.744' N, 88° 00.443' W), in the Gulf of Alaska (*GoA—R/V Melville*, August 2013; Station 004—49° 34.194' N, 138° 40.188' W; Station 015—58° 59.968' N, 140° 59.956' W; Station 033—49° 57.343' N, 132° 40.094' W), and from coastal surface waters from a floating dock at Marsh Landing at the mouth of the Duplin River (a tidal creek draining 1200 ha of salt marsh) adjacent to Sapelo Island, Georgia,

USA (Marsh Landing—August 2011 and 2012, September 2013; 31° 25.068' N, 81° 17.721' W). Two water masses (Antarctic Surface Water at 10–75 m and Circumpolar Deep Water at 300 m) were sampled in the Southern Ocean for comparison as these have shown different Thaumarchaeota populations in a previous study (Kalanetra et al., 2009). For GoM and GoA samples, water was collected at 200 m as this depth was likely to have high abundance of Thaumarchaeota relative to other organisms, except for GoA Stn 015 (50 m), which was from a more shallow, coastal station (max 180 m depth). Additional Marsh Landing samples were collected over a 24 h period at high tide and low tide (Supplementary Table 3) from Marsh Landing (August 2012, September 2013) and from a dock at Hunt Camp (August 2012; 31° 28.012' N, 81° 16.006' W), on the Duplin River upstream of Marsh Landing. We collected 0.22 μm filtered sample water for nutrient analysis, including the measurement of ammonia concentration (Supplementary Tables 2, 3) using previously described methods (Holmes et al., 1999). Photosynthetically-active radiation (PAR) data was gathered to pair to samples collected over 24 h at Marsh Landing (2012,



**FIGURE 2 |** Locations of sample sites where H<sub>2</sub>O<sub>2</sub> experiments were performed. **(A)** Southern Ocean west of the Antarctic Peninsula; **(B)** Gulf of Mexico; **(C)** Gulf of Alaska, **(D)** Marsh Landing on Sapelo Island, Georgia.

2013) from the NOAA National Estuarine Research Reserve System (NERRS) meteorological station on Sapelo Island near the floating dock we sampled at Marsh Landing (data accessed from the NOAA NERRS Centralized Data Management Office website: <http://www.nerrdata.org/>; accessed 12 Oct 2014).

## Experimental Setup

Seawater from each site (Figure 2) was collected from Niskin bottles attached to CTD rosettes (Southern Ocean, GoM, GoA) or directly from surface water (Marsh Landing) into 4 L (Southern Ocean) or 10 L (GoM, GoA, Marsh Landing) carboys and kept at *in situ* temperature (on-board Percival incubator—Southern Ocean; seawater flow-through tank—GoM, GoA, Marsh Landing) in the dark. <sup>15</sup>N-labeled ammonium (<sup>15</sup>NH<sub>4</sub>Cl; Cambridge Isotope Laboratories) was added at a concentration of 50 nM (Santoro et al., 2010; Beman et al., 2012) to determine ammonia oxidation (AO) rates, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; Fisher Scientific—Southern Ocean; J. T. Baker—GoM, GoA, Marsh Landing) was added to experimental treatments at

target concentrations designated as either “low” (10–100 nM) or “high” (30–300 nM) amendments; actual H<sub>2</sub>O<sub>2</sub> additions varied by sample site (Table 1, Supplementary Table 4). For these addition experiments, H<sub>2</sub>O<sub>2</sub> and <sup>15</sup>N-labeled ammonium were added directly to carboys for incubation; however, in the Southern Ocean <sup>15</sup>NH<sub>4</sub>Cl was added to duplicate, 250 mL subsamples after an initial incubation (6 h) with H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> concentrations in stock solutions were determined by measuring absorbance at 240 nm using the measured molar absorptivity of 38.1 M<sup>-1</sup> cm<sup>-1</sup> (Miller and Kester, 1988). Initial [H<sub>2</sub>O<sub>2</sub>] were also estimated by modeling H<sub>2</sub>O<sub>2</sub> decay (Figure 3, Supplementary Table 4) when possible. We did not add H<sub>2</sub>O<sub>2</sub> to samples collected for diurnal studies at Marsh Landing and Hunt Camp (2012, 2013), as we were more interested in the impact on the *in situ* [H<sub>2</sub>O<sub>2</sub>] on AO rates and the variability over a day and tidal cycle. We also took advantage of a rainstorm during sample collection (Marsh Landing 2012, 2013) to gather rainwater (collected over 15 min in a square plastic container) for use in an incubation experiment to compare the effects of H<sub>2</sub>O<sub>2</sub> from chemical vs. natural sources.

**TABLE 1 | Summary of ROS incubation experiments.**

Sample	Depth (m)	<i>in situ</i> [H <sub>2</sub> O <sub>2</sub> ]	Nominal [H <sub>2</sub> O <sub>2</sub> ] Addn.	Modeled <sup>#</sup> [H <sub>2</sub> O <sub>2</sub> ] Addn.	AO Rate (% control)	<i>p</i> -value ( <i>t</i> -test)
Southern Ocean 600.160	10	ND	30 nM	ND	0.61	<b>&lt;0.001</b>
			100 nM	ND	-0.13	<b>&lt;0.001</b>
	55	ND	30 nM	ND	-0.91	<b>&lt;0.001</b>
			100 nM	ND	-0.28	<b>&lt;0.001</b>
	300	ND	30 nM	ND	3.4	<b>&lt;0.001</b>
			100 nM	ND	0.85	<b>&lt;0.001</b>
Southern Ocean 600.080	10	14.3	30 nM	ND	-8.5	<b>0.002</b>
			100 nM	ND	-1.7	<b>0.007</b>
	75	2.9	30 nM	ND	32	0.13
			100 nM	ND	-12	<b>0.007</b>
	300	7.0	30 nM	ND	8.4	<b>0.001</b>
			100 nM	ND	-50	<b>&lt;0.001</b>
GoM C4	200	16.3	100 nM	163	76	0.38
			300 nM	464	76	0.12
GoM B4	200	28.1	100 nM	234	75	0.17
			300 nM	731	34	<b>0.037</b>
GoM A6	200	14.6	100 nM	197	74	0.26
			300 nM	556	34	<b>0.032</b>
GoA Stn 004	200	6.9	100 nM	112	99	0.93
			300 nM	312	68	0.37
GoA Stn 010	200	8.8	30 nM	73.4	170	0.089
			100 nM	168	103	0.87
			300 nM	419	40	<b>0.036</b>
GoA Stn 015	50	15.6	30 nM	52.9	93	0.26
			100 nM	160	94	0.37
			300 nM	433	94	0.41
GoA Stn 033	200	14.3	30 nM	69.2	63	<b>0.034</b>
Marsh Landing 2011	<1	ND	100 nM	ND	150	0.091
			300 nM	ND	33	<b>0.023</b>
Marsh Landing 2012	<1	201	100 nM	176	91	0.69
			300 nM	248	63	0.24
			100 nM Rain	221	80	0.44
Marsh Landing 2013	<1	108	10 nM	61.1	99	0.90
			30 nM	80.1	96	0.54
			100 nM	138	96	0.36
			300 nM	297	91	0.32
			100 nM Rain	167	86	0.16

All [H<sub>2</sub>O<sub>2</sub>] in nM; *t*-test against control rates, *p*-values in bold are significant (*p* < 0.05). ND, not determined. Rates are reflected as a percentage of the control; negative rates (italicized) reflect δ<sup>15</sup>N values that were higher at *T* = 0 than at the end of incubation (these rates are essentially zero or the limit of detection, defined as 0.001 nM d<sup>-1</sup>). *t*-tests were re-calculated using the detection limit instead of the negative rate, which had no effect on the significance of these results. <sup>#</sup>Modeled [H<sub>2</sub>O<sub>2</sub>] (nM) at time = 0 h for each experiment determined from *y*-int of the regression as discussed in Materials and Methods.

Rainwater was filtered prior to [H<sub>2</sub>O<sub>2</sub>] measurement and addition to prevent decay, and diluted to target nM additions. For comparison to general microbial community activity, leucine

incorporation rates were measured in samples from all sites using <sup>13</sup>C- or <sup>3</sup>H-labeled leucine (See Supplementary Methods for details).

## Nucleic Acid Extraction and Quantification

After incubation for 6 h with H<sub>2</sub>O<sub>2</sub>, samples were filtered directly onto 47 mm (Southern Ocean) or 142 mm (GoM, GoA, Marsh Landing) 0.22 μm pore size GVWP filters (Millipore), which were frozen in RNAlater (Ambion) at −20°C (GoM, GoA) or −80°C (Southern Ocean, Marsh Landing) before transport back to the laboratory, where they were kept frozen at −80°C until extraction. RNA was extracted following previously described methods (Poretsky et al., 2008; Gifford et al., 2011) with the replacement of 200 μm zirconium beads (OPS Diagnostics) for the initial bead-beating step. DNA was removed after extraction with the TURBO DNase-Free Kit (Ambion) following manufacturer's instructions with an additional enzyme treatment at 2X concentration. RT-qPCR targeting *amoA* transcripts was performed using the One-Step RT-qPCR SYBR Mix (BioRad), Archaeal *amoA* primers (Wuchter et al., 2006), and 1–2 μL of RNA template. AOB *amoA* transcripts were not detected in any of our samples (data not shown). qPCR reaction conditions follow previous methods (Kalanetra et al., 2009) with the addition of a 15 min incubation step at 50°C for reverse transcription of RNA to cDNA prior to the initial denaturation at 95°C. Raw transcript abundance (copies μL<sup>-1</sup> of RNA extract) was converted to transcripts L<sup>-1</sup> using the volume filtered and an elution volume of 100 μL, assuming 100% extraction efficiency. DNA extraction with phenol:chloroform, quantification of Archaeal, and Bacterial genes using qPCR, and calculation of gene copies L<sup>-1</sup> were performed as previously described (Tolar et al., 2013). Further characterization of the diversity of Thaumarchaeota communities from this study was done using next-generation sequencing (See Supplementary Methods for details).

## Ammonia Oxidation Rate Measurements

Ammonia oxidation (AO) rates were measured during dark incubations at *in situ* temperature for 24 h (GoM, Marsh Landing), 48 h (GoA), or 96 h (Southern Ocean) with 50 nM <sup>15</sup>NH<sub>4</sub>Cl added (mean = 12%, range = 1.7–45% of total [NH<sub>4</sub>]; Supplementary Table 2). We did not find significant effects on longer incubation time for AO rate measurements made in the Southern Ocean at the same time (Tolar et al., 2016a). For each rate measurement, an initial sample was filtered and frozen immediately after addition of tracer; for samples from the Southern Ocean, parallel control incubations were performed using the addition of tracer to filtered sample water. Incubations were terminated by freezing samples at −20 or −80°C and samples remained frozen at −80°C until analysis. Samples were analyzed using the “denitrifier” method (Sigman et al., 2001) as described previously (Popp et al., 1995; Dore et al., 1998). Briefly, NO<sub>2</sub> and NO<sub>3</sub> present in each sample were converted to N<sub>2</sub>O gas through denitrification by *Pseudomonas aureofaciens*, transferred from the reaction vial, cryofocused, and separated from other gases using a CP-PoraBOND Q capillary column (0.32 mm inner diameter × 25 m × 5 μm) at 20–25°C. δ<sup>15</sup>N values of N<sub>2</sub>O were measured using a MAT-252 mass spectrometer (Finnigan) and used to determine how much <sup>15</sup>NH<sub>3</sub> tracer was introduced to the NO<sub>x</sub> pool through AO (Beman et al., 2008; Christman et al., 2011). Catalase (Sigma Aldrich) was added to thawed samples (10 μL of 150 units L<sup>-1</sup> catalase per 50 mL sample) that

were incubated at room temperature for 60 min to prevent any inhibition of *P. aureofaciens* growth or denitrification ability by residual H<sub>2</sub>O<sub>2</sub> (no difference observed in N<sub>2</sub>O production with catalase addition; *t*-test, *p* = 0.43; Supplementary Table 5). AO rates were calculated from δ<sup>15</sup>N values as previously described (Christman et al., 2011; Beman et al., 2012); the detection limit for this measurement (0.001 nM d<sup>-1</sup>) was determined by using the detection limit for [NH<sub>4</sub>] and [NO<sub>x</sub>], as well as the uncertainty in stable isotope measurements with this setup (Beman et al., 2011).

## Hydrogen Peroxide Concentrations

H<sub>2</sub>O<sub>2</sub> was measured in the Southern Ocean using the POHPAA method (Miller and Kester, 1988) on samples that were first derivatized, then frozen at −20 to −80°C for subsequent measurement on an Aqualog fluorometer (Horiba). Since H<sub>2</sub>O<sub>2</sub> concentrations were below the limit of detection (5 nM) in some of these samples (Supplementary Table 6), we switched to a more sensitive method (King et al., 2007) using a FeLume chemiluminescence system (Waterville Analytical; Supplementary Figure 1) for the remaining experiments (GoM, Marsh Landing, GoA). Full details of the setup for the FeLume system are available in the Supplementary Methods. Briefly, H<sub>2</sub>O<sub>2</sub> reacts with 10-methyl-0-(*p*-formylphenyl)-acridinium carboxylate trifluoromethanesulfonate (acridinium ester; provided by James Kiddle, University of Western Michigan) at alkaline pH, forming a chemiluminescent product that can be quantified using flow injection analysis (Powers et al., 2015); See Supplementary Methods for more details. A 2 mM H<sub>2</sub>O<sub>2</sub> stock solution was prepared from 30% H<sub>2</sub>O<sub>2</sub> (J. T. Baker) in ultrapure water and checked spectrophotometrically as above. Standards were prepared in aged 0.2 μm filtered seawater and checked for low H<sub>2</sub>O<sub>2</sub> with H<sub>2</sub>O<sub>2</sub> blanks of aged seawater with added catalase (20 μL of 100 units L<sup>-1</sup> catalase to 20 mL seawater). Under these conditions, the H<sub>2</sub>O<sub>2</sub> detection limit, defined as three times the standard deviation of the blank, was 2–5 nM.

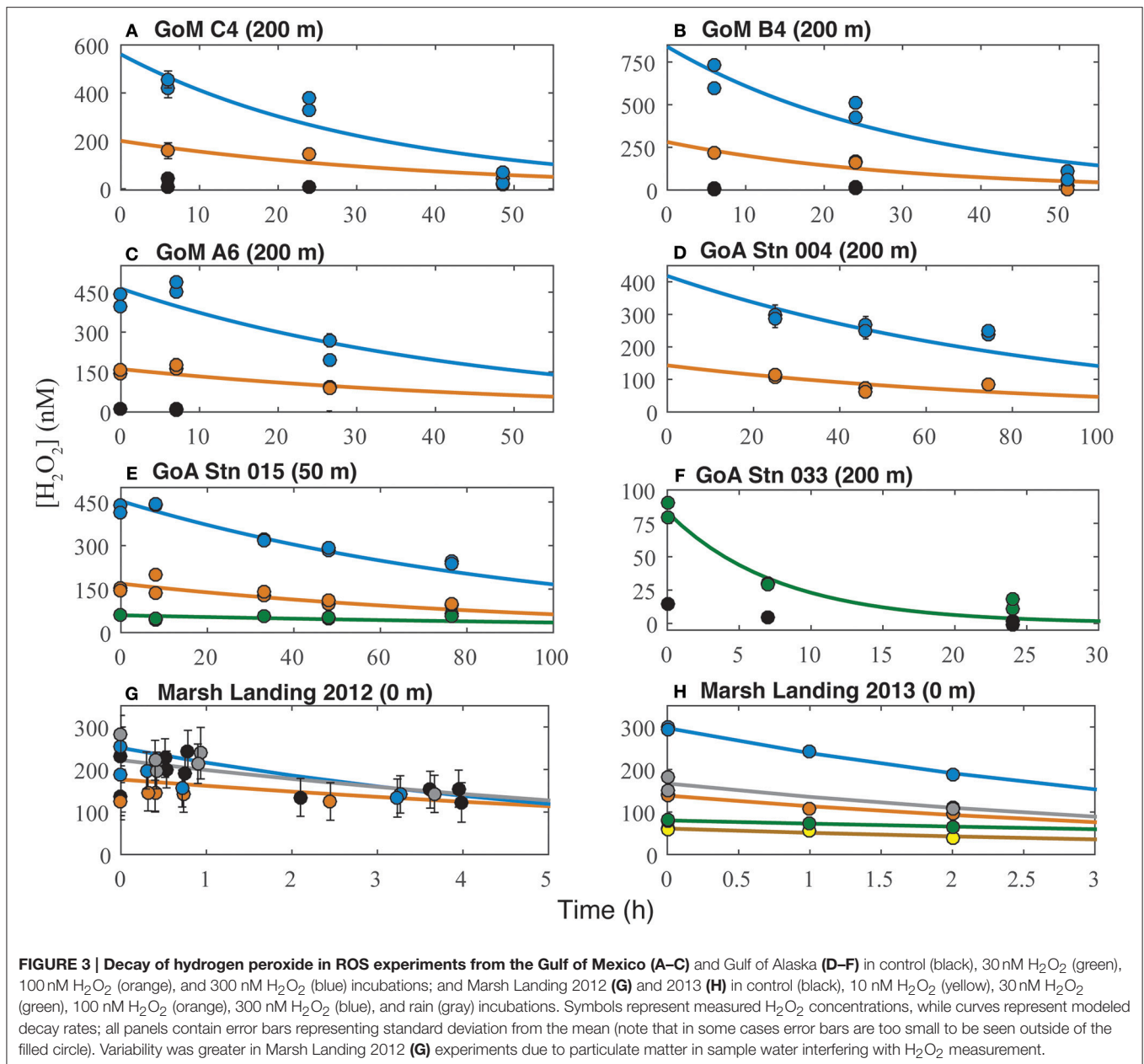
While the overall decay of [H<sub>2</sub>O<sub>2</sub>] has been reported as second order (Zepp et al., 1987), our experiments generally followed first-order kinetics with respect to hydrogen peroxide concentration, using the calculations from Zepp et al. (1987)

$$\frac{d[\text{H}_2\text{O}_2]}{dt} = -k_{\text{obs}}[\text{H}_2\text{O}_2] \quad (1)$$

where *k*<sub>obs</sub> is the observed rate constant (h<sup>-1</sup>). By integrating Equation (1), H<sub>2</sub>O<sub>2</sub> decay can be modeled with the following equation:

$$[\text{H}_2\text{O}_2] = [\text{H}_2\text{O}_2]_0 e^{-k_{\text{obs}}t} \quad (2)$$

where [H<sub>2</sub>O<sub>2</sub>]<sub>0</sub> is the initial peroxide concentration. For each experiment, H<sub>2</sub>O<sub>2</sub> data were fit to Equation (2) with a non-linear regression of H<sub>2</sub>O<sub>2</sub> vs. time using the curve fitting toolbox in MATLAB® (MathWorks) and both [H<sub>2</sub>O<sub>2</sub>]<sub>0</sub> and *k*<sub>obs</sub> were used as fitting parameters. Decay rates for GoM samples were determined using H<sub>2</sub>O<sub>2</sub> concentration data from the first 24 h of incubation; GoA decay rates from Stations 004 and 015 were determined over 76 h, Station 010 over 147 h, and Station 033 over 24 h (note that all AO rate incubation samples were



terminated at 48 h by subsampling the incubation bottle; the remaining sample was incubated for longer periods as necessary to detect complete decay of H<sub>2</sub>O<sub>2</sub>); and Marsh Landing 2012 decay rates were determined over 3.5 h, while Marsh Landing 2013 decay rates were determined over 2 h. Half-lives varied for each environment, ranging from ~3 to 8 h (Marsh Landing) to over 120 h (GoA; Supplementary Table 4).

## Statistical Analysis

Statistical tests were performed with R (<http://www.r-project.org/>) using the commands “aov” for one-way analysis of variance (ANOVA), “TukeyHSD” for the Tukey’s honestly significant difference (HSD) test, “t-test” for Student’s *t*-test, and “cor.test” for calculations of Pearson’s

correlation coefficient (all from the stats package included with R). Stripcharts were used to assess normal distribution of data and a Bartlett test was run to evaluate equality of variance (both within R). Rate data was log-transformed (after scaling to remove negative values) to prevent bias from higher rates obtained in some samples. Percent reduction of rates (AO and leucine incorporation) was calculated by dividing a given rate by the average rate from a non-H<sub>2</sub>O<sub>2</sub> addition control.

## RESULTS

A summary of results from all experiments is available in **Table 1**. AO rates were below detection in the Southern Ocean

following the addition of 6–80 nM H<sub>2</sub>O<sub>2</sub> to samples above *in situ* concentrations (**Figures 4A,B; Table 1**, Supplementary Table 6), indicating that AOO in this region are extremely sensitive to H<sub>2</sub>O<sub>2</sub>. Concentrations of H<sub>2</sub>O<sub>2</sub> in Southern Ocean samples were low (2.9–14 nM; Supplementary Table 6) and match previous measurements (Resing et al., 1993; Yocis, 1995; Sarthou et al., 1997). Despite this relatively small increase in [H<sub>2</sub>O<sub>2</sub>], AO rates decreased 67 to >100% of the control rate (**Table 1**; negative rates indicate samples where the δ<sup>15</sup>N value was higher at *T* = 0, and are considered zero or the limit of detection). The change in abundance of AOA *amoA* transcripts did not reflect the drastic decrease in AO rates observed in treatments with H<sub>2</sub>O<sub>2</sub> additions (this was observed at all sites sampled; **Figure 5**). Southern Ocean leucine incorporation rates at Station 600.160 were also reduced with increasing H<sub>2</sub>O<sub>2</sub> concentration (**Figure 6A**), but this reduction was only significant at 10 m (100 nM addition only) and 55 m (both 30 and 100 nM additions; Supplementary Table 2). The reduction in leucine incorporation (mean = 17%) was significantly less than that of AO rates (mean = 67%; *t*-test, *p* < 0.01).

The AO response to H<sub>2</sub>O<sub>2</sub> additions was variable in both the Gulf of Mexico (GoM; **Figure 4C, Table 1**; average 38% AO reduction) and the Gulf of Alaska (GoA; **Figure 4D, Table 1**; average 8.5% AO reduction), with the greatest inhibition observed at Station B4 (GoM) and Station 033 (GoA). At the furthest offshore GoM site (Station C4), there was no difference in AO between samples with 0 or 100 nM added H<sub>2</sub>O<sub>2</sub>, and no change was observed with amendments of up to 300 nM H<sub>2</sub>O<sub>2</sub> in the coastal GoA sample (Station 015, 50 m). An analysis of Thaumarchaeota 16S rRNA and *amoA* genes (Supplementary Figure 2) showed that the clades found in the GoA sample most resilient against H<sub>2</sub>O<sub>2</sub> additions (Station 015) were the least similar to other GoA communities. Inhibition of leucine incorporation (protein synthesis) also varied with sample location; for example, we found no significant difference with increased [H<sub>2</sub>O<sub>2</sub>] at Stations C4 or A6 (GoM; **Figure 6B**), whereas AO rates were reduced at both sites (24–66%; **Figure 4C, Table 1**).

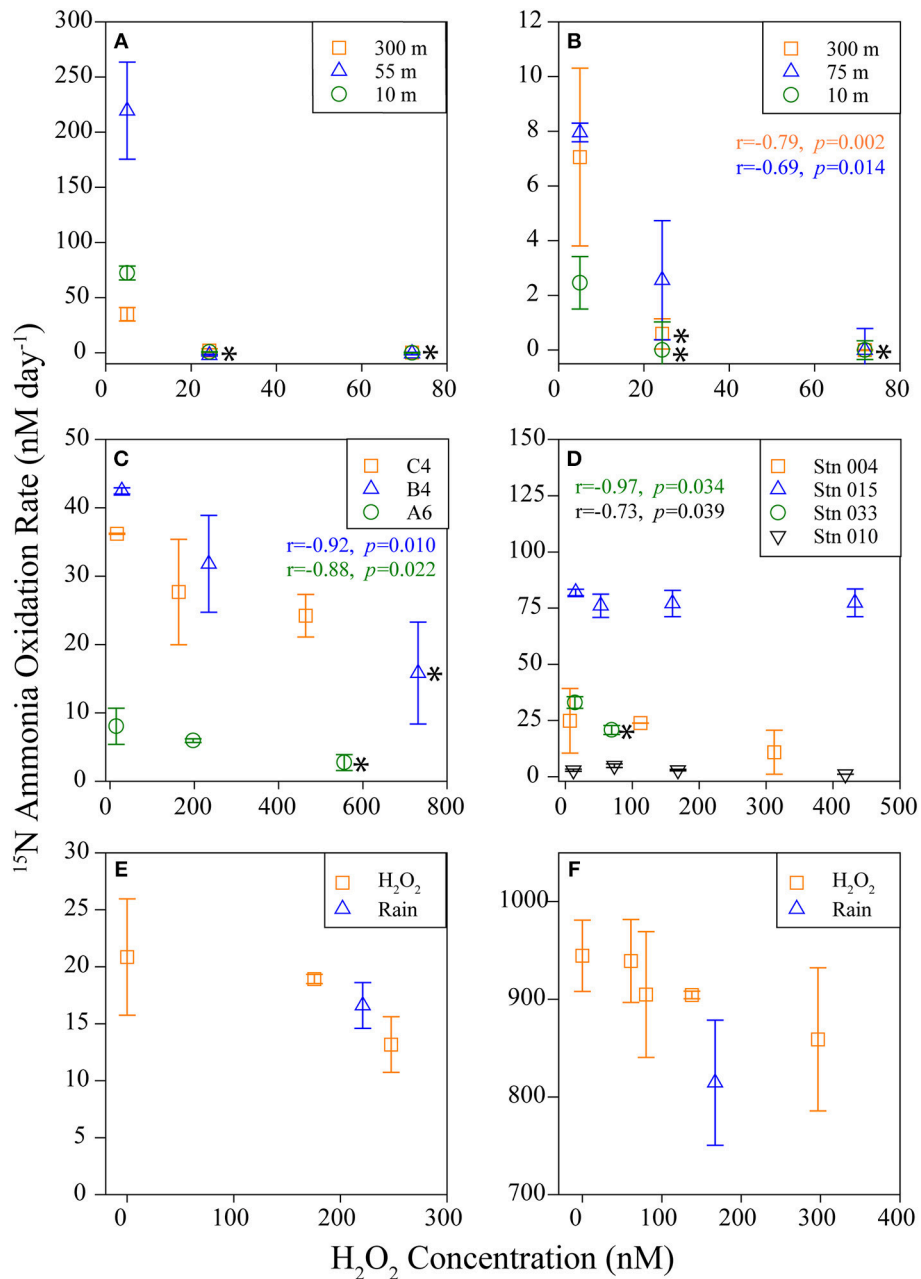
Samples from Marsh Landing were the least inhibited by H<sub>2</sub>O<sub>2</sub> with only one out of ten measurements showing a significant reduction in AO across all 3 years sampled (**Figures 4E,F**, Supplementary Figure 3): the addition of 300 nM H<sub>2</sub>O<sub>2</sub> in 2011 (67% reduction of AO rate; **Table 1**; *t*-test, *p* < 0.05). These coastal microbial communities were capable of reducing H<sub>2</sub>O<sub>2</sub> concentrations ~6X more quickly (**Figures 3G,H**) than communities from open ocean sites (Supplementary Table 4, **Figures 3A–F**), and were markedly distinct in both Thaumarchaeota 16S rRNA and *amoA* diversity (Supplementary Figure 2). Time courses (2011; Supplementary Figure 3) revealed that 300 nM H<sub>2</sub>O<sub>2</sub> additions significantly reduced AO within 6 h (*t*-test, *p* < 0.03; Supplementary Table 2), with no additional reduction or recovery of AO rates after 24 h (*p* < 0.05; Supplementary Table 2). As with other experiments, Marsh Landing *amoA* transcript abundance was not reduced by H<sub>2</sub>O<sub>2</sub> (**Figures 5E,F**). Leucine incorporation in these samples was also significantly reduced by H<sub>2</sub>O<sub>2</sub> additions after 6 h (*t*-test, *p* < 0.03; **Figures 6D–F**, Supplementary Table 2), but the

reduction was not significantly different from the reduction in AO rates (mean = 18 vs. 5% reduction; *t*-test, *p* ≥ 0.2). We also found that additions of rainwater inhibited AO rates (**Figures 4E,F**), and a rain event during the 24 h sampling study in 2012 further suppressed rates at one site, despite the absence of PAR and thus photochemical H<sub>2</sub>O<sub>2</sub> production (Supplementary Figure 4B). Rainwater H<sub>2</sub>O<sub>2</sub> concentrations were 30–50 μM (diluted to target nM concentrations for experiments; Supplementary Table 3), consistent with previous measurements (e.g., Zika et al., 1982; Cooper et al., 1987; Willey et al., 1996).

Additional samples collected from Marsh Landing and Hunt Camp over 24 h (**Figure 7**, Supplementary Figure 4) revealed a daily increase in [H<sub>2</sub>O<sub>2</sub>] from 10 to 500 nM [Supplementary Table 3; corresponding to increase in sunlight or “photosynthetically active radiation,” (PAR)]—1 to 2 orders of magnitude greater than what was detected in the Southern Ocean. Samples collected at and immediately after the time of greatest [H<sub>2</sub>O<sub>2</sub>] showed decreased AO rates (**Figure 7**, Supplementary Figure 4), although there was higher variability due to impact of mixing and other processes inherent to an estuarine tidal creek. There was also no clear pattern based on tide sampled (Supplementary Table 3), with no significant difference in rates measured in August 2012 at Marsh Landing except between the two low tides sampled (*t*-test, *p* = 0.043; Supplementary Figure 4A), equal rates at high and low tide at Hunt Camp (*p* = 0.21; Supplementary Figure 4B), and the highest rate in September 2013 at the high tide sampled during the night (*p* < 0.005 for all comparisons; **Figure 7**). There was no discernable trend based on time of day or [H<sub>2</sub>O<sub>2</sub>]. Thaumarchaeota *amoA* genes did not vary for experiments in 2012 (Supplementary Figure 4), but *amoA* transcripts peaked at dawn at Marsh Landing (Supplementary Figure 4A) and showed no trend at Hunt Camp (Supplementary Figure 4B). For the Marsh Landing 2013 diurnal study, there was greater variation across the 24-h period than in 2012; *amoA* gene abundance peaked after noon and midnight, while *amoA* transcripts were most abundant right after sunset and before sunrise (**Figure 7**).

Our data show that nanomolar concentrations of H<sub>2</sub>O<sub>2</sub> can be harmful to open-ocean AOO, with the most sensitive communities found in polar waters (**Figure 4, Table 1**). In contrast, AOO in coastal samples from Marsh Landing appear to be more resistant to H<sub>2</sub>O<sub>2</sub> toxicity, as do AOO in samples from the coastal GoA. Although the results were variable apart from the Southern Ocean experiments, a significant reduction of AO rates following H<sub>2</sub>O<sub>2</sub> amendments was observed when the entire dataset was evaluated together (ANOVA, *n* = 140, *F* = 11.2, *p* < 0.001). The same analysis revealed no significant difference in leucine incorporation rates (all sites: *n* = 169, *F* = 2.24, *p* = 0.09; excluding Marsh Landing: *n* = 128, *F* = 2.6, *p* = 0.06), suggesting that AO is more sensitive to H<sub>2</sub>O<sub>2</sub> toxicity than general microbial metabolism. Comparing the percent reduction in AO rates based on site (ANOVA, *n* = 140, *F* = 18.7, *p* < 0.001) revealed a significant difference between samples from the Southern Ocean (100 ± 19%) and all other sites [Marsh Landing (12 ± 30%), GoM (38 ± 21%), GoA (8.5 ± 36%); Tukey’s test, *p* < 0.001 for all three pairs). This indicates that our hypothesis that ROS



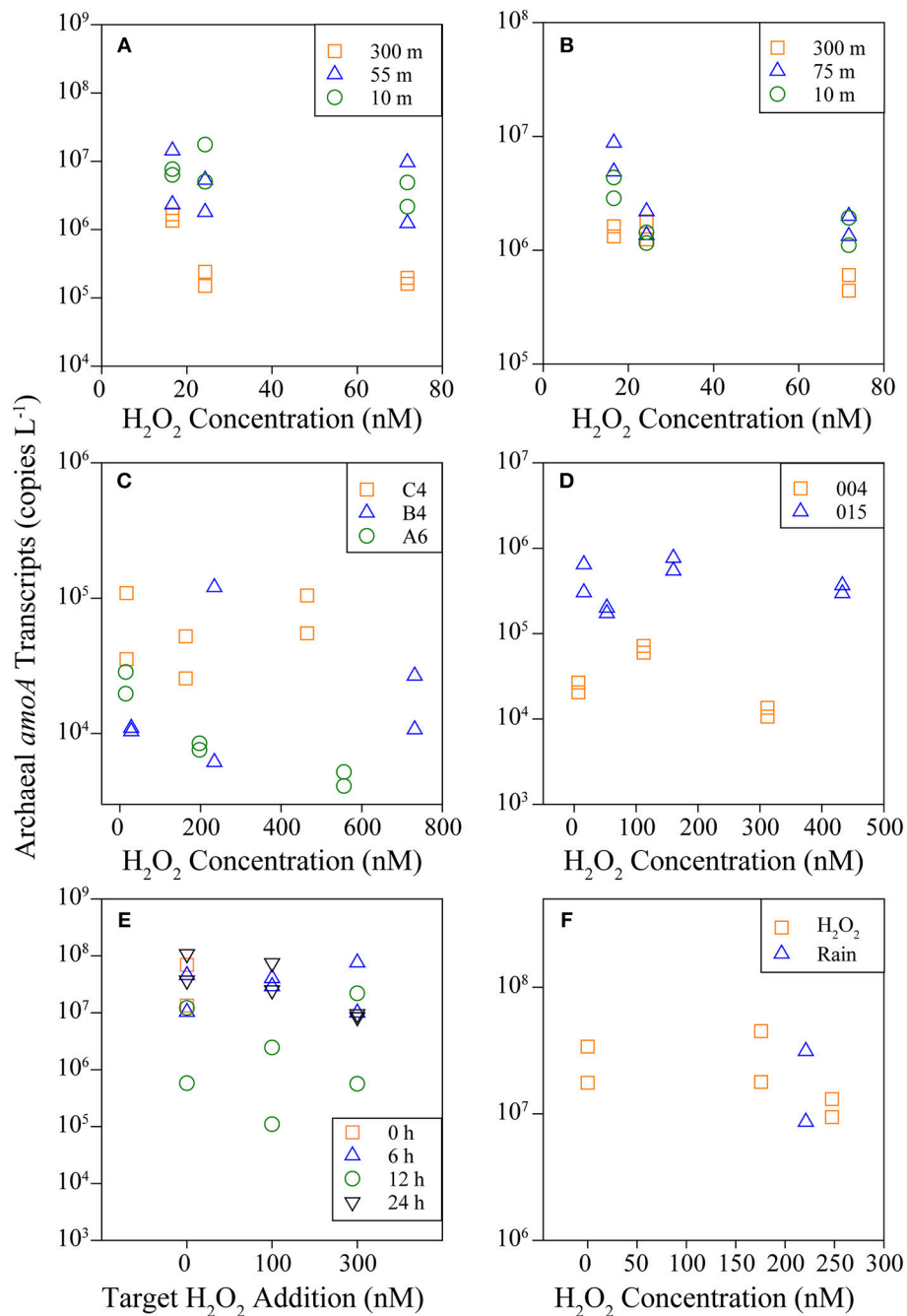


**FIGURE 4 | <sup>15</sup>N-Ammonia oxidation rates.** AO rates measured following H<sub>2</sub>O<sub>2</sub> amendments of samples from the Southern Ocean (**A** Station 600.160, **B** Station 600.080; symbols represent sample depth), Gulf of Mexico and Gulf of Alaska (**C,D**, respectively; symbols represent stations sampled; sample depth was 200 m except at Station 015, which was sampled at 50 m), and coastal waters from Marsh Landing in (**E**) 2012 and (**F**) 2013 (symbols represent H<sub>2</sub>O<sub>2</sub> origin, either chemical H<sub>2</sub>O<sub>2</sub> or from rainwater; surface water samples collected). Error bars represent standard deviation from the mean of replicate AO measurements [ $n = 4$  (**A,B**), 2 (**C-F**)]. An \* to the right of a point indicates a significant difference ( $t$ -test,  $p < 0.05$ ) from the control rate (for **A,B**, overlapping points were all significantly different, and are represented by only one \*). H<sub>2</sub>O<sub>2</sub> concentrations measured for each individual sample; non-control sample [H<sub>2</sub>O<sub>2</sub>] includes added H<sub>2</sub>O<sub>2</sub>. Pearson's correlation coefficient ( $r$ ) and associated  $p$ -value are shown for those samples with a significant regression between rate and [H<sub>2</sub>O<sub>2</sub>], color-coded to match legend for each panel (note that regressions were not done for Station 600.160 (**A**) as [H<sub>2</sub>O<sub>2</sub>] was not measured).

can inhibit AO is valid only in certain ocean regions, which warrants further investigation, as site-specific environmental factors or overall microbial community composition may play a role.

## DISCUSSION

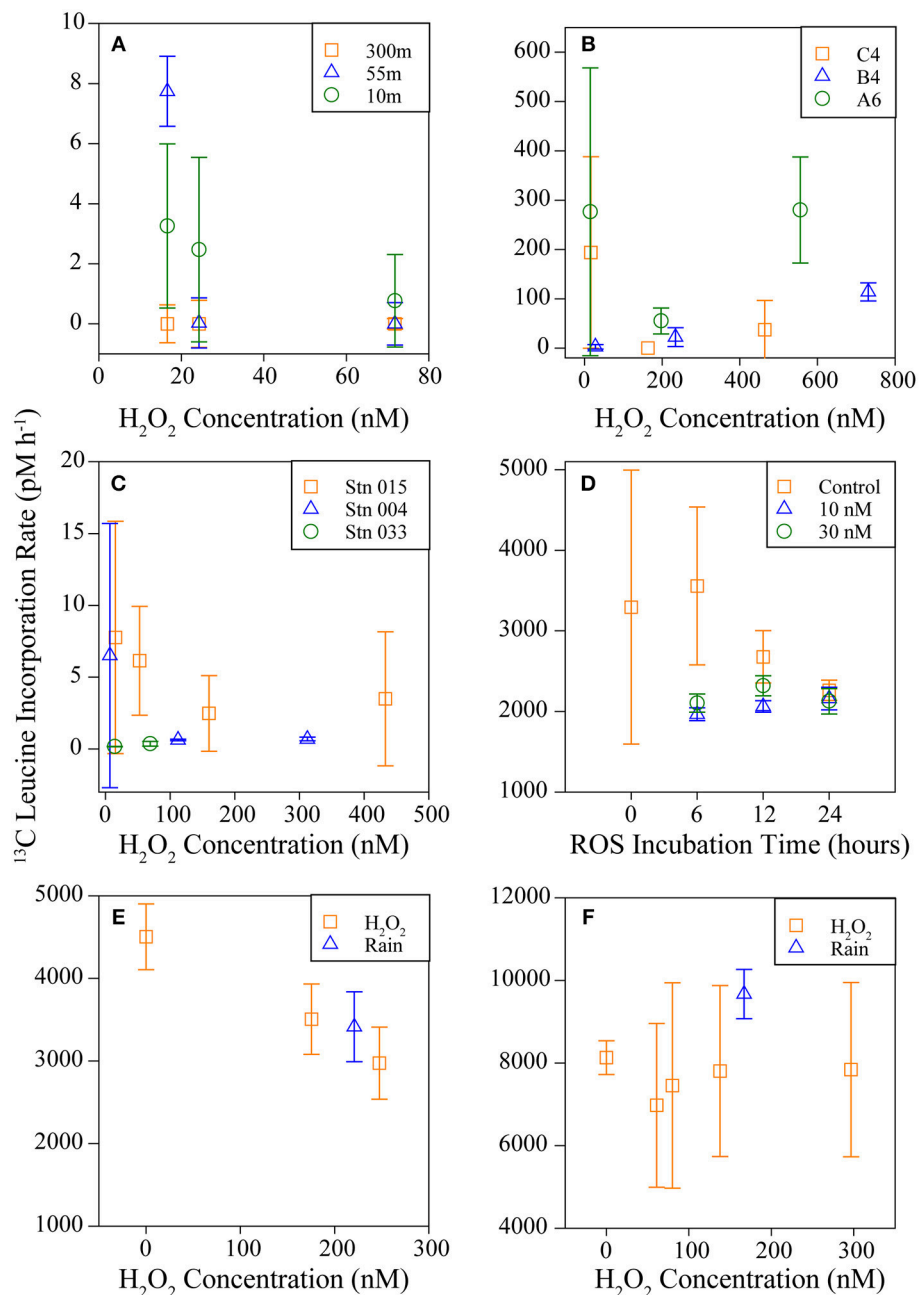
Culture-based experiments testing light inhibition have indicated that AO by both AOB (Hooper and Terry, 1973; Horrigan



**FIGURE 5 |** Archaeal *amoA* transcripts quantified by RT-qPCR using RNA collected from the Southern Ocean (A,B; symbols represent sample depth), Gulf of Mexico and Gulf of Alaska (C,D, respectively; symbols represent station sampled), and Marsh Landing in 2011 (E) and 2012 (F). Symbols indicate H<sub>2</sub>O<sub>2</sub> origin, either chemical H<sub>2</sub>O<sub>2</sub> or from rainwater.

and Springer, 1990; Hyman and Arp, 1992; Guerrero and Jones, 1996a) and AOA (Merbt et al., 2012; Qin et al., 2014) is decreased in the presence of increased light; yet none of these studies measured ROS generation during their incubations, which can occur at rates of 10–600 nM H<sub>2</sub>O<sub>2</sub> h<sup>-1</sup> under simulated light conditions (Kieber et al., 2003, 2014; Powers and Miller, 2015). However, Hooper and Terry (1974) postulated

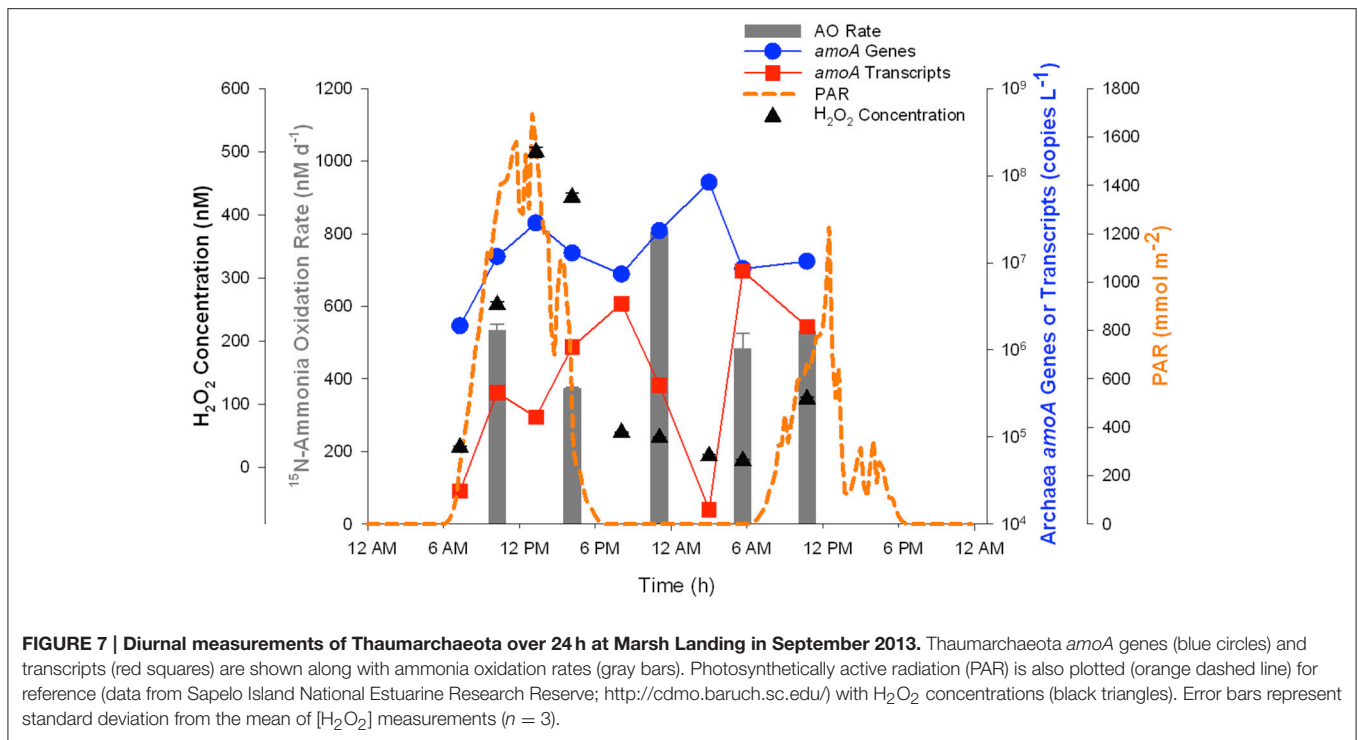
that the mechanism of this damage was likely due to ROS (“a peroxide or free radical”), based on their observation that reduced photoinhibition was observed when oxygen levels were low or absent in their experiments. Additionally, a very recently published study showed that H<sub>2</sub>O<sub>2</sub> concentrations ≥200 nM completely inhibited AO by a newly isolated marine thaumarchaeote, strain DDS1 (Kim et al., 2016).



**FIGURE 6 | Bacterial and archaeal incorporation of L-leucine measured in the Southern Ocean (A; symbols represent sample depth), the Gulf of Mexico, and the Gulf of Alaska (B,C, respectively; symbols represent station sampled), and Marsh Landing in 2011 (D; symbols represent H<sub>2</sub>O<sub>2</sub> addition), 2012, and 2013 (E,F, respectively; symbols represent H<sub>2</sub>O<sub>2</sub> origin, either chemical H<sub>2</sub>O<sub>2</sub> or from rainwater). Incubations were performed using <sup>3</sup>H-labeled (A,D) or <sup>13</sup>C-labeled (B,C,E,F) L-leucine. Error bars represent standard deviation from the mean of replicate measurements [*n* = 6 (A), 3 (C,D), 2 (B,E,F)].**

We have shown here that nanomolar [H<sub>2</sub>O<sub>2</sub>] can inhibit AO in bacterioplankton where Thaumarchaeota are the dominant AOO, and can also inhibit protein synthesis by the overall microbial community, which includes other nitrogen-cycling organisms. H<sub>2</sub>O<sub>2</sub> is produced by abiotic and biotic processes in all sunlit marine waters where it can enter and damage cells that do not have detoxifying enzymes (Figure 1). Surface water

[H<sub>2</sub>O<sub>2</sub>] ranges from 8 nM to 4.5 μM in marine environments (reviewed in Kieber et al., 2003), and varies from <10 to 250 nM in open ocean waters (e.g., Yuan and Shiller, 2005). Generally, lower H<sub>2</sub>O<sub>2</sub> concentrations (10–20 nM) occur in areas with both low solar irradiance and CDOM like the Southern Ocean (Resing et al., 1993; Yocis, 1995; Sarthou et al., 1997), while higher [H<sub>2</sub>O<sub>2</sub>] occurs in coastal regions with both increased irradiance and



**FIGURE 7 | Diurnal measurements of Thaumarchaeota over 24 h at Marsh Landing in September 2013.** Thaumarchaeota *amoA* genes (blue circles) and transcripts (red squares) are shown along with ammonia oxidation rates (gray bars). Photosynthetically active radiation (PAR) is also plotted (orange dashed line) for reference (data from Sapelo Island National Estuarine Research Reserve; <http://cdmo.baruch.sc.edu/>) with H<sub>2</sub>O<sub>2</sub> concentrations (black triangles). Error bars represent standard deviation from the mean of [H<sub>2</sub>O<sub>2</sub>] measurements ( $n = 3$ ).

CDOM, such as in the Amazon River plume (up to 300 nM; Yuan and Shiller, 2001), Orinoco River plume (140–470 nM; Moore et al., 1993), or coastal Gulf of Mexico (90–240 nM; Zika et al., 1985; Cooper et al., 1987). Our study examined Thaumarchaeota populations from both coastal and offshore marine waters that covered this range of natural H<sub>2</sub>O<sub>2</sub> concentrations. Our observation of increased sensitivity in Southern Ocean samples could reflect microorganisms that can survive without ROS detoxification systems in the typically low [H<sub>2</sub>O<sub>2</sub>] present there (2.1–33 nM; Resing et al., 1993; Yocis, 1995; Sarthou et al., 1997). Although H<sub>2</sub>O<sub>2</sub> is only one type of ROS, it can both be produced from superoxide and form the hydroxyl radical via reactions with metals, and also is the longest-lived of the ROS species (Kieber et al., 2003, and references therein). Therefore, focusing our work on H<sub>2</sub>O<sub>2</sub> can inform the potential impacts of ROS in general on these communities.

Recent work has shown that Thaumarchaeota are active and oxidize ammonia in sunlit surface waters (Smith et al., 2014a), and culture-based Thaumarchaeota photoinhibition experiments indicated that the specific intensity of light that was inhibitory depended on the strain used (Merbt et al., 2012; Qin et al., 2014). This was also true for AOB, with different strains showing different light intensities needed to inhibit growth (Horrigan and Springer, 1990; Guerrero and Jones, 1996a). Additionally, the wavelength of light used also showed a variable inhibitory effect, with UV wavelengths (<400 nm) the most damaging to AO by AOB cultures (Hooper and Terry, 1974; Guerrero and Jones, 1996a). Recovery after photoinhibition was also found to be wavelength-dependent, as AOB exposed to longer wavelengths (>400 nm) regained activity faster than those

exposed to shorter wavelengths or sunlight (Guerrero and Jones, 1996b). Strong absorption of UV radiation (280–400 nm) leads to the production of H<sub>2</sub>O<sub>2</sub> (Miller, 2000; Yocis et al., 2000), with only 10–20% of H<sub>2</sub>O<sub>2</sub> produced by wavelengths >400 nm (Miller, 2000). Therefore, observed wavelength-dependent biological photosensitivity may simply reflect higher [H<sub>2</sub>O<sub>2</sub>] production.

In one AOA photoinhibition study, Thaumarchaeota strains did not recover growth in the dark following light exposure (Merbt et al., 2012); yet in another, Qin et al. (2014) observed recovery in two strains, one of which was included in the Merbt et al. (2012) experiments (“*Candidatus Nitrosopumilus maritimus*” SCM1). Additionally, while Merbt et al. (2012) saw 82% of growth inhibition at 15  $\mu\text{E m}^{-2} \text{s}^{-1}$  for strain SCM1, a 12-fold increase in light intensity (180  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) was required to match this inhibition in experiments performed by Qin et al. (2014). Although the authors had no explanation for this discrepancy, a recent study by Kim et al. (2016) has provided a potential answer. Thaumarchaeota cultures have shown enhanced growth with additions of  $\alpha$ -ketoglutarate (Tourna et al., 2011; Qin et al., 2014), which was determined to scavenge endogenously-produced H<sub>2</sub>O<sub>2</sub> that otherwise would inhibit growth (Kim et al., 2016), much like the inhibition of AO shown here. The study by Qin et al. (2014) is the first to report the supplement of  $\alpha$ -ketoglutarate to media for growth of this particular strain, and its addition could explain their photoinhibition experimental results by protecting SCM1 against H<sub>2</sub>O<sub>2</sub>. Interestingly, the two other thaumarchaeote strains examined Qin et al. (2014) were unable to grow without addition of  $\alpha$ -ketoglutarate, indicating enhanced sensitivity to

ROS and highlighting differences even amongst closely related isolates.

The sensitivity of ammonia oxidation by AOA to H<sub>2</sub>O<sub>2</sub> could explain their typical absence from oceanic surface waters, consistent with early theories for photoinhibition of AO by AOB (Alleman et al., 1987). A mid-summer bloom of Thaumarchaeota in Georgia coastal waters (Hollibaugh et al., 2014) despite high *in situ* [H<sub>2</sub>O<sub>2</sub>] (up to 500 nM) probably reflects the lower sensitivity of this population of AOA to H<sub>2</sub>O<sub>2</sub>, and is likely related to the distinct “cotype” of Thaumarchaeota that resides in coastal Marsh Landing waters compared to open-ocean sites (Supplementary Figure 2). AO has been shown to exhibit less photoinhibition in an estuarine AOB isolate than in marine isolates (Horrihan and Springer, 1990), which is consistent with our findings. However, as shown here, the dynamics of Thaumarchaeota populations in a coastal estuary are more complex (Figure 7, Supplementary Figure 4), with compounding influences of sunlight, tides, physical mixing, and storm events all impacting results. Consequently, it is important to collect samples at shorter time scales to reveal how such complicating factors may individually affect the data. For example, we included incubations with rainwater-derived H<sub>2</sub>O<sub>2</sub> that showed an inhibitory effect on AO matching chemical additions (Figures 4E,F) indicating that storm events could impact bacterioplankton and nitrification that occurs in coastal surface waters (Smith et al., 2014a). Although our diurnal experiments were done in the dark after initial sample collection, we were able to observe natural variation in AO rates and archaeal *amoA* genes and transcripts under *in situ* conditions that included H<sub>2</sub>O<sub>2</sub> concentration. We did not find clear patterns between time of day or tide and H<sub>2</sub>O<sub>2</sub> inhibition in these samples, but can now compare our results to future experiments at *in situ* light levels and sample shorter time scales during the transition between tides.

On a broader scale, our results were location-dependent, with the most sensitive communities found in the Southern Ocean and the most resilient taken from coastal surface waters at Marsh Landing, suggesting that sample location—and thus inherently microbial community composition—is important for predicting ROS sensitivity of AOO. We also observed inconsistencies between sample sites in both the Gulf of Mexico and the Gulf of Alaska, conceivably due to the efficiency with which the surrounding microbial community removes H<sub>2</sub>O<sub>2</sub> (Morris et al., 2012), although differences in net decay rate (Supplementary Table 4, Figures 3A–F) do not fully explain this variability. Alternatively, this may indicate a difference in the composition of thaumarchaeote populations where some “ecotypes” could be more or less resistant to ROS stress.

The question remains as to why some Thaumarchaeota would be sensitive to H<sub>2</sub>O<sub>2</sub> (especially compared to Bacteria), given that they likely evolved in an oxygenated world (Spang et al., 2010; Kelly et al., 2011). Most Thaumarchaeota reside in deep waters with very low *in situ* H<sub>2</sub>O<sub>2</sub> concentrations, but some clades have been found in surface waters, primarily at higher latitudes (Murray et al., 1998, 1999a; Wuchter et al., 2006). However, we have documented an abundant and active community of Thaumarchaeota in coastal Georgia surface waters (Hollibaugh et al., 2011, 2014), where [H<sub>2</sub>O<sub>2</sub>] is high

(10–500 nM), especially during summer (Supplementary Tables 2, 3, Figure 7, Supplementary Figure 4). These Thaumarchaeota are comparably less sensitive to H<sub>2</sub>O<sub>2</sub> (Figures 2E,F, Table 1), and the overall community at Marsh Landing is distinct from open-ocean “ecotypes” (Supplementary Figure 2) and more similar to “*Ca. N. maritimus*” SCM1 (Hollibaugh et al., 2011, 2014). Thus, a more direct test of H<sub>2</sub>O<sub>2</sub> sensitivity using isolates or enrichments could assist in division of Thaumarchaeota into meaningful ecotypes, and may also support the previously described phylogenetic differences between “Group A” (near-surface) and “Group B” (deep) clades (Francis et al., 2005; Beman et al., 2008, 2010; Luo et al., 2014) and their activity (Smith et al., 2014a).

We also observed a discrepancy between H<sub>2</sub>O<sub>2</sub> sensitivity of AO rates vs. *amoA* transcripts, where rates were reduced despite unchanged transcript levels (Figure 5). mRNA has a much shorter half-life (minutes) compared to protein (hours) such as enzymes, which coordinate biochemical reactions measured as rates (reviewed in Moran et al., 2013). Despite this disconnect, some correlations have been found between rates and gene abundances (Smith et al., 2014a), but most have been weak (Beman et al., 2008; Tolar et al., 2016a,b) or non-existent (Santoro et al., 2010). We do not understand cell-level regulation of transcription or translation in these organisms well enough to predict why this disconnect occurs, although it may simply be due to biases introduced by sample collection or processing time (Feike et al., 2012; Moran et al., 2013). Here, we have minimized the effect of such biases by including non-addition controls, and so any inhibition observed is relative to the control. As *amoA* transcript abundance was less impacted by H<sub>2</sub>O<sub>2</sub> additions, this may indicate that ammonia monooxygenase is not transcriptionally inhibited by ROS, but rather by damage to the enzyme itself. In *Nitrosomonas europaea*, direct inhibition of the ammonia monooxygenase enzyme by light has been observed and recovery only occurred if *de novo* protein synthesis was possible, indicating that in AOB transcription was not affected (Hyman and Arp, 1992).

Mechanisms of H<sub>2</sub>O<sub>2</sub>-induced stress include the Fenton reaction, which depends on the presence of reduced Fe and can cause oxidative damage to Thaumarchaeota by the formation of the toxic hydroxyl radical (Imlay, 2008) and inactivation of iron-containing enzymes (Sobota and Imlay, 2011; Anjem and Imlay, 2012). This process occurs within cells; however, membrane-bound enzymes or transporters could also be damaged by exogenous ROS. Damage to enzymes can be reversed (or prevented) by replacement of Fe(III) with a divalent metal, such as manganese (Anjem and Imlay, 2012), and analysis of Thaumarchaeota genomes indicates widespread replacement of Fe by Cu in the active site of many enzymes, including ammonia monooxygenase and proteins in the electron transport system (Walker et al., 2010; Amin et al., 2013). Additionally, the only catalase found in Thaumarchaeota genomes has a manganese cofactor instead of the typical Fe (heme). It is therefore possible that Thaumarchaeota evolved to use alternative metal cofactors in place of Fe to reduce the burden of ROS stress, rather than as a response to low Fe bioavailability (Amin et al., 2013).

We have shown here that ammonia oxidation in the open-ocean is more sensitive to exogenous H<sub>2</sub>O<sub>2</sub> than in coastal environments (average 57% reduction in AO, excluding Marsh Landing; **Table 1**), and that distinct Thaumarchaeota communities inhabit these samples (Supplementary Figure 2). Yet, we do not have enough physiological information on individual Thaumarchaeota “ecotypes” to understand what makes them different, only correlations of diversity with depth (Francis et al., 2005), habitat (Biller et al., 2012), or ammonia concentration (Sintes et al., 2013). It is therefore possible that ROS sensitivity could be a defining trait for division of Thaumarchaeota into meaningful ecotypes, although further work with a diverse group of cultures is necessary. Mesocosm-based manipulation studies on environmental samples should provide a useful approach and crucial first step for our understanding of how AOA will respond to a changing environment.

## AUTHOR CONTRIBUTIONS

BT, LP, WM, and JH generated hypotheses and planned experiments. WM and LP provided methods and equipment for H<sub>2</sub>O<sub>2</sub> analysis, and BT and LP performed experiments and measured H<sub>2</sub>O<sub>2</sub>. BP and NW provided laboratory support and training for <sup>15</sup>N-ammonia oxidation measurements, and NW assisted BT with <sup>15</sup>N-ammonia oxidation sample processing. BT and JH analyzed the data and wrote the paper.

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The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmars.2016.00237/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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