



Acidification Enhances Hybrid N₂O Production Associated with Aquatic Ammonia-Oxidizing Microorganisms

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Ammonia-oxidizing microorganisms are an important source of the greenhouse gas nitrous oxide (N₂O) in aquatic environments. Identifying the impact of pH on N₂O production by ammonia oxidizers is key to understanding how aquatic greenhouse gas fluxes will respond to naturally occurring pH changes, as well as acidification driven by anthropogenic CO₂. We assessed N₂O production rates and formation mechanisms by communities of ammonia-oxidizing bacteria (AOB) and archaea (AOA) in a lake and a marine environment, using incubation-based nitrogen (N) stable isotope tracer methods with ¹⁵N-labeled ammonium (¹⁵NH₄⁺) and nitrite (¹⁵NO₂⁻), and also measurements of the natural abundance N and O isotopic composition of dissolved N₂O. N₂O production during incubations of water from the shallow hypolimnion of Lake Lugano (Switzerland) was significantly higher when the pH was reduced from 7.54 (untreated pH) to 7.20 (reduced pH), while ammonia oxidation rates were similar between treatments. In all incubations, added NH₄⁺ was the source of most of the N incorporated into N₂O, suggesting that the main N₂O production pathway involved hydroxylamine (NH₂OH) and/or NO₂⁻ produced by ammonia oxidation during the incubation period. A small but significant amount of N derived from exogenous/added ¹⁵NO₂⁻ was also incorporated into N₂O, but only during the reduced-pH incubations. Mass spectra of this N₂O revealed that NH₄⁺ and ¹⁵NO₂⁻ each contributed N equally to N₂O by a “hybrid-N₂O” mechanism consistent with a reaction between NH₂OH and NO₂⁻, or compounds derived from these two molecules. Nitrifier denitrification was not an important source of N₂O. Isotopomeric N₂O analyses in Lake Lugano were consistent with incubation results, as ¹⁵N enrichment of the internal N vs. external N atoms produced site preferences (25.0–34.4‰) consistent with NH₂OH-dependent hybrid-N₂O production. Hybrid-N₂O formation was also observed during incubations of seawater from coastal Namibia with ¹⁵NH₄⁺ and NO₂⁻. However, the site preference of dissolved N₂O here was low (4.9‰), indicating that another mechanism, not captured during the incubations, was important. Multiplex sequencing of 16S rRNA revealed distinct ammonia oxidizer communities: AOB dominated numerically in Lake Lugano, and AOA dominated in the seawater. Potential for hybrid N₂O formation exists among both communities, and at least in AOB-dominated environments, acidification may accelerate this mechanism.

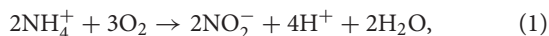
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INTRODUCTION

Ammonia oxidizing bacteria (AOB) and archaea (AOA) are a source of the greenhouse gas nitrous oxide (N₂O) (Goreau et al., 1980; Santoro et al., 2011; Löscher et al., 2012) in soils and aquatic environments. The rate at which these microorganisms produce N₂O depends on the rate at which they carry out chemosynthetic reactions that oxidize ammonia (NH₃) to nitrite (NO₂⁻). However, other environmental factors can enhance their N₂O production rate, such as reduced oxygen (O₂) concentrations (Goreau et al., 1980; Löscher et al., 2012), higher NO₂⁻ concentrations, and higher densities of ammonia-oxidizing cells (Frame and Casciotti, 2010). In soils, pH is another factor that influences N₂O production, with acidic soils generally producing more N₂O than alkaline soils (Martikainen, 1985). Certain lakes and marine environments also experience pH decreases, which may occur naturally as a result of rapid respiration of organic carbon to carbon dioxide (CO₂), or by the dissolution of acid-forming gases (e.g., CO₂, sulfur dioxide, and nitrogen oxides) produced by human activities.

There are several ways in which reducing the pH of aquatic environments (i.e., acidification) may affect the rate of N₂O production by ammonia oxidizers. Some evidence suggests that acidification will cause ammonia oxidation rates to decline. Specifically, the ammonia monooxygenase enzyme (AMO), which catalyzes conversion of NH₃ to the intermediate hydroxylamine (NH₂OH), is thought to act on the free base form of the substrate (NH₃), rather than the protonated form, ammonium (NH₄⁺) (Suzuki et al., 1974; Stein et al., 1997). In the pH range of many natural aquatic systems (pH 6–8) NH₄⁺/NH₃ is mostly present as NH₄⁺ (pK_a = 9.25 at 25°C). Any acidification will further reduce the fraction of NH₄⁺/NH₃ that is present as NH₃, and thus reduce the substrate concentration for ammonia oxidizers.

The net effect of ammonia oxidation is also acidifying, releasing protons (H⁺) to the surrounding environment:



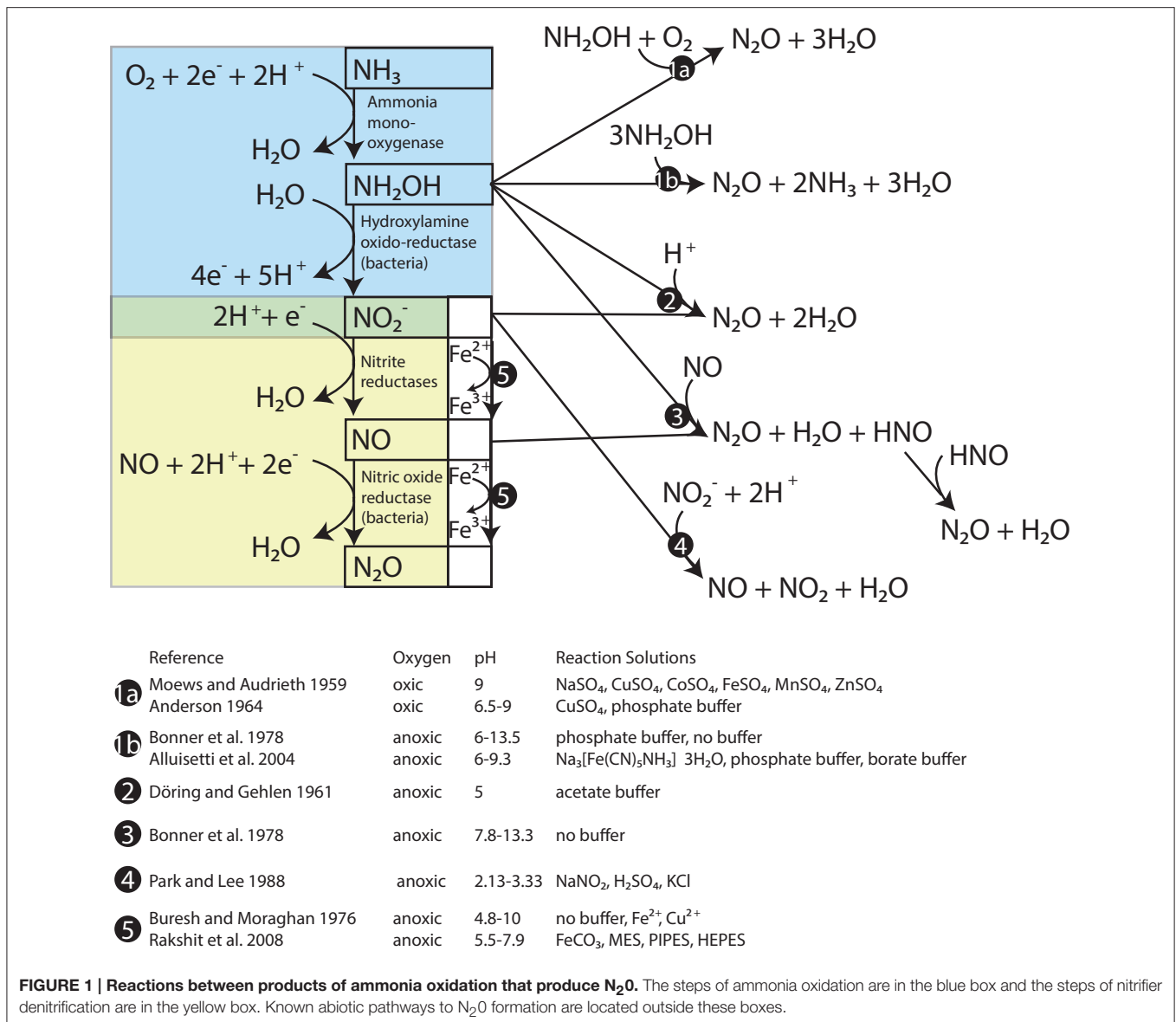
so that, for example, AOB batch cultures that are actively consuming NH₃ are normally exposed to pH decreases as they grow. In these cultures, once the pH drops below ~6.5, further ammonia oxidation is inhibited (Allison and Prosser, 1993; Jiang and Bakken, 1999b). However, the reason for this may not be decreased substrate availability, since decreases in the activity of AOB are not necessarily correlated with reductions in the NH₃ concentration (Jiang and Bakken, 1999a). It is more likely that inhibition is caused by other factors, such as toxic buildup of nitrous acid (HNO₂), nitric oxide (NO), and nitrogen dioxide (NO₂) under acidic conditions (Schmidt and Bock, 1997; Stein and Arp, 1998; Schmidt et al., 2002; Udert et al., 2003; Park and Bae, 2009). Recent environmental studies suggest that ammonia oxidation rates may not have a single relationship to pH. For example, ammonia oxidation rates in the open ocean are inhibited by acidification (from pH 8.1–8.2 down to pH 7.6–7.8; Beman et al., 2011; Rees et al., 2016), whereas

sedimentary ammonia oxidation rates do not seem to be sensitive to acidification (from pH 8 down to 6; Kitidis et al., 2011).

AOA may not be subject to the same growth inhibition as AOB at lower pH ranges. For example, an obligately acidophilic AOA with an optimum pH range of 4–5 was discovered in acidic soil (Lehtovirta-Morley et al., 2011), and in soil pH manipulation experiments, archaeal *amoA* transcript abundances outnumbered those of AOB in acidic soils (Nicol et al., 2008), suggesting that AOA may outcompete AOB in acidic environments. Marine AOA, which are generally regarded as more important than AOB to ammonia oxidation in the ocean (Wuchter et al., 2006), may also be more tolerant of acidic conditions. For example, certain marine AOA strains are capable of maintaining near-maximal growth rates down to a pH of 5.9 (Qin et al., 2014), perhaps because they express NH₄⁺-transport proteins that actively transport NH₄⁺ into AOA cells, thus supplying AMO with NH₃ under acidic conditions (Lehtovirta-Morley et al., 2011, 2016).

Unlike NO₂⁻, N₂O is not the major nitrogenous product of ammonia oxidation, and it is not known to what degree the reactions that produce N₂O are convolved with the main energy-harnessing reactions of ammonia oxidizers (i.e., NH₃ oxidation to NH₂OH and then to NO₂⁻). This means that the impact of pH on the N₂O production rate may be decoupled from its impact on the ammonia oxidation rate. That is, even if acidification decreases the ammonia oxidation rate, the N₂O production rate may not necessarily also decrease proportionally. In fact, many of the reactive nitrogen oxides produced during ammonia oxidation undergo N₂O-forming reactions over relevant timescales, with or without enzyme catalysis, and with their own pH-dependencies.

One of these nitrogen oxides is NH₂OH, which is the enzymatic product of NH₃ oxidation by AMO in both AOB and AOA (Figure 1, blue box; Hofman and Lees, 1953; Vajjala et al., 2013). Although most NH₂OH is converted to NO₂⁻ during active ammonia oxidation, NH₂OH is also subject to abiotic autoxidation (Figure 1, pathway 1a) and disproportionation reactions (Figure 1, pathway 1b) that produce N₂O as well as nitrogen (N₂), nitric oxide (NO), and NH₃/NH₄⁺. N₂O yields during these reactions vary with alkalinity (Bonner et al., 1978), redox conditions (Moews and Audrieth, 1959; Pacheco et al., 2011), and the presence of certain transition metals (Anderson, 1964; Alluisetti et al., 2004). NH₂OH may also react with NO₂⁻/HNO₂ to produce N₂O (Figure 1, pathway 2). This reaction occurs abiotically at a rate that accelerates as pH decreases (Döring and Gehlen, 1961; Bonner et al., 1983). It can also be catalyzed by the copper- and iron-containing NO₂⁻ reductases of certain denitrifying bacteria (Iwasaki et al., 1963; Kim and Hollocher, 1984), as well as soluble enzyme extracts of AOB that have an acidic optimum pH (Hooper, 1968). A reaction such as pathway 2 could explain the “hybrid” N₂O production observed in AOA cultures, where one NO₂⁻-derived N atom and one NH₃-derived N atom (e.g., from NH₂OH) were combined into the same N₂O molecule (Stieglmeier et al., 2014b). Furthermore, Harper et al. (2015) found that this hybrid reaction between NH₂OH and NO₂⁻ was responsible for most of the N₂O produced by activated sludge during bioreactor experiments. NH₂OH may also react abiotically with NO to form N₂O and



N₂ in proportions that are pH-dependent (Figure 1, pathway 3; Bonner et al., 1978). In terms of tracing the source compounds contributing N to N₂O, NO can be derived abiotically from HNO₂ through a disproportionation reaction (Figure 1, pathway 4; Park and Lee, 1988), and the reaction of HNO₂-derived NO with NH₂OH could also produce a hybrid type N₂O. However, abiotic disproportionation HNO₂ tends to be most important only in very acidic environments (pK_a HNO₂ = 2.8; Riordan et al., 2005).

Reduction of NO₃⁻ and NO₂⁻ by trace metal ions (Buresh and Moraghan, 1976) and metal-containing minerals (e.g., Rakshit et al., 2008) is known as chemodenitrification (Figure 1, pathway 5). In this process, reduced metal species, particularly Fe²⁺ (and possibly also Mn²⁺) are oxidized, and NO, N₂O, and N₂ are produced (Picardal, 2012). This pathway has a recognized importance in soils (Zhu-Barker et al., 2015), but is less studied in seawater and eutrophic lake water, which typically have

much lower metal concentrations (Morel et al., 2003) than soil. Reducing sediments along productive continental margins may support significant rates of chemodenitrification (Scholz et al., 2016).

Enzymatic reduction of NO₂⁻ to NO and N₂O in AOB is known as nitrifier denitrification (Figure 1, yellow box). This pathway produces N₂O whose N atoms are both derived from NO₂⁻.¹ The existence of this pathway in AOB was confirmed in cultures of *Nitrosomonas europaea* by the production of N₂O with a molecular mass of 46 (⁴⁶N₂O = ¹⁵N¹⁵N¹⁶O) after tracer additions of ¹⁵NO₂⁻ (Poth and Focht, 1985). However, in similar experiments with AOA cultures, Stieglmeier et al. (2014b) observed no ⁴⁶N₂O production, even at low O₂ concentrations that are thought to stimulate

¹For clarity in this paper, we will reserve the term nitrifier denitrification for this specific chain of enzymatic reactions and will not use it for other forms of reductive N incorporation from NO₂⁻ into N₂O.

nitrifier denitrification in AOB (Goreau et al., 1980). Similarly, microrespirometry measurements of *Nitrososphaera viennensis* cultures indicate that this AOA does not reduce NO₂⁻ to N₂O (Kozłowski et al., 2016).

In AOB, NO produced by nitrite reduction is converted to N₂O by a membrane-bound NO reductase (NOR) that reduces 2NO to N₂O (Figure 1, yellow box; Beaumont et al., 2004b; Kozłowski et al., 2014). In some denitrifiers, the NOR homolog that carries out the same reduction of NO to N₂O, has a neutral to acidic pH optimum (5–7.6; Hoglen and Hollocher, 1989) raising the possibility that this step in nitrifier denitrification also has a slightly acidic pH optimum. Among AOA, however, no homologs for the catalytic subunit of bacterial NOR (*norB*) have been found in any sequenced genomes to date (Santoro et al., 2015), confirming tests of AOA cultures that indicate that nitrifier denitrification does not occur in these organisms (Stieglmeier et al., 2014b; Kozłowski et al., 2016).

NO is a precursor of N₂O during bacterial nitrifier denitrification, but its production and consumption may be involved in other processes in ammonia oxidizers. For example, NO is an intermediate in the catalytic cycle of hydroxylamine oxidoreductase (HAO) (Cabail and Pacheco, 2003), an enzyme that oxidizes NH₂OH to NO₂⁻ in AOB (Figure 1, blue box). NO production is also required for AOA to carry out their ammonia oxidation cycle (Shen et al., 2013; Martens-Habbena et al., 2015; Kozłowski et al., 2016), though no HAO homologs have been identified among AOA (Hallam et al., 2006; Walker et al., 2010).

Field studies assessing the importance of N₂O production pathways in aquatic environments have relied on two approaches to date: (1) ¹⁵N tracer incubation studies that track the incorporation of N derived from ¹⁵N-labeled precursor molecules, and (2) dissolved N₂O measurements of the bulk O and N stable isotopic composition as well as the intramolecular distribution of ¹⁵N and ¹⁴N between the internal and external N atoms of the linear, asymmetrical N₂O molecule (known as site preference; SP = δ¹⁵N^{internal} - δ¹⁵N^{external}; Toyoda and Yoshida, 1999). The SP signature can be useful for distinguishing N₂O production pathways because it is often (but not always) independent of the isotopic composition of the starting compounds (Yang et al., 2014). Using the first approach, Nicholls et al. (2007) and Trimmer et al. (2016) may have observed hybrid N₂O formation by an ammonia oxidizer community immediately above the oxygen minimum zone (OMZ) of the Arabian Sea and in the Eastern Tropical North Pacific, respectively, where they observed ⁴⁵N₂O but not ⁴⁶N₂O production during tracer incubations with ¹⁵NH₄⁺ and NO₂⁻ with a natural abundance (NA) isotopic composition. In studies using the second approach, profiles of the SP of N₂O have been used to distinguish N₂O produced by NH₂OH-dependent pathway(s), which have a distinctly higher SP (~34‰; e.g., Sutka et al., 2006; Heil et al., 2014; Frame and Casciotti, 2010) than N₂O that is formed during denitrification and nitrifier denitrification, which has a much lower SP (0 to -5‰; Toyoda et al., 2005; Sutka et al., 2006; Yamazaki et al., 2014).

Here we have used profiles of dissolved inorganic N concentrations (NH₄⁺, NO₃⁻, NO₂⁻, and N₂O) and the natural abundance isotopic composition of NO₃⁻, NO₂⁻, and N₂O to

locate depths where ammonia oxidation and/or N₂O production are important in the water columns of Lake Lugano, a human-impacted lake in southern Switzerland, and the marine upwelling zone off the Namibian coast of southwestern Africa. N₂O isotope and site preference profiles were used to identify the likely pathways of N₂O production and the involved substrates/intermediates in the two environments. Short (24–30 h) incubations with ¹⁵N-tracers (¹⁵NH₄⁺ and ¹⁵NO₂⁻) at targeted depths revealed that hybrid N₂O formation occurred in both the shallow hypolimnion of Lake Lugano, as well as in water from the Namibian upwelling zone. Furthermore, N₂O yields produced during incubations of Lake Lugano water were significantly higher when the pH was reduced experimentally. The isotopic composition of the N₂O that was produced indicated that the increase was due, at least in part, to enhanced incorporation of N derived from exogenous NO₂⁻. Multiplex sequencing of microbial 16S rRNA from the incubation locations indicated that AOB numerically dominated the ammonia-oxidizing community in Lake Lugano whereas AOA dominated in the Namibian Upwelling zone. The lines of evidence presented here suggest that there is potential, at least over the short term, for acidification to enhance hybrid N₂O formation in aquatic environments.

METHODS

Sampling

Lake Lugano is separated into a permanently stratified northern basin and a monomictic southern basin. This study focuses on the 95 m-deep southern basin. Water samples and incubation water were collected with a 5L Niskin bottle at the Figino Station (45.95°N, 8.90°E) during a sampling campaign on November 5, 2013. Profiles of dissolved O₂, temperature, salinity, and pH were collected by a conductivity, temperature, and depth sensor (CTD). O₂ profiles were calibrated by Winkler titration. Water from the Namibian Upwelling zone was collected by hydrocast with a 10L-Niskin bottle rosette at station 89 (20.65°S, 10.95°E) on January 28, 2014 during the NamUFil cruise of the R/V *Meteor*.

Geochemical Profiles

Water samples for NH₄⁺ and NO₃⁻ concentrations, as well as NO₃⁻ isotope measurements were immediately filtered through 0.22 μm-pore sterivex filters (Millipore) and then frozen within 2 h of sampling. NH₄⁺ concentrations were measured fluorometrically (Holmes et al., 1999). NO₂⁻ concentrations were determined by converting NO₂⁻ present in 10 ml of sample water to N₂O by azide reduction (McIlvin and Altabet, 2005) and then quantifying the amount of N₂O in each sample by gas chromatography-isotope ratio mass spectrometry (GC-IRMS, see below). NO₂⁻ concentration standards were prepared in 10 ml of distilled water and in lake-water or seawater, and were analyzed by GC-IRMS along with the samples. For NO₃⁻ concentration and isotopic measurements, sulfamic acid was used to remove NO₂⁻ prior to analysis (Granger and Sigman, 2009). NO₃⁻ concentrations were measured by reduction to NO with Vanadium (III) and chemiluminescence detection (Braman

and Hendrix, 1989). Nitrate N and O isotope measurements of duplicate samples were performed by conversion of NO₃⁻ to N₂O using the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002) and subsequent purification and analysis of this N₂O with a modified purge-and-trap gas bench GC-IRMS (Thermo Finnigan DeltaV Plus) system. Isotopic calibration was performed by concurrent analysis of NO₃⁻ isotope standards USGS 32, USGS 34, and USGS 35 (Casciotti et al., 2008). N and O isotopic data are reported on the permil (‰) scale referenced to air N₂ and Vienna Standard Mean Ocean Water (VSMOW), respectively ($\delta^{15}\text{N} = ([^{15}\text{N}]/[^{14}\text{N}]_{\text{sample}} / [^{15}\text{N}]/[^{14}\text{N}]_{\text{air-N}_2} - 1) \times 1000\text{‰}$ and $\delta^{18}\text{O} = ([^{18}\text{O}]/[^{16}\text{O}]_{\text{sample}} / [^{18}\text{O}]/[^{16}\text{O}]_{\text{VSMOW}} - 1) \times 1000\text{‰}$).

Samples for N₂O concentration and isotope analyses were taken by overfilling 160 ml glass sample bottles twice from the bottom through a plastic hose connected to the Niskin outlet. The Lake Lugano N₂O samples were preserved by adding 100 µl of saturated mercuric chloride solution (HgCl₂) after a headspace was added by pipetting 1 ml of water off the top of each bottle. Each bottle was then sealed with a butyl rubber septum (VWR, 5483369) and aluminum crimps (CS Chromatographie, 300219). The marine samples were preserved by adding 5 ml of 10 M sodium hydroxide (NaOH) to the bottom of each bottle with a syringe (Mengis et al., 1997), pipetting 1 ml of water off the top for headspace, sealing with butyl septa and aluminum crimps, and then shaking vigorously to distribute the NaOH. Lake Lugano N₂O samples were analyzed within 1 week of collection. Marine samples were analyzed within 3 months of collection. The total N₂O in each sample was purged with carrier helium directly into a customized purge-and-trap system (McIlvin and Casciotti, 2010) and analyzed by continuous-flow GC-IRMS. Duplicate N₂O samples at each depth were collected for the Lake Lugano profile and one sample from each depth was analyzed for the Namibian Upwelling profile. N₂O isotope ratios were referenced to N₂O injected from a reference N₂O tank ($\geq 99.9986\%$, Messer) calibrated on the Tokyo Institute of Technology scale (Mohn et al., 2012) for bulk and site-specific isotopic composition by J. Mohn (EMPA, Switzerland). Ratios of m/z 45/44, 46/44, and 31/30 signals were converted to $\delta^{15}\text{N-N}_2\text{O}$ (referenced to N₂_AIR), $\delta^{18}\text{O-N}_2\text{O}$ (referenced to Vienna Standard Mean Ocean Water), and site-specific $\delta^{15}\text{N}^\alpha$ and $\delta^{15}\text{N}^\beta$ -N₂O according to Frame and Casciotti (2010), with an additional two-point correction (Mohn et al., 2014) using measurements of two isotopic mixtures of N₂O in synthetic air (CA-06261 and 53504; kindly provided by J. Mohn). N₂O concentrations were calculated by converting the N₂O sample peak areas measured by GC-IRMS to N₂O standards prepared by converting NO₃⁻ to a known quantity of N₂O by the denitrifier method (McIlvin and Casciotti, 2010). At each depth, temperature and salinity data were used to calculate the N₂O concentrations at equilibrium with the atmosphere according to Weiss and Price (1980), based on atmospheric partial pressures reported by NOAA ESRL Global Monitoring Division (<http://esrl.noaa.gov/gmd/>). The saturation disequilibrium ($\Delta\text{N}_2\text{O}$) was calculated as the difference between the measured N₂O concentration and the atmospheric equilibrium concentration, with positive values corresponding to oversaturation.

Incubations

A list of all incubation treatments is provided in **Table 1**. Water for the Lake Lugano incubations that was collected at 17 m depth was poured into opaque 10 L HDPE canisters (Huber, 15.0250.03), stored in the dark for ~5 h during transport back to the laboratory, and then amended with ¹⁵N-labeled incubation reagents. Water for the seawater incubations was drawn from 200 m depth and immediately mixed with the ¹⁵N-labeled substrates inside 3.4 L LDPE drinking water containers (Campmor, 81027). A dilution (1:2) of 30% hydrochloric acid (Fluka TraceSelect, 96208) with milliQ water was added to water for reduced-pH incubations of Lake Lugano water and mixed immediately before NH₄⁺ and NO₂⁻ substrates were added. Tracer ¹⁵NH₄Cl (98.5%) and Na¹⁵NO₂ (99.2%) purchased from Cambridge Isotope Laboratories (NLM-658 and NLM-467) were paired, respectively, with NaNO₂ and NH₄Cl with natural abundance (NA) isotopic compositions, so that each incubation received either 1 µM ¹⁵NH₄⁺ + 1 µM NA NO₂⁻ or 1 µM NA NH₄⁺ + 1 µM ¹⁵NO₂⁻. For each set of experimental conditions (**Table 1**), 10 acid-washed 160 ml glass incubation bottles (Wheaton, 223748) were rinsed with milliQ and lake or sea water, and then filled with 120 ml of incubation water and closed with gray fluorobutyl PTFE-lined septa (National Scientific, C4020-36AP) and aluminum crimps. Headspace O₂ concentrations of the reduced-O₂ incubations were adjusted by displacement with either high purity (99.999%) helium for the Lake Lugano incubations, or N₂ for the Namibian Upwelling incubations. O₂ in the headspace was quantified using a gas chromatograph with an electron-capture detector (SRI 8610C), and O₂ concentrations were calculated according to Weiss and Price (1980). During the Lake Lugano incubations, one bottle was sacrificed at the beginning of the incubation, and three bottles each were sacrificed after ~5, 17, or 30 h. During the Namibian Upwelling incubations, one bottle for each set of experimental conditions was sacrificed at the beginning of each incubation, and three bottles were sacrificed after ~12 and ~24 h. Bottles were incubated in the dark at 20–21°C for both experiments.

Immediately before an incubation bottle was sacrificed, 40 ml of liquid was withdrawn by syringe for colorimetric pH measurements with phenol red (Robert-Baldo et al., 1985) or frozen at -80°C and then stored at -20°C prior to measurements of concentration and dissolved N isotope composition. Seawater incubation samples were also filtered through polycarbonate membrane filters (Whatman nuclepore) with 0.22 µm pores before freezing. NH₄⁺ and NO₃⁻ concentration measurements were made as described above. The initial ¹⁵N atom fraction ($^{15}\text{F} = [^{15}\text{N}]/[^{15}\text{N} + ^{14}\text{N}]$) of NH₄⁺ was calculated at the beginning of the incubation using the added tracer concentration and the ambient concentration of NH₄⁺ before tracer addition, and assuming that the $\delta^{15}\text{N}$ of ambient NH₄⁺ was 0‰. The ¹⁵F of NH₄⁺ was not measured during the incubations. The ¹⁵F of NO₂⁻ during the incubations was measured using the azide reduction/GC-IRMS method (McIlvin and Altabet, 2005), and the ¹⁵F of NO₃⁻ was measured using the denitrifier method (Sigman et al., 2001) after removal of NO₂⁻ with sulfamic acid. NO_x⁻-derived N₂O with a ¹⁵N composition <15% was analyzed using the IRMS manufacturer's resistor (3

TABLE 1 | Summary of experimental conditions for the Lake Lugano and Namibian Upwelling experiments.

Location	Name	pH	[O ₂] μM	Tracers	Timepoints (hours)
Lake Lugano	control-pH, control-O ₂	7.54 ± 0.02	290 ± 14	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 5, 17, 30
Lake Lugano	control-pH, reduced-O ₂	7.54 ± 0.02	70 ± 10	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 5, 17, 30
Lake Lugano	Reduced-pH, control-O ₂	7.20 ± 0.02	290 ± 14	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 5, 17, 30
Lake Lugano	reduced-pH, reduced-O ₂	7.20 ± 0.02	70 ± 9	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 5, 17, 30
Namibian upwelling	control-O ₂	–	220 ± 12	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 24
Namibian upwelling	reduced-O ₂	–	50 ± 10	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 24
Namibian upwelling	reduced-O ₂	–	20 ± 10	1) 1 μM ¹⁵ NH ₄ ⁺ + 1 μM NA NO ₂ ⁻ 2) 1 μM NA NH ₄ ⁺ + 1 μM ¹⁵ NO ₂ ⁻	0, 24

× 10¹⁰ Ω) and capacitor pairing on the m/z 45 Faraday cup. For more ¹⁵N-enriched N₂O, the resistance on the m/z 45 cup was reduced to 3 × 10⁸. Internal isotope standards for ¹⁵FNO₂⁻ and ¹⁵FNO₃⁻ were prepared in triplicate by mixing NA NaNO₂ or KNO₃ of known δ¹⁵N values with either 99.2% Na¹⁵NO₂ or 98.5% Na¹⁵NO₃ (Cambridge Isotope Laboratories, NLM-157). The total N₂O in each incubation bottle was analyzed for concentration and m/z ion ratios 45/44 and 46/44 as described for natural abundance stable isotope measurements of water column N₂O samples. Additional information about converting IRMS measurements to ⁴⁵N₂O and ⁴⁶N₂O production rates is provided in the Supplementary Material S.1.

DNA Extraction

Water from the incubation depth was filtered through a single 0.22 μm-pore size 47 mm polycarbonate Nuclepore filter (Whatman). The volume of water filtered from the Namibian Upwelling was 4 L and from the Lake Lugano was 0.3 L. Filters were frozen immediately and stored at ≤ -80°C until extraction. DNA from either environment was extracted using the FastDNA spin kit for soil (MP Biomedicals).

Illumina 16S rRNA Library Generation

The polymerase chain reaction (PCR) was used to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene of prokaryotes using universal 16S rRNA V4 primers F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3'; Caporaso et al., 2011). Forward and reverse primers were barcoded and appended with Illumina-specific adapters (Kozich et al., 2013). PCR amplifications were carried out using BioReagents™ exACTGene™ Complete PCR Kit and Core Reagent Sets (Thermo Fisher Scientific) for 30 cycles (Caporaso et al., 2012). Agarose gel electrophoresis was used to separate

PCR products of the correct size that were then band-excised and recovered using a QIAquick gel extraction kit (Qiagen). For each library, triplicate PCR products were combined and quantified with a Qubit fluorometric assay (ThermoFisher Scientific) and pooled at equimolar ratios. The final pool was analyzed on an Agilent 2100 Bioanalyzer System (Agilent Technologies) using a High Sensitivity DNA chip to determine the average size of the amplicon pool. Quantitative PCR was performed on the pool using a Biorad IQ5 real time thermocycler (Bio-Rad Laboratories) and Illumina quantification standards (KAPA Biosystems). The samples were sequenced on an Illumina MiSeq (<http://www.illumina.com/systems/miseq.ilmn>) using 201 nucleotide paired-end multiplex sequencing with 5% PhiX spiked into the run. The library was sequenced on a single flow cell using V2 sequencing reagents, generating paired reads of ~400 bp, with ~150 bp overlap between forward and reverse reads.

Bioinformatic Analyses

Raw 16S rRNA sequence data were initially processed in Basespace using Illumina's metagenomic pipeline (<https://basespace.illumina.com/home/index>). Merging the paired reads and further analyses were carried out using Axiome with installed PANDAseq and the Quantitative Insights Into Microbial Ecology (QIIME v.1.8.0) software pipeline, including taxaplot for overall bacterial diversity, and calculations of Chao1 to estimate taxon richness and rarefaction curves to calculate species richness for a given number of individual sequences sampled (Caporaso et al., 2010; Masella et al., 2012; Lynch et al., 2013). On PANDAseq, the minimum overlap length was set at the threshold of 0.9. The read length maximum for all sequences was 253 bp. Since the V4 region of the 16S rRNA gene is conserved,

reads were removed from further analysis if at least one of the following criteria was met: reads were ≥ 4 bp shorter than the maximum mentioned above, the number of ambiguous bases was ≥ 1 , homopolymers with > 4 bp were present, or sequences did not match any sequences in the database by more than 97% based on the percent coverage in BLAST. The clustering of the sequences into operational taxonomic units (OTUs) was initially performed using UCLUST (Edgar, 2010; Edgar et al., 2011) with a cutoff value of 97% sequence identity. The taxonomic identity of a representative sequence from each cluster was classified using the RDP classifier (Wang et al., 2007) and Greengenes datafiles compiled in October 2012 (downloaded from: <http://greengenes.lbl.gov/>), which includes chimera screening based on 16S rRNA gene records from GenBank (DeSantis et al., 2006). The confidence threshold was set at the default cutoff value of 80% in order to retrieve potential sequences from the nitrifying taxa Thaumarchaeota and Nitrosomonadaceae.

Phylogenetic Analyses

Phylogenetic trees were constructed using MEGA 7 (Kumar et al., 2016). The 16S rRNA gene sequences of representative AOA and AOB were downloaded from GenBank (Benson et al., 2015) for all phylogenetic analyses. Gene sequences were aligned using the MUSCLE algorithm in MEGA 7 and manually inspected. The final alignments consisted of 253 characters, comprising 481 and 108 taxa for sequences related to AOA and AOB, respectively. Phylogenetic reconstruction was implemented using Maximum Parsimony (MP) and Maximum Likelihood (ML). MP was implemented with complete deletion if gaps were present, and tree-bisection-reconnection (TBR) utilized for tree generation. The resulting trees were obtained via random stepwise addition of sequences, at MP search level 1, with 25 initial trees generated from a heuristic search. ML was implemented using Tamura-Nei model of nucleotide substitution rates, with tree inference based on Nearest-Neighbor-Interchange (NNI). Statistical support for MP and ML trees were obtained from 1000 bootstrap replicates under the same initial settings (only bootstrap values $> 50\%$ are reported). Pairwise base comparisons of OTUs and their closest relatives were determined using BLAST (Altschul et al., 1990) and reported as % identity values.

Statistical and Ecological Analyses

The abundances (frequencies) of ammonia oxidizer OTUs in the Namibian Upwelling and Lake Lugano samples were counted using a program written in Python (v.2.7) (<https://www.python.org/download/releases/2.7>) to search for each OTU that was verified by the above phylogenetic analyses (Lau et al., 2015). The Shannon-Weiner Index and Pielou Evenness were calculated using the BiodiversityR package (Kindt and Coe, 2005) in R version 3.2.2 (<http://www.R-project.org/>). All partial 16S rRNA gene sequence data are available through the European Bioinformatics Institute (EBI) with project accession number PRJEB11492. The nucleotide sequences for the partial 16S rRNA genes have been deposited in EBI under individual accession numbers (LN908279-LN908785).

RESULTS

Geochemical Profiles

Lake Lugano

The 17-m incubation depth in Lake Lugano corresponded to the shallow local minima in both pH (7.47) and O₂ concentration (58 μM ; **Figures 2A,B**). Below 15 m, the respective NH₄⁺ and NO₂⁻ concentrations dropped from their surface values of ~ 3.8 μM and ~ 1.2 μM to ~ 0.9 μM and ≤ 0.3 μM , respectively (**Figures 2C,D**). In contrast, N₂O concentrations increased steadily from a surface concentration of 11 nM to 37 nM at 50 m ($\Delta\text{N}_2\text{O} = 22$ nM; **Figure 2E**). The $\delta^{15}\text{N}\text{-N}_2\text{O}$ dropped from a surface value of 4.9‰ to a minimum of 2.4‰ at 15 m and then increased again to 4.9‰ at 50 m (**Figure 2F**). In contrast, the $\delta^{18}\text{O}\text{-N}_2\text{O}$ and SP increased in parallel, from the surface values of 44.5‰ and 15.3‰, respectively, to values of 47.2‰ and 34.4‰, respectively, at 50 m (**Figures 2G,H**). Like N₂O, NO₃⁻ concentrations increased between the surface (58 μM) and 50 m (92 μM ; **Figure 2I**) and the $\delta^{15}\text{N}\text{-NO}_3^-$ profile had a distinct minimum around 15–17 m (**Figure 2J**), corresponding with the $\delta^{15}\text{N}\text{-N}_2\text{O}$ minimum. However, the shape of the $\delta^{18}\text{O}\text{-NO}_3^-$ profile was the mirror opposite of the $\delta^{18}\text{O}\text{-N}_2\text{O}$ profile, with $\delta^{18}\text{O}\text{-NO}_3^-$ decreasing from 6.7‰ in the surface to 1.6‰ at 50 m (**Figure 2K**). Surface NO₂⁻ was depleted in ¹⁵N relative to both NO₃⁻ and N₂O, with $\delta^{15}\text{N}\text{-NO}_2^-$ values between -29% and -27% (**Figure 2L**).

With the onset of seasonal anoxia in the south basin of Lake Lugano (Lehmann et al., 2004; Bleses et al., 2014), the sediments (95 m) and deep redox transition zone (70–90 m) become important in the production and consumption of deep N₂O in this system (Freymond et al., 2013; Wenk et al., 2016). Here we have restricted our discussion to the top 50 m of the water column. See Wenk et al. (2014) and Wenk et al. (2016) for details about N cycle dynamics in the redox transition zone.

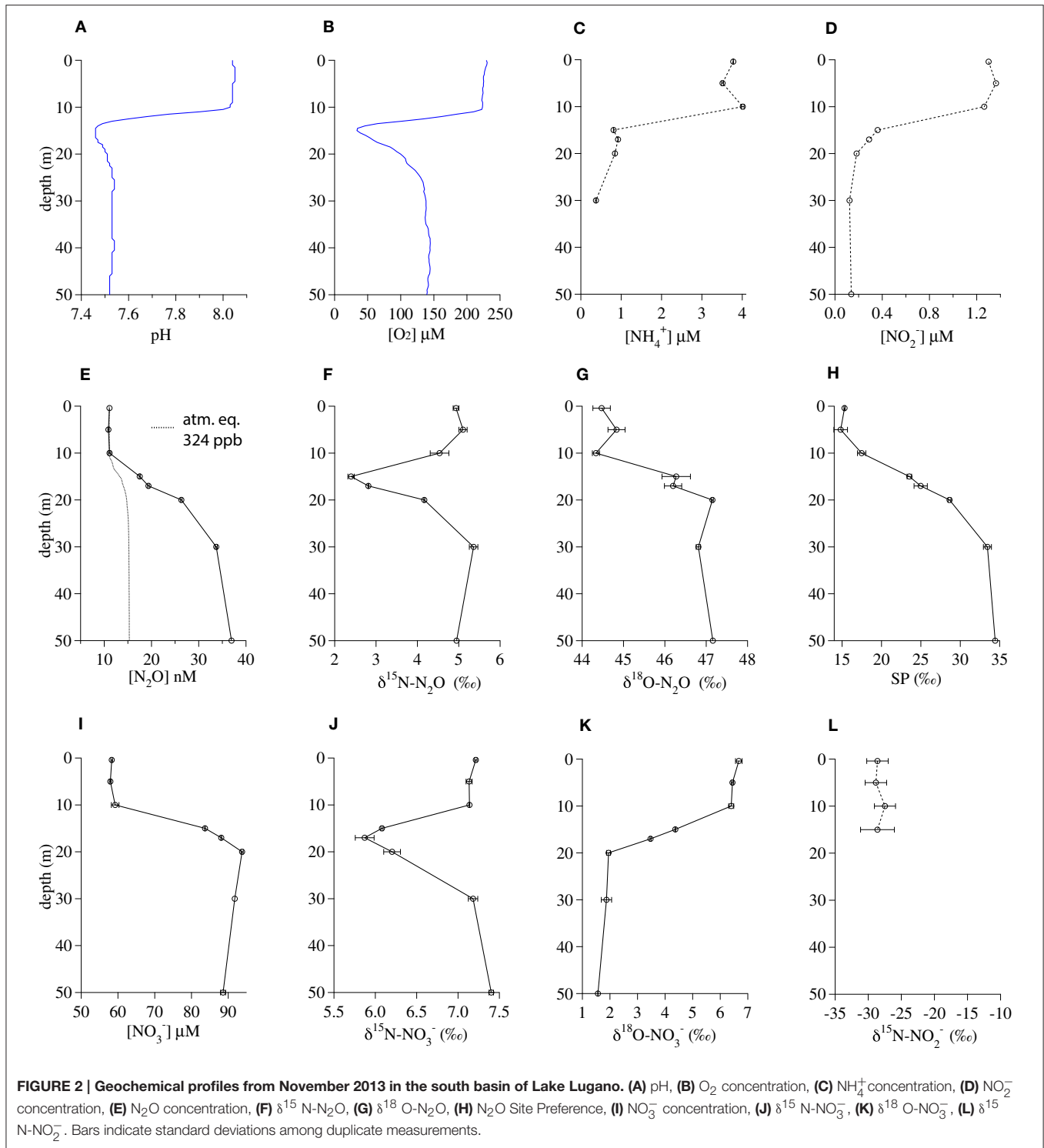
Namibian Upwelling Zone

The salinity (35.06) and potential temperature (11.74°C) of the water at the 200-m incubation depth (**Figure 3A**) was characteristic of a deeper tropical branch of South Atlantic Central Water that flows northwestward along the African continental shelf at this latitude (Brea et al., 2004). The *in situ* O₂ concentration (56.8 μM) at 200 m fell within a broader O₂-depleted zone extending from 150 to 500 m (**Figure 3B**). The incubation depth was well-below the depth of the NH₄⁺ concentration maximum (30 m, **Figure 3C**) and the NO₂⁻ maximum (50 m, **Figure 3D**). N₂O oversaturation ($\Delta\text{N}_2\text{O}$) was greatest between 200 and 400 m (**Figure 3E**). At 200 m, there were minima in $\delta^{15}\text{N}\text{-N}_2\text{O}$ ($5.1 \pm 0.02\%$, **Figure 2F**), $\delta^{18}\text{O}\text{-N}_2\text{O}$ ($39.3 \pm 0.24\%$, **Figure 3G**), and SP ($4.9 \pm 0.4\%$, **Figure 3H**). NO₃⁻ concentrations were near zero in the surface water and increased with depth to a near-maximal concentration of 29 μM at 200 m (**Figure 3I**). At this depth, NO₃⁻ had a $\delta^{15}\text{N}$ ($5.3 \pm 0.2\%$, **Figure 3J**) similar to that of N₂O and a $\delta^{18}\text{O}$ ($3.0 \pm 0.3\%$, **Figure 3K**) that was much lower than that of N₂O.

Nitrification Rates

Lake Lugano

Ammonia oxidation rates were estimated in two ways, using data from either the ¹⁵NH₄⁺ incubations or the ¹⁵NO₂⁻ incubations.



The first method was by linear regression of the [¹⁵N_{NOX-}] produced over time during the ¹⁵NH₄⁺ incubations (**Figure 4A**) multiplied by an isotopic dilution factor ($1/^{15}F_{NH_4+0} = [^{15}NH_4+ + ^{14}NH_4+]/[^{15}NH_4+]$) calculated from the added tracer, the concentration of NH₄⁺ present in the water before tracer addition, and assuming that this NH₄⁺ had a δ¹⁵N of 0‰.

The pH reduction from 7.54 to 7.20 was associated with a 12% decrease in ammonia oxidation rates in incubations at the untreated-O₂ concentrations (rates calculated in this way were $0.356 \pm 0.006 \mu\text{M/day}$ in the untreated-pH incubations and $0.314 \pm 0.027 \mu\text{M/day}$ in the reduced-pH incubations). Among the reduced-O₂ incubations, those with a reduced pH had 14%

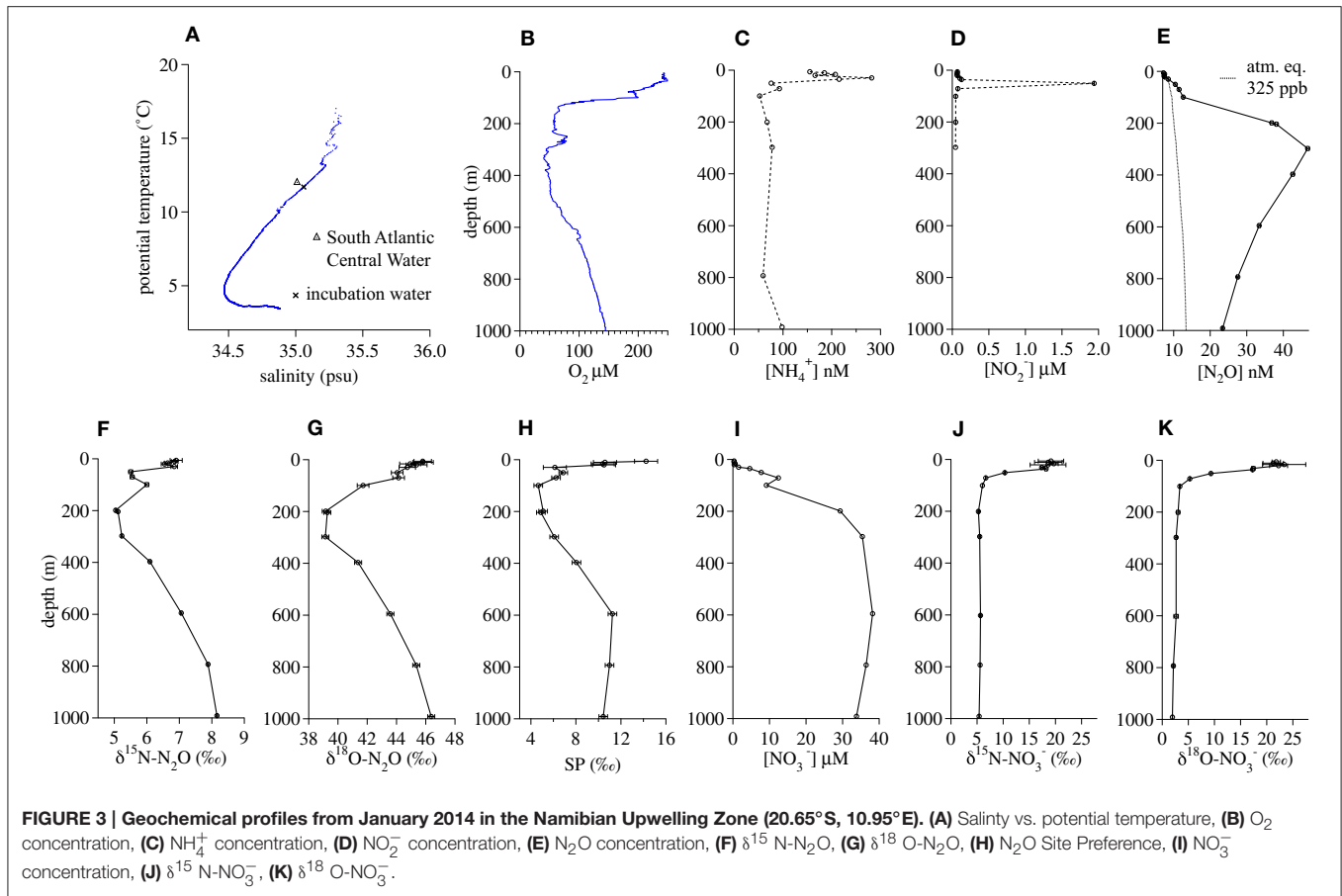


FIGURE 3 | Geochemical profiles from January 2014 in the Namibian Upwelling Zone (20.65°S, 10.95°E). (A) Salinity vs. potential temperature, (B) O₂ concentration, (C) NH₄⁺ concentration, (D) NO₂⁻ concentration, (E) N₂O concentration, (F) δ¹⁵N-N₂O, (G) δ¹⁸O-N₂O, (H) N₂O Site Preference, (I) NO₃⁻ concentration, (J) δ¹⁵N-NO₃⁻, (K) δ¹⁸O-NO₃⁻.

lower ammonia oxidation rates than those at the untreated pH (0.325 ± 0.004 μM/day and 0.380 ± 0.005 μM/day, respectively). Because we did not measure the ¹⁵F_{NH4+} over the course of the ¹⁵NH₄⁺ incubations, we were not able to account for the dilution of the tracer ¹⁵NH₄⁺ over time by regeneration of NH₄⁺, or removal of NH₄⁺ from the system by processes other than ammonia oxidation (Ward and Kilpatrick, 1990). Therefore, in a second approach, we used the results of the incubations with ¹⁵NO₂⁻ and NA NH₄⁺ to calculate zero-order rates of ammonia oxidation (R_{amm_ox}) and nitrite oxidation (R_{nit_ox}). Specifically, rates were calculated for each timepoint (t) using measurements of the ¹⁵N atom fractions of NO₂⁻ (¹⁵F_{NO₂⁻}, Figure 4B) and NO₃⁻ (¹⁵F_{NO₃⁻}, Figure 4C), and the total concentrations of NO₂⁻ ([NO₂⁻], Figure 4D) and NO₃⁻ ([NO₃⁻], Figure 4E) to solve the following equations:

$$[^{14}\text{NO}_2^-](t) = R_{\text{amm_ox}} \times t - (1 - ^{15}\text{F}_{\text{NO}_2^-}) \times R_{\text{nit_ox}} \times t + [^{14}\text{NO}_2^-]_0 \quad (2)$$

$$[^{15}\text{NO}_2^-](t) = -(^{15}\text{F}_{\text{NO}_2^-}) \times R_{\text{nit_ox}} \times t + [^{15}\text{NO}_2^-]_0 \quad (3)$$

$$[^{14}\text{NO}_3^-](t) = (1 - ^{15}\text{F}_{\text{NO}_2^-}) \times R_{\text{nit_ox}} \times t + [^{14}\text{NO}_3^-]_0 \quad (4)$$

$$[^{15}\text{NO}_3^-](t) = (^{15}\text{F}_{\text{NO}_2^-}) \times R_{\text{nit_ox}} \times t + [^{15}\text{NO}_3^-]_0 \quad (5)$$

Ammonia oxidation rates calculated this way were similar but somewhat higher than those derived from the ¹⁵NH₄⁺

incubation data. Average rates calculated for incubations terminated at the middle timepoint (~17 h) and final timepoint (~30 h) were 0.50 ± 0.02 μmol/day for the untreated-O₂—untreated-pH incubations, 0.48 ± 0.02 μM/day for the reduced-O₂—untreated-pH incubations, 0.54 ± 0.01 μM/day for the untreated-O₂—reduced-pH incubations, and 0.54 ± 0.02 μM/day for the reduced-O₂—reduced-pH incubations. Rates are given with the standard deviation among the calculated rates for each of the 17 and 30-h time points. Values of R_{nit_ox} calculated for the ¹⁵NO₂⁻ incubations were higher than R_{amm_ox} under all conditions. The average R_{nit_ox} was 0.64 ± 0.06 μmol/day among the untreated-O₂—untreated-pH incubations, 0.59 ± 0.05 μmol/day among the reduced-O₂—untreated-pH incubations, 0.72 ± 0.03 μM/day among the untreated-O₂—reduced-pH incubations, and 0.72 ± 0.05 μM/day among the reduced-O₂—reduced-pH incubations. Thus, there was net consumption of NO₂⁻ of 0.1–0.2 μM/day.

Namibian Upwelling Zone

In the Namibian Upwelling incubations, ammonia-oxidation rates calculated from linear regressions of ¹⁵NO_x⁻ measured at 12 and 24 h during ¹⁵NH₄⁺ incubations were two orders of magnitude lower than during the Lake Lugano incubations. Average rates were 3.0 ± 0.3 nM/day (r² = 0.96) at 220 μM O₂, 2.4 ± 0.3 nM/day (r² = 0.91) at 50 μM O₂, and 2.3 ± 0.2 nM/day (r² = 0.98) at 20 μM O₂ (Table 2).

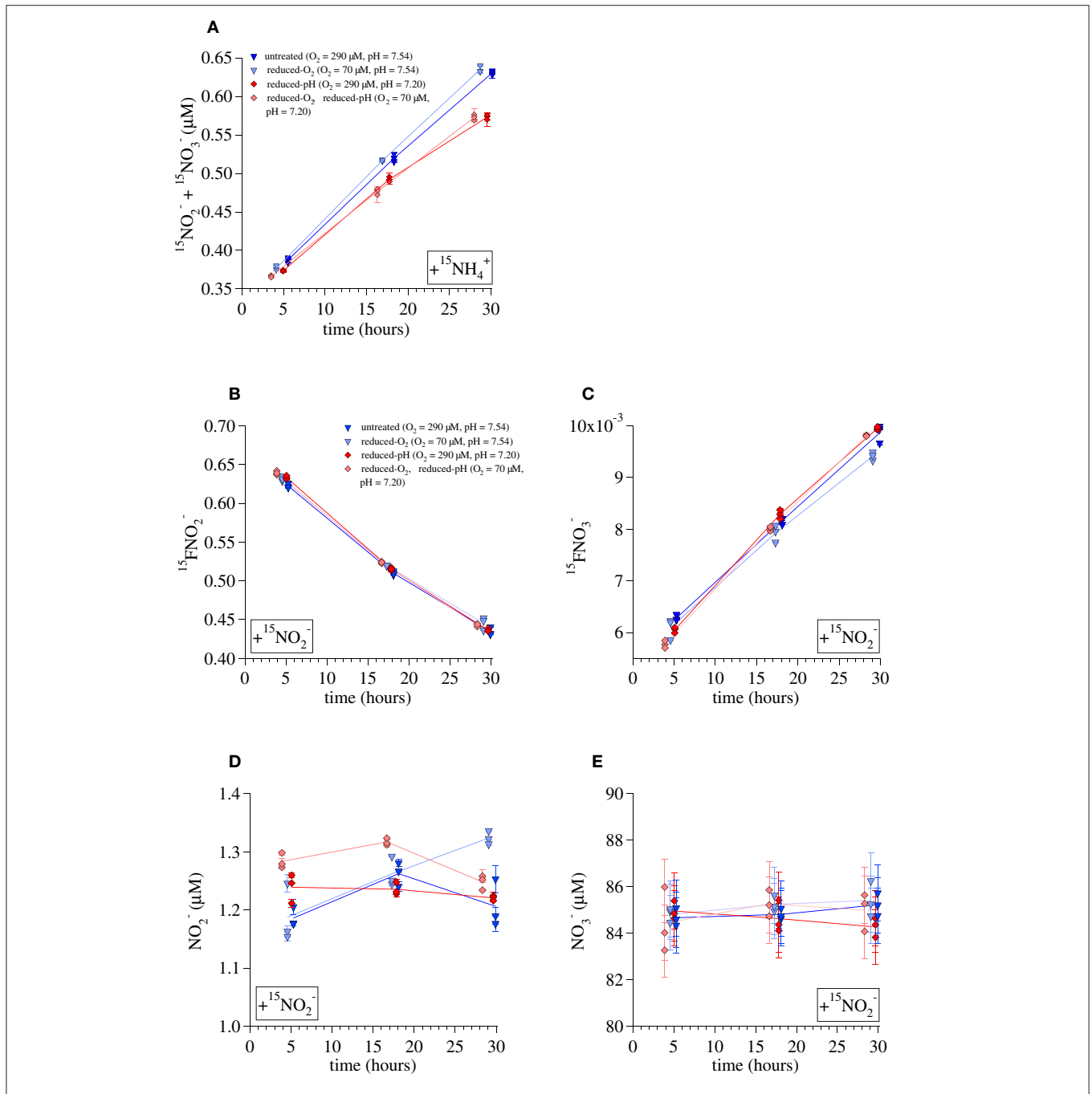


FIGURE 4 | Data used to calculate ammonia-oxidation rates during the Lake Lugano incubations. (A) $^{15}\text{NO}_X^-$ production during $^{15}\text{NH}_4^+$ incubations, **(B)** $^{15}\text{FNO}_2^-$ during $^{15}\text{NO}_2^-$ incubations, **(C)** $^{15}\text{FNO}_3^-$ during $^{15}\text{NO}_2^-$ incubations, **(D)** NO_2^- concentrations during $^{15}\text{NO}_2^-$ incubations, and **(E)** NO_3^- concentrations during $^{15}\text{NO}_2^-$ incubations. Lines are drawn between averages of triplicate incubations at each time point. Error bars represent one standard deviation from the average of duplicate measurements.

N₂O Production Rates and Yields Lake Lugano

Incorporation of tracer ^{15}N into N_2O over the course of the incubation can produce an excess of either m/z 45 N_2O (total $^{45}\text{N}_2\text{O} = ^{15}\text{N}^{14}\text{N}^{16}\text{O} + ^{14}\text{N}^{15}\text{N}^{16}\text{O} + ^{14}\text{N}^{14}\text{N}^{17}\text{O}$) or m/z 46 N_2O (total $^{46}\text{N}_2\text{O} = ^{15}\text{N}^{15}\text{N}^{16}\text{O} + ^{14}\text{N}^{14}\text{N}^{18}\text{O}$).

During the Lake Lugano incubations with $^{15}\text{NH}_4^+$ + NA NO_2^- , significant amounts of both $^{45}\text{N}_2\text{O}$ and $^{46}\text{N}_2\text{O}$ were produced (**Figures 5A,B**). The quantities of $^{45}\text{N}_2\text{O}$ and $^{46}\text{N}_2\text{O}$ present in each incubation bottle were calculated by converting the calibrated molecular ratios ($^{45}\text{R} = [^{45}\text{N}_2\text{O}]/[^{44}\text{N}_2\text{O}]$ and $^{46}\text{R} = [^{46}\text{N}_2\text{O}]/[^{44}\text{N}_2\text{O}]$) to molecular fractions ($^{45}\text{F} =$

TABLE 2 | Results of incubations in the Namibian Upwelling zone.

[O ₂] μM	N tracers	Incubation time (hours)	Ammonia oxidation rate (nM/day)	δ ¹⁵ N-N ₂ O (‰)	stddev δ ¹⁵ N-N ₂ O (‰)	δ ¹⁸ O-N ₂ O (‰)	stddev δ ¹⁸ O-N ₂ O (‰)	Total N ₂ O (nmoles)	Error total N ₂ O (nmoles)	n
Kill controls	¹⁵ NH ₄ ⁺ , NA NO ₂ ⁻	0		7.0	0.9	42.8	0.7			3
220 ± 12	¹⁵ NH ₄ ⁺ , NA NO ₂ ⁻	24.0	3.0 ± 0.3	10.8	1.5	43.3	0.5	2.7	0.08	2
50 ± 10	¹⁵ NH ₄ ⁺ , NA NO ₂ ⁻	26.0	2.4 ± 0.3	13.8	0.9	44.0	0.2	2.0	0.05	3
20 ± 10	¹⁵ NH ₄ ⁺ , NA NO ₂ ⁻	24.1	2.3 ± 0.2	20.8	2.0	46.0	0.5	1.7	0.05	3
Kill controls	NA NH ₄ ⁺ , ¹⁵ NO ₂ ⁻	0		6.8	0.2	45.5	0.5			3
220 ± 12	NA NH ₄ ⁺ , ¹⁵ NO ₂ ⁻	23.9	-	7.3	0.1	45.6	0.1	2.5	0.08	2
50 ± 10	NA NH ₄ ⁺ , ¹⁵ NO ₂ ⁻	25.2	-	8.0	0.5	45.2	0.2	1.9	0.06	3
20 ± 10	NA NH ₄ ⁺ , ¹⁵ NO ₂ ⁻	24.0	-	11.3	1.8	44.5	0.5	1.5	0.04	3

$$[^{45}\text{N}_2\text{O}]/[^{44}\text{N}_2\text{O} + ^{45}\text{N}_2\text{O} + ^{46}\text{N}_2\text{O}] \text{ and } ^{46}\text{F} = [^{46}\text{N}_2\text{O}]/[^{44}\text{N}_2\text{O} + ^{45}\text{N}_2\text{O} + ^{46}\text{N}_2\text{O}]$$

$$^{45}\text{F} = 1/(1 + ^{46}\text{R}/^{45}\text{R} + 1/^{45}\text{R}) \tag{6}$$

$$^{46}\text{F} = 1/(1 + ^{45}\text{R}/^{46}\text{R} + 1/^{46}\text{R}) \tag{7}$$

and then multiplying the molecular fractions by the total N₂O:

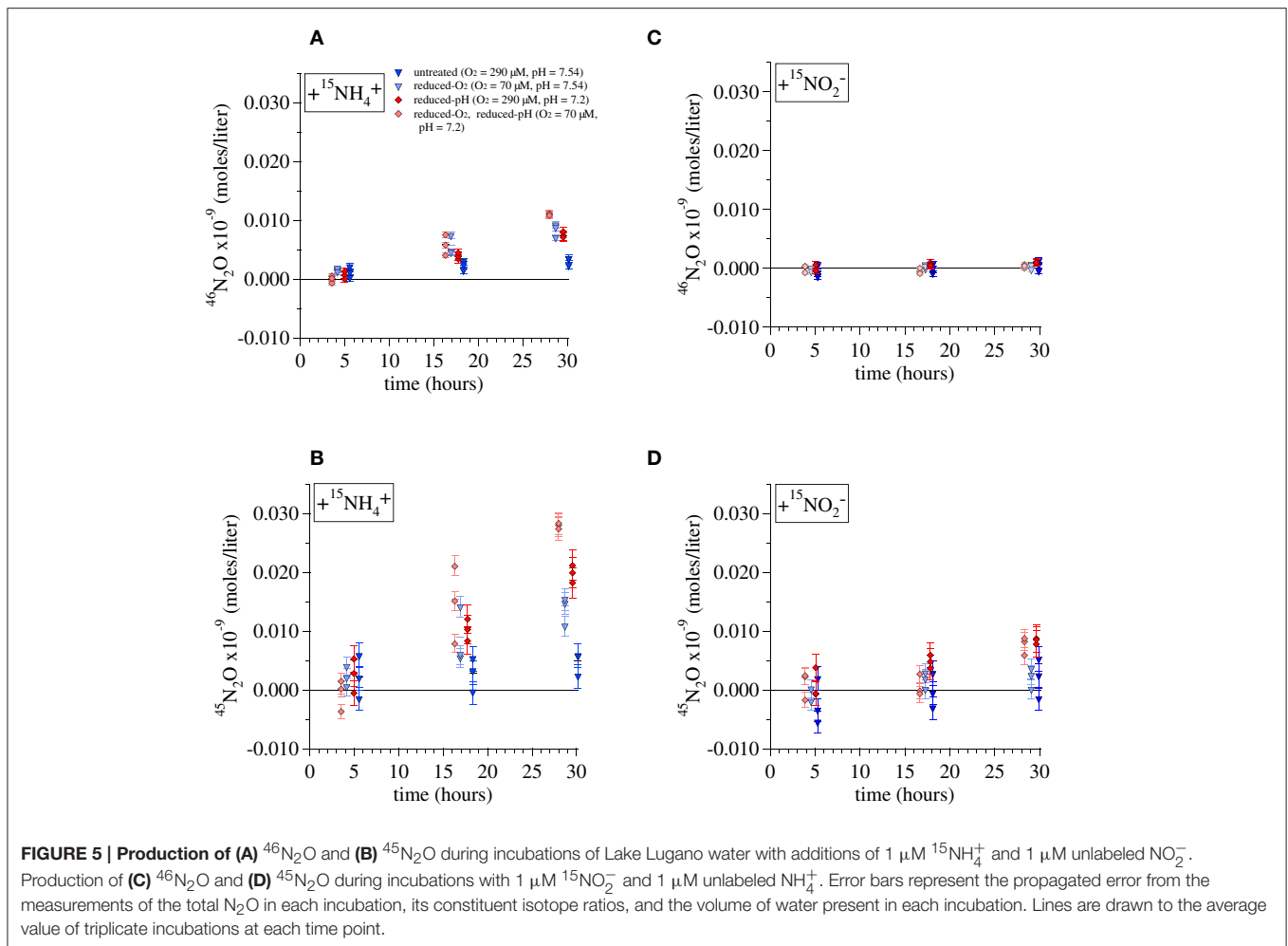
$$^{45}\text{N}_2\text{O} = ^{45}\text{F} \times [\text{total N}_2\text{O}] \tag{8}$$

$$^{46}\text{N}_2\text{O} = ^{46}\text{F} \times [\text{total N}_2\text{O}]. \tag{9}$$

For each set of experimental conditions, the total ⁴⁵N₂O and ⁴⁶N₂O measured in incubations killed immediately after tracer addition (t₀) was subtracted from the measurements of incubations killed at all subsequent time points. The ⁴⁵R and ⁴⁶R measured in incubations killed at t₀ were similar to those of the background N₂O, indicating that our preservation methods prevented further N₂O production from either ¹⁵NH₄⁺ or ¹⁵NO₂⁻. Error bars in **Figure 5** represent the propagated error from measurements of the total N₂O in each incubation, its isotope ratios, and the total volume of water and background N₂O present in each incubation bottle. Total daily incorporation of ¹⁵N into N₂O during ¹⁵NH₄⁺ incubations was calculated from these daily rates as (Rate_{45N2O} + 2 × Rate_{46N2O}). Rates of ¹⁵N incorporation were 0.0086 ± 0.003 nM-N/day for the untreated-O₂—untreated-pH ¹⁵NH₄⁺ incubation, 0.025 ± 0.005 nM-N/day for the reduced-O₂—untreated-pH incubations, 0.028 ± 0.003 nM-N/day for the untreated-O₂—reduced-pH incubations, and 0.043 ± 0.001 nM-N/day for the reduced-O₂—reduced-pH incubation, where we have indicated ± one standard deviation from the daily average calculated using the final three incubations. Assuming that NH₄⁺ is the ultimate source of all the N₂O produced during these incubations, multiplying the rates of ¹⁵N incorporation by their respective isotope dilution factors (1/¹⁵F_{NH₄⁺0}) would increase total N₂O production rates by factors of 1.50 for the untreated-O₂—untreated-pH and reduced-O₂—untreated-pH incubations, and 1.61 for the untreated-O₂—reduced-pH and reduced-O₂—reduced-pH incubations.

Multiplying rates by 1/¹⁵F_{NH₄⁺0} does not account for incorporation of N from exogenous NO₂⁻ into N₂O, which probably also contributed to N₂O production, particularly in the reduced-pH incubations. Indeed, during incubations with ¹⁵NO₂⁻, significant amounts of ⁴⁵N₂O formed during both the reduced-pH and reduced O₂—reduced-pH incubations (*t*-test, *p* = 0.012 and 0.022, respectively; **Figure 5D**), indicating that ¹⁵N derived from tracer ¹⁵NO₂⁻ also contributed to N₂O production. However, no significant ⁴⁶N₂O production was observed among any of the ¹⁵NO₂⁻ incubations (**Figure 5C**), including those that produced significant amounts of ⁴⁵N₂O.

The yield of N₂O during the ¹⁵NH₄⁺ incubations was calculated as the rate of incorporation of ¹⁵N into N₂O relative to the rate of ¹⁵NO_x⁻ production. The average yields (mol ¹⁵N-N₂O/mol ¹⁵N-NO_x⁻) were 3.64 × 10⁻⁵ for the untreated-O₂—untreated-pH incubations, 10.0 × 10⁻⁵ for the reduced-O₂—untreated-pH incubations, 14.0 × 10⁻⁵ for the untreated-O₂—reduced-pH incubations and 21.0 × 10⁻⁵ for the reduced-O₂—reduced-pH incubations.



Namibian Upwelling Zone

Among the Namibian Upwelling incubations, there was detectable production of $^{45}\text{N}_2\text{O}$ when $1\ \mu\text{M}\ ^{15}\text{NH}_4^+$ + $1\ \mu\text{M}$ NA NO_2^- was added, but not when $1\ \mu\text{M}$ NA NH_4^+ + $1\ \mu\text{M}\ ^{15}\text{NO}_2^-$ was added. The increases in $^{45}\text{N}_2\text{O}$ were too small to be converted to significant daily rates of $^{45}\text{N}_2\text{O}$ production, and therefore results are reported in **Table 2** using the more sensitive delta notation where the $\delta^{15}\text{N}\text{-N}_2\text{O}$ signal increases in proportion to $[\text{N}_2\text{O}^{15}]/[\text{N}_2\text{O}^{44}]$, and the $\delta^{18}\text{O}\text{-N}_2\text{O}$ signal increases in proportion to $[\text{N}_2\text{O}^{46}]/[\text{N}_2\text{O}^{44}]$. Increases in $\delta^{15}\text{N}\text{-N}_2\text{O}$ over 24 h were higher during the $^{15}\text{NH}_4^+$ incubations ($10.8 \pm 1.5\%$ to $20.8 \pm 2\%$) than during the $^{15}\text{NO}_2^-$ incubations ($7.3 \pm 0.1\%$ to $11.3 \pm 1.8\%$). During the $^{15}\text{NH}_4^+$ incubations, increases in $\delta^{18}\text{O}\text{-N}_2\text{O}$ ($43.3 \pm 0.5\%$ to $46.0 \pm 0.5\%$) were smaller than the increases in $\delta^{15}\text{N}\text{-N}_2\text{O}$. The increases in $\delta^{18}\text{O}\text{-N}_2\text{O}$ during the 20 and $50\ \mu\text{M}\text{-O}_2$ incubations were significantly higher than the $\delta^{18}\text{O}$ of the background N_2O (two-tailed $p = 0.003$ and 0.0462 , respectively for the $20\ \mu\text{M}$ - and $50\ \mu\text{M}\text{-O}_2$ incubations). The $^{15}\text{NH}_4^+$ incubations with the lower O_2 concentrations showed the largest increases in $\delta^{15}\text{N}\text{-N}_2\text{O}$. This was partly due to the fact that headspace O_2 displacement with He and N_2 also reduced background N_2O

in the reduced- O_2 incubations (**Table 2**). However, correction for the differences in background N_2O among O_2 treatments explains less than half of the increase in $\delta^{15}\text{N}\text{-N}_2\text{O}$ during the $20\ \mu\text{M}\text{-O}_2$ incubations as compared to the $220\ \mu\text{M}\text{-O}_2$ incubations.

Nitrifier Community Composition Overall Microbial Diversity and Abundances

The OTU abundances and their taxonomic affiliation, closest known cultured relatives, and the number of reads assigned to each OTU are summarized in Tables S1, S2. Three separate reactions with DNA extracted from the Lake Lugano water (17 m depth) yielded 382,374, 77,700, and 287,306 partial 16S rRNA gene sequence reads. Three separate reactions with DNA from the Namibian Upwelling incubation water yielded 246,743, 23,288, and 154,829 reads. These reads underwent quality filtration and de-noising to produce a total of 271,425 unique OTUs that were ~ 253 bp long. Figure S5 summarizes the bacterial and archaeal phyla into which these OTUs fall. Comparison of the observed taxon richness to Chao1-estimated richness revealed that multiplex sequencing coverage was $47.9 \pm 1.4\%$ in the Lake Lugano sample and

52.3 ± 3.7% in the Namibian Upwelling sample. Rarefaction analyses that assess taxon richness in the Namibian Upwelling and Lake Lugano samples were generated with the QIIME pipeline (see Figure S4; Caporaso et al., 2012). The Shannon-Weiner diversity index (H), which is directly proportional to the number of taxa and inversely proportional to the number of sequences falling into each taxon, was an order of magnitude higher for AOB in Lake Lugano than for AOB in the Namibian Upwelling, whereas this index was higher for AOA in the Namibian Upwelling than in Lake Lugano (Figure S6).

AOA and AOB Diversity and Abundances

A total of 442 unique OTUs related to AOA and 65 unique OTUs related to the AOB family Nitrosomonadaceae were identified. The AOA OTUs constitute 0.3 and 31.2% of total microbial OTUs in Lake Lugano and the Namibian Upwelling site, respectively. AOB OTUs constituted 0.6% of total microbial OTUs in the Lake Lugano sample, but were extremely rare (< 0.01%) in the Namibian Upwelling sample. Both ML and MP trees were nearly identical in their placement of AOA and AOB OTUs with respect to their closest relatives. The 65 AOB OTUs fell into a monophyletic cluster that included cultured members of the family Nitrosomonadaceae (Figure 6A). The closest cultured representative to 27 of the 65 OTUs was the predominantly terrestrial species *Nitrosospira briensis* (with 91–100% 16S rRNA sequence identity), although their closest relatives were all uncultured freshwater organisms (see Figure 6A; Table S1). Most of the AOB OTUs (62) were detected only in the Lake Lugano sample and not the Namibian Upwelling sample, and the remaining 3 OTUs were present in both the Lake Lugano and Namibian Upwelling samples (Table S1).

The 442 unique AOA sequences fell into a clade with members of the Thaumarchaea (marine group I archaea) such as *Nitrosopumilus maritimus* SCM1 and *Nitrososphaera* sp. JG1, with moderate bootstrap support (Figure 6B). The closest cultured relatives of these OTUs were mainly found in seawater, with the majority most closely related to *Candidatus Nitrosopelagicus brevis* strain CN25 (with 88–100% sequence identity), and a large number most closely related to *Candidatus Nitrosopumilus* sp. NF5 and *Candidatus Nitrosopumilus* sp. D3C (with 86–98% sequence identity; see Figure 6B; Table S2). With a few exceptions, the uncultured closest relatives of these OTUs were also detected in seawater (Figure 6B). Of the 442 AOA OTUs identified, 339 were detected only in the Namibian Upwelling sample, 18 were detected only in Lake Lugano, and 85 were detected in both locations (Table S2). Among the AOA OTUs unique to Lake Lugano, their closest cultured relatives include *Candidatus Nitrosopelagicus brevis* strain CN25 (Santoro et al., 2015) and *Candidatus Nitrosopumilus* sp. HCA1 (KF957663.1) (Bayer et al., 2016), which are both marine, and also *Nitrososphaera viennensis* EN76 (Stieglmeier et al., 2014a) and *Candidatus Nitrososphaera evergladensis* SR1 (Zhalnina et al., 2014), which were both found in soil.

DISCUSSION

Geochemical Profiles

The profiles of the N₂O concentration and isotopic composition were useful as qualitative indicators of the depths of rapid nitrification in Lake Lugano. Although the depth of the water used for the Lake Lugano experiments (17 m) was shallower than the N₂O concentration maximum, its coincidence with a clear minimum in the δ¹⁵N-N₂O profile (Figure 2F) suggests that there was rapid *in situ* N₂O production there. However, the absence of a corresponding extremum in the SP profile (Figure 2H) at this depth suggests that the δ¹⁵N-N₂O minimum probably reflects a minimum in the δ¹⁵N of the precursor N molecule, rather than a change in the mechanism of N₂O formation at this depth. While there is also a minimum in the δ¹⁵N-NO₃⁻ profile at the incubation depth, the δ¹⁸O-NO₃⁻ profile indicates that NO₃⁻ was probably not the precursor of N₂O at this depth. More precisely, at this depth, the δ¹⁸O-N₂O was 44‰ higher than the δ¹⁸O-NO₃⁻, whereas the combination of a 42‰ branching isotope effect (Casciotti et al., 2007; Frame et al., 2014) and a -22‰ kinetic isotope effect (Granger et al., 2006) associated with NO₃⁻ reduction to N₂O, should have produced N₂O with a δ¹⁸O that was at most only 20‰ higher than the δ¹⁸O-NO₃⁻.

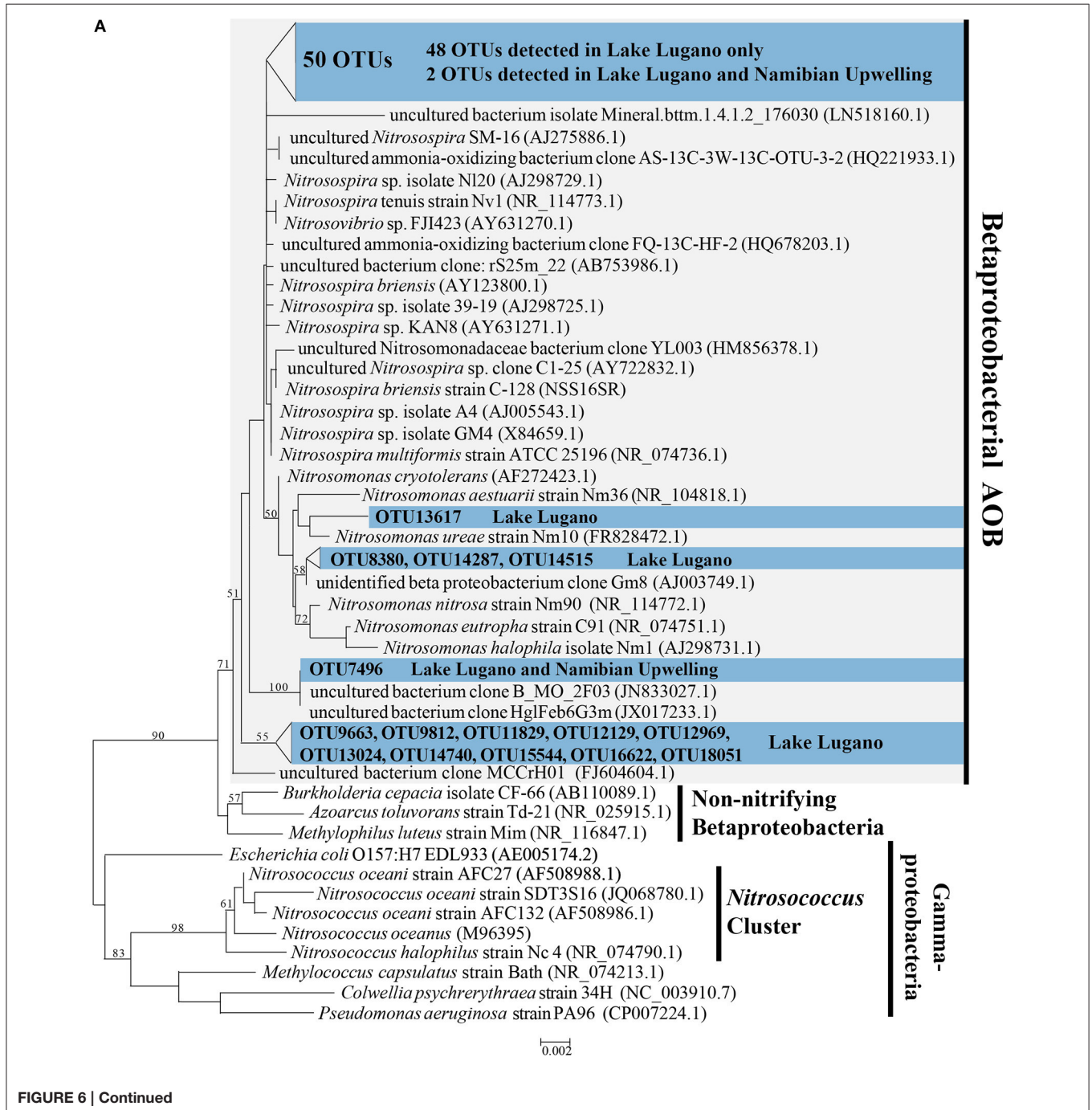
The similarity of the δ¹⁵N-N₂O and δ¹⁵N-NO₃⁻ profiles suggests that both compounds are derived from a shared pool of relatively low-δ¹⁵N precursor N, which could be either NH₄⁺ or NO₂⁻. The δ¹⁵N-NO₂⁻ in the top 15 m ranged between -29‰ and -27‰ (Figure 2L). The NH₃ oxidized to NO₂⁻ at these depths could have been relatively depleted in ¹⁵N because of rapid remineralization of isotopically lighter organic N. The 20‰ equilibrium isotope effect between NH₄⁺ and NH₃ (Hermes et al., 1985) and/or expression of the isotope effect(s) associated with ammonia oxidation (Casciotti et al., 2003) may have also contributed to production of ¹⁵N-depleted N₂O by ammonia oxidizers at this depth.

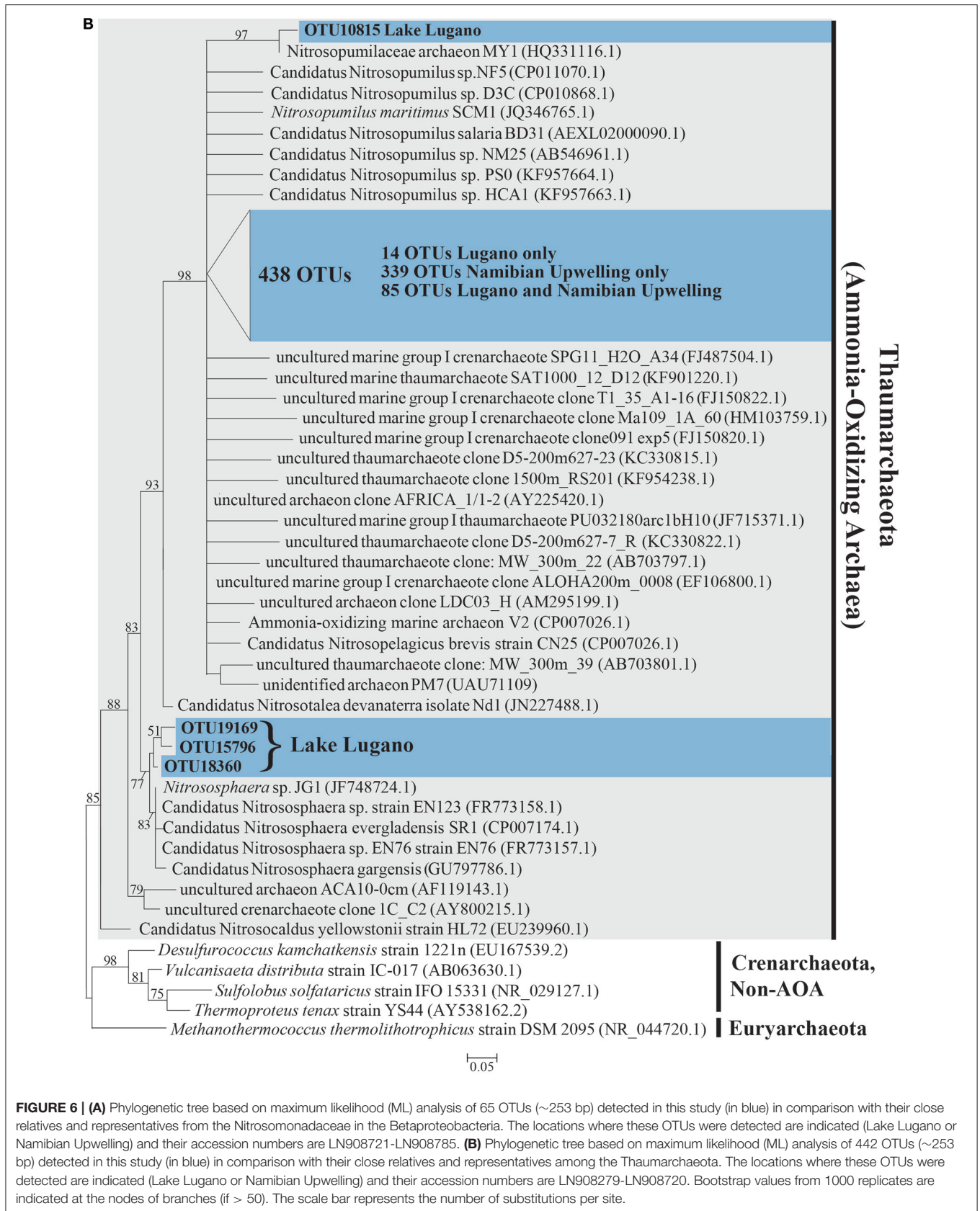
N₂O production in the deeper water of this basin has been linked to a NH₂OH-decomposition pathway, largely by the SP value of the N₂O, which approaches a value of ~34‰ in the oxic water between 30 and 70 m (Wenk et al., 2016 and Figure 2H). This particular N₂O formation mechanism happens during ammonia oxidation in aerobic conditions (Sutka et al., 2006; Frame and Casciotti, 2010; Santoro et al., 2011). Interestingly, such a high SP value is also observed in N₂O that is formed abiotically by either the hybrid reaction of NH₂OH with HNO₂/NO₂⁻ or by the oxidation of NH₂OH (Figure 1, pathways 1 and 2; Heil et al., 2014). Thus the production of high SP (~34‰) N₂O often observed among AOB and AOA cultures may not distinguish a pathway involving only NH₂OH from a hybrid pathway where N₂O is formed via the reduction of HNO₂/NO₂⁻ by NH₂OH. To our knowledge, no data has been reported on the SP of N₂O produced by the enzyme-catalyzed reaction of NH₂OH and HNO₂ described by Hooper (1968), but it seems reasonable to assume that it may also be ~34‰. Thus, while the SP of the N₂O present in the shallower depths of Lake Lugano increases steadily between 5 and 50 m (Figure 2H), suggesting a NH₂OH-dependent N₂O formation mechanism, we

cannot use SP alone to distinguish N₂O produced by the reaction between NH₂OH and NO₂⁻ from N₂O produced by NH₂OH autoxidation or disproportionation.

The high concentration of N₂O that had accumulated at the depth of the Namibian Upwelling incubation had a relatively low SP (Figure 3H), suggesting that the source of this N₂O was either NO_x⁻ reduction by denitrification or NO₂⁻ reduction by nitrifier denitrification (Toyoda et al., 2005; Frame and Casciotti, 2010). However, in our incubations we observed

hybrid N₂O formation rather than denitrification or nitrifier denitrification. The absence of denitrification and nitrifier denitrification during the incubations is unsurprising, given the relatively high O₂ concentrations (20, 50, or 220 μM), all of which were well above thresholds that limit transcription of *norB* in denitrifiers (Dalsgaard et al., 2014) and initiation of nitrifier denitrification by AOB (Frame and Casciotti, 2010). The lower O₂ concentrations tested during these incubations were similar to the *in situ* O₂ concentration at this depth (56.8 μM),





suggesting that if denitrification or nitrifier denitrification had occurred in this water mass, it was not happening at the time and location where we sampled it. Frame et al. (2014) have argued that in this region of the South Atlantic, transport and mixing of continental shelf water that is O₂-depleted and contains N₂O produced by anaerobic or suboxic processes with relatively O₂-rich offshore water, can produce water that contains relatively high O₂ concentrations and also N₂O with isotopic signatures that are characteristic of low-O₂ processes like denitrification or nitrifier denitrification.

Nitrification Rates

The difference in ammonia oxidation rates ($R_{\text{amm_ox}}$) calculated during incubations with $^{15}\text{NH}_4^+$ vs. those with $^{15}\text{NO}_2^-$ is probably the result of dilution and loss of the added $^{15}\text{NH}_4^+$ due to rapid NH_4^+ regeneration and uptake, which would both tend to reduce our estimate of $R_{\text{amm_ox}}$ during the $^{15}\text{NH}_4^+$ incubations. It also suggests that the rate reduction that we observed in the reduced-pH $^{15}\text{NH}_4^+$ incubations does not necessarily reflect an actual reduction in the rate of ammonia oxidation, but instead a more rapid reduction in the $^{15}\text{F}_{\text{NH}_4^+}$ over time as compared to the control-pH incubations.

In both Lake Lugano and the Namibian Upwelling, zero-order reaction kinetics were assumed for ammonia oxidation, rather than first-order or Michaelis-Menten kinetics. This was probably a reasonable assumption in the Namibian Upwelling experiments, because AOA have an extremely high affinity for $\text{NH}_3/\text{NH}_4^+$, with half-saturation constants (K_m) on the order of 100 nM (Martens-Habbenha et al., 2009; Horak et al., 2013). In contrast, AOB have a lower affinity for NH_4^+ than marine assemblages of AOA (Horak et al., 2013; Newell et al., 2013). The lowest reported K_m among cultivated AOB representatives is 6 μM , and the typical range for cultivated AOB is 0.05–14 mM (Knowles et al., 1965; Keener and Arp, 1993; Martens-Habbenha et al., 2009; Jiang and Bakken, 1999b). NH_4^+ concentrations during the incubations remained well above the K_m of AOA but below the K_m reported for AOB. Since both AOB and AOA were present in the Lake Lugano incubations, we used the results of the $^{15}\text{NO}_2^-$ incubations to model first-order rate constants for ammonia oxidation that were $0.46 \pm 0.04 \text{ M}^{-1} \text{ day}^{-1}$ for the untreated-O₂—untreated-pH incubations, $0.45 \pm 0.04 \text{ M}^{-1} \text{ day}^{-1}$ for the reduced-O₂—untreated pH incubations, $0.47 \pm 0.04 \text{ M}^{-1} \text{ day}^{-1}$ for the untreated-O₂—reduced-pH incubations, and $0.42 \pm 0.04 \text{ M}^{-1} \text{ day}^{-1}$ for the reduced-O₂—reduced-pH incubations. Using the observed concentration of NH_4^+ (0.92 μM) at 17 m in Lake Lugano and assuming that this concentration is in steady-state, the actual ammonia oxidation rate may be slightly lower than what we calculated using the zero-order reaction model.

N₂O Yields and Mechanisms of N₂O Formation in Lake Lugano

Total N₂O yields measured in Lake Lugano (3.64×10^{-5} to $21.0 \times 10^{-5} \text{ mol } ^{15}\text{N-N}_2\text{O} / \text{mol } ^{15}\text{N-NO}_2^-$) were comparable to those observed by Yoshida et al. (1989) in the western North Pacific, also using ^{15}N tracer techniques (8 to 54×10^{-5}). They were at the low end of the range observed during growth of batch cultures

of the AOB *Nitrosomonas marina* (10 to 60×10^{-5}) at similar O₂ concentrations (Frame and Casciotti, 2010) and were lower than those observed for batch cultures of the AOA *N. maritimus* (60 to 100×10^{-5}) and *N. viennensis* (140 to 180×10^{-5}) in media buffered to pH 7.5 (Stieglmeier et al., 2014b). The N₂O formed during the Lake Lugano incubations was largely derived from intermediates or products of the ammonia oxidation reactions, and relatively little N was incorporated from exogenous NO_2^- , as indicated by the higher rates of $^{15}\text{N-N}_2\text{O}$ formation during the $^{15}\text{NH}_4^+$ incubations than during the $^{15}\text{NO}_2^-$ incubations (Figures 5A–D).

During all of the $^{15}\text{NH}_4^+$ incubations, the measured ratio of $^{46}\text{N}_2\text{O}:$ $^{45}\text{N}_2\text{O}$ production (0.38–0.67) was lower than the expected ratio produced by random pairing of N derived from NH_4^+ with the isotope ratio $^{15}\text{F}_{\text{NH}_4^+}$ (expected $^{46}\text{N}_2\text{O}/^{45}\text{N}_2\text{O} = (^{15}\text{F}_{\text{NH}_4^+})^2 / (2 \times ^{15}\text{F}_{\text{NH}_4^+} \times (1 - ^{15}\text{F}_{\text{NH}_4^+})) = 1.0$ for the untreated-O₂—untreated-pH and reduced-O₂—untreated-pH incubations and 0.82 for the untreated-O₂—reduced-pH and reduced-O₂—reduced-pH incubations). Interestingly, Jung et al. (2014) also report relatively high $^{45}\text{N}_2\text{O}$ production compared to $^{46}\text{N}_2\text{O}$ production during tracer incubations of AOB and soil AOA cultures in the presence of 99% $^{15}\text{NH}_4^+$ and excess N.A. NO_2^- . Because they were working with laboratory cultures, their experiments started with almost no background $^{14}\text{NH}_4^+$ and contained no ammonium regenerating processes that would increase $^{14}\text{NH}_4^+$ over the course of the incubations, allowing them to attribute the $^{45}\text{N}_2\text{O}$ production to a reaction between $^{15}\text{NH}_4^+$ -derived N and NO_2^- -derived N.

In the present study, incubations with $^{15}\text{NH}_4^+$ at the reduced pH produced lower ratios of $^{46}\text{N}_2\text{O}:$ $^{45}\text{N}_2\text{O}$ than incubations at the untreated pH. Three factors may account for the difference: (1) the $^{15}\text{F}_{\text{NH}_4^+}$ was lower among the reduced-pH incubations (0.62) than it was among incubations at the untreated pH (0.67), (2) there may have been differences in the regeneration rates of NH_4^+ among experimental treatments, which would have progressively reduced the $^{15}\text{F}_{\text{NH}_4^+}$ at different rates in the two different pH treatments, and (3) an increased contribution of N from an unlabeled N pool (i.e., exogenous NO_2^-) enhanced $^{45}\text{N}_2\text{O}$ production. Although we lack knowledge of the evolution of $^{15}\text{F}_{\text{NH}_4^+}$ during our experiments that would allow us to rule out factors (1) and (2), we can be certain that the more rapid production of $^{45}\text{N}_2\text{O}$ during the reduced-pH $^{15}\text{NO}_2^-$ incubations was at least partly the result of additional N-incorporation from exogenous NO_2^- , given the production of $^{45}\text{N}_2\text{O}$ in the $^{15}\text{NO}_2^-$ -amended experiment (Figure 5D), and that this argues in favor of factor (3) discussed above.

Nitrifier denitrification is unlikely to have contributed to N₂O production during the Lake Lugano experiments. During the $^{15}\text{NO}_2^-$ incubations with untreated-O₂—reduced-pH and reduced-O₂—reduced-pH, the formation of $^{45}\text{N}_2\text{O}$ and not $^{46}\text{N}_2\text{O}$ (Figures 5C,D) suggests that nitrifier denitrification was not important. Although cultured representatives of *Nitrosospira*, the most abundant AOB genus in the Lake Lugano, are known to contain *norB* homologs (Garbeva et al., 2007), whose enzyme products reduce NO to N₂O during nitrifier denitrification

reactions (**Figure 1**, yellow box; Schmidt et al., 2004; Kozłowski et al., 2014), the incubation conditions, such as the relatively high O₂ concentrations (70 and 290 μM), were unlikely to have stimulated nitrifier denitrification. Rather, a hybrid N₂O formation mechanism (**Figure 1**, pathway 2) that combines one N derived from exogenous NO₂⁻ with one N derived from a different, unlabeled N pool, explains the formation of ⁴⁵N₂O in the absence of ⁴⁶N₂O formation during the ¹⁵NO₂⁻ incubations. Stieglmeier et al. (2014b) also observed hybrid N₂O formation by AOA cultures, and have suggested that this other pool of N is NH₂OH. NH₂OH is known to form N₂O in the presence of NO₂⁻, both enzymatically (Hooper, 1968) and abiotically (Döring and Gehlen, 1961). Since both NO₂⁻ and NH₂OH form in the periplasm (Hollocher et al., 1982), a reaction between these two compounds during the ¹⁵NH₄⁺ incubations is also consistent with the relatively high rates of both ⁴⁵N₂O and ⁴⁶N₂O production (**Figures 5A,B**). However, without knowledge of ¹⁵F_{NH4+} over the course of the incubations, we cannot rule out formation of some ¹⁵N-N₂O through NH₂OH autoxidation or disproportionation (**Figure 1**, pathway 1). A third possibility is that two intracellular NO₂⁻ molecules react with each other to form N₂O via nitrifier denitrification in a system where mixing between endogenous (i.e., periplasmic) NO₂⁻ and exogenous NO₂⁻ occurs so slowly that the two NO₂⁻ pools have distinct isotopic compositions. In the ¹⁵NH₄⁺ incubations, nitrifier denitrification of the relatively ¹⁵N-enriched periplasmic NO₂⁻ would produce more ⁴⁵N₂O and ⁴⁶N₂O than we would predict based on the measured isotopic composition of the total NO₂⁻. In the ¹⁵NO₂⁻ incubations, nitrifier denitrification of periplasmic NO₂⁻ would mainly produce ⁴⁴N₂O, and only a small influx of exogenous ¹⁵NO₂⁻ would be reduced to ⁴⁵N₂O after mixing with unlabeled periplasmic NO₂⁻ (as we observed during the reduced-pH incubations; **Figure 5D**). In this way, it would be possible for nitrifier denitrification to produce ⁴⁵N₂O and not ⁴⁶N₂O during the ¹⁵NO₂⁻ incubations. We believe that this pathway is unlikely given the relatively high O₂ concentrations in our incubations, but without more detailed knowledge of the size of the periplasmic NO₂⁻ pool maintained by the ammonia-oxidizing cells, and the rate at which this pool exchanges with the external NO₂⁻ pool, we cannot completely exclude the possibility of nitrifier denitrification.

Possible Explanations for the Influence of pH on N₂O Production

Reducing the pH during the Lake Lugano incubations increased ¹⁵N₂O production in both the ¹⁵NH₄⁺ experiments and the ¹⁵NO₂⁻ experiments. There may be several reasons for this, including structural (e.g., the outer cell membrane may exchange HNO₂/NO₂⁻ more rapidly between the periplasm and the outer environment at a lower pH), enzymatic (e.g., a shift toward the optimal pH of the N₂O-producing enzymes in the periplasm), transcriptional (regulation of the genes encoding the enzymes involved in N₂O production may be pH-sensitive), and chemical (due to acceleration in the rates of abiotic reactions that produce N₂O from precursor molecules made by AOB). As discussed

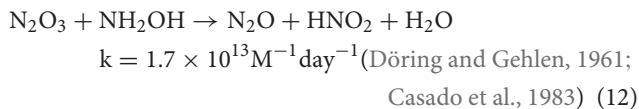
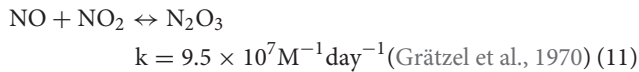
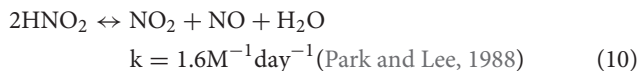
below, the most likely explanations are a shift toward the optimal pH of enzymes that catalyze N₂O production and/or the involvement of a non-biological catalyst that accelerates the abiotic reactions that form N₂O.

The majority of ammonia oxidizers in Lake Lugano are Gram-negative bacteria, which means that they have a periplasmic space that is bounded by inner and outer cell membranes. In other Gram-negative species such as *Escherichia coli*, the pH of the periplasm rapidly changes to reflect that of the external environment (Wilks and Slonczewski, 2007). To our knowledge, it is unknown whether AOB regulate the pH of their periplasm or not, but decreases in the periplasmic pH are likely to enhance the rates of the N₂O-forming reactions of NH₂OH and/or NO₂⁻. Furthermore, if there are differences in the rate at which HNO₂ vs. NO₂⁻ cross the outer cell membrane, as suggested by Hollocher et al. (1982), then a pH shift could also alter the rate at which ¹⁵N from the tracer ¹⁵NO₂⁻ enters the periplasm and the rate at which the NO₂⁻ formed during ammonia oxidation is expelled from the periplasm into the outer environment. This effect would not necessarily change the actual rate of N₂O production, just our ability to observe it with ¹⁵N tracers. However, if this occurs, it is unlikely to be the dominant effect, because we observed increased ¹⁵N-N₂O production during the reduced-pH incubations with ¹⁵NH₄⁺, and in this case, more rapid exchange of NO₂⁻/HNO₂ across the outer cell membrane in the reduced-pH incubations would dilute the periplasmic concentration of ¹⁵NO₂⁻, and therefore decrease the rate of ¹⁵N-N₂O production relative to total N₂O production.

The results of the ¹⁵NH₄⁺-incubations are consistent with an acceleration of the periplasmic reactions that form N₂O. Formation of ⁴⁶N₂O, which is composed only of ¹⁵NH₄⁺-derived N (and should therefore be relatively independent of any influx of external NO₂⁻ into the periplasm), was also faster at the reduced pH (**Figure 5A**). At the enzyme level, Hooper (1968) observed acceleration of N₂O formation with decreasing pH: AOB enzyme extracts converted NH₂OH + HNO₂ to N₂O with an optimum pH of 5.75, via a reaction whose rate increased steadily as the pH dropped from 7.5 to 6. Assuming that a similar reaction also occurs in intact AOB cells, the reaction rate increase observed by Hooper (1968) was large enough to explain the pH effect observed during the Lake Lugano incubations. Decreases in pH also have effects at the level of transcription and protein expression. For example expression of enzymes involved in handling nitrogen oxides in AOB, increases as the pH of the growth medium decreases from 8.2 to 7.2 (Beaumont et al., 2004a). The mechanism for this appears to depend on a transcriptional regulator whose repression is reversed by the presence of NO₂⁻ at lower pH values (Beaumont et al., 2004a).

Without some form of catalysis, the rate constants reported for the abiotic hybrid N₂O formation reaction between NH₂OH and NO₂⁻ are too small for these reactions to have contributed significantly to N₂O production during our incubations. In particular, the set of reactions thought to produce N₂O that contains one NH₂OH-derived N and one HNO₂-derived N, has a second-order dependence on [HNO₂] (Döring and Gehlen, 1961;

Bonner et al., 1983; Schreiber et al., 2012):



This rate-limiting step (i.e., Equation 10, the formation of NO by disproportionation of HNO₂) becomes important at pH values <4.5 (Hooper, 1968), but it is not fast enough to explain ⁴⁵N₂O production during the incubations, given the low rate constant for HNO₂ disproportionation and the low [HNO₂] during the incubations (1.8 × 10⁻¹¹ M at the untreated pH and 4.0 × 10⁻¹¹ M at the reduced pH). Thus a role for a catalyst, whether enzymatic or non-biological, is indicated.

It is important to note that the NO reacting in Equation (11) may be formed through mechanisms other than the rate-limiting abiotic HNO₂ disproportionation step. As mentioned earlier, a number of processes in ammonia oxidizers release NO. For example, nitrite reductases can convert NO₂⁻ to NO. In AOB, NO is an intermediate in the catalytic cycle of hydroxylamine oxidoreductase (HAO) (Cabail and Pacheco, 2003) and may be released from NH₂OH in HAO enzyme preparations (Hooper and Nason, 1965; Ritchie and Nicholas, 1972; Hooper and Terry, 1979). In AOA, NO is needed to oxidize NH₂OH to NO₂⁻ (Kozłowski et al., 2016). Abiotic reactions between NH₂OH and NO observed by Bonner et al. (1978) (pH 7.8, anaerobic conditions), produced N₂O that was ~75% composed of equal proportions of NH₂OH-derived N and NO-derived N, and ~25% composed of only NO-derived N. If this reaction occurs during ammonia oxidation, then depending on whether NO is derived from NH₂OH, NO₂⁻, or both, these reactions could produce N₂O that is entirely derived from NH₂OH, or some mixture of hybrid N₂O and N₂O that is entirely derived from NO₂⁻.

Chemodenitrification, the reduction of NO₃⁻ or NO₂⁻ coupled to oxidation of ferrous iron (Fe²⁺) (Buresh and Moraghan, 1976; Rakshit et al., 2008; Picardal, 2012) was an unlikely source of N₂O during the Lake Lugano incubations. In the top 20 m of the lake, concentrations of metals involved in chemodenitrification (Fe and possibly manganese, Mn) were less than the 1.7 μM detection limit when measured by induction coupled plasma optical emission spectrometry (J. Tischer, U. Basel, unpublished data). Furthermore, the ¹⁵F_{NO₂- values during incubations with ¹⁵NO₂⁻ (Figure 4B) were high enough that if chemodenitrification of NO₂⁻ to N₂O had been significant, the observed production of ⁴⁵N₂O (Figure 5C) would have been accompanied by detectable ⁴⁶N₂O production (Figure 5D), and it was not. The ¹⁵F_{NO₃- values were lower than ¹⁵F_{NO₂- values (Figure 4C), so that mixed reduction of both NO₂⁻ and NO₃⁻ by Fe²⁺ could explain detectable ⁴⁵N₂O production in the absence of detectable ⁴⁶N₂O production. However, NO₃⁻ reduction by Fe²⁺ is much slower than oxidation by NO₂⁻, except when Cu²⁺ > 1.6 μM (Buresh and Moraghan, 1976; Picardal, 2012). We did}}}

not measure Cu concentrations in Lake Lugano, but the total dissolved Cu measured in Lake Greifen, a similarly eutrophic lake in northeastern Switzerland, were much lower than this (0.5–2.8 × 10⁻⁸ M; Xue and Sigg, 1993).

Although trace metal concentrations were low in Lake Lugano, metal ions could have played a role in accelerating the hybrid N₂O reaction. Harper et al. (2015) have reported that Cu²⁺ can drive abiotic hybrid N₂O formation rates in activated sludge that are faster than the biologically-catalyzed reactions. Furthermore, NH₂OH disproportionation and oxidation reactions may be driven by the presence of copper and iron ions (Anderson, 1964; Alluisetti et al., 2004).

Evidence for Hybrid N₂O Formation in the Namibian Upwelling Zone

The relationship between the changes in δ¹⁵N-N₂O and δ¹⁸O-N₂O during the Namibian Upwelling incubations were too small to convert to N₂O production rates, but they still contain information about the mechanism of N₂O formation. In particular, in light of the high degree of ¹⁵N labeling of the NH₄⁺ pool (¹⁵F_{NH₄+₀ = 0.94) that was achieved during the ¹⁵NH₄⁺ incubations, random pairing of NH₄⁺-derived N atoms cannot explain the large increase in δ¹⁵N-N₂O relative to δ¹⁸O-N₂O. In the Supplementary Material S.2 we demonstrate that the change in δ¹⁵N-N₂O relative to the change in δ¹⁸O-N₂O observed during these incubations is not consistent with the formation of N₂O composed only of N derived from NH₄⁺. Specifically, N₂O produced with a binomial distribution of ¹⁵N and ¹⁴N derived from NH₄⁺ with this ¹⁵F_{NH₄+₀ would produce a much larger increase in δ¹⁸O-N₂O (i.e., more ⁴⁶N₂O) relative to the increase in δ¹⁵N-N₂O (⁴⁵N₂O) than what was observed. In contrast, hybrid N₂O formation (for example, by reaction of highly ¹⁵N-labeled NH₂OH with unlabeled NO or NO₂⁻) would produce a much larger increase in δ¹⁵N-N₂O with almost no change in δ¹⁸O-N₂O (Figure S3).}}

If hybrid N₂O formation produced ⁴⁵N₂O during the ¹⁵NH₄⁺ incubations, from which N pool was the ¹⁴N atom derived? ¹⁵N was more rapidly incorporated into N₂O during the ¹⁵NH₄⁺ incubations than during the ¹⁵NO₂⁻ incubations, as indicated by the larger increase in δ¹⁵N-N₂O (⁴⁵N₂O) during the ¹⁵NH₄⁺ incubations (Table 2). If both NH₄⁺ and exogenous NO₂⁻ had contributed equally to N₂O formation, then we would expect approximately equal increases in δ¹⁵N-N₂O when either ¹⁵NH₄⁺ or ¹⁵NO₂⁻ was added (¹⁵F_{NO₂-₀ = 0.95). The fact that the δ¹⁵N-N₂O increased by less during the ¹⁵NO₂⁻ incubations than during the ¹⁵NH₄⁺ incubations suggests that exogenous NO₂⁻ could not have been the sole source of ¹⁴N to ⁴⁵N₂O produced during the ¹⁵NH₄⁺ incubations. Possibly, an intracellular pool of unlabeled NO₂⁻ was held over from before the ¹⁵NH₄⁺ incubation started. If there was enough of this holdover NO₂⁻ to dilute any endogenous ¹⁵NO₂⁻ produced by ¹⁵NH₄⁺ oxidation, then reactions between ¹⁵NH₂OH with the holdover ¹⁴NO₂⁻ would produce an increase in δ¹⁵N-N₂O (⁴⁵N₂O) without increasing δ¹⁸O-N₂O (⁴⁶N₂O).}

Previous studies in aquatic systems have reported some variation in the importance of NH₄⁺-derived N vs. NO₂⁻-derived

N to N₂O production. Similar results to ours are reported for incubations of suboxic Black Sea water, where Westley et al. (2006) also found production of ¹⁵N-N₂O with the addition of ¹⁵NH₄⁺ but not ¹⁵NO₂⁻. In the Eastern Tropical South Pacific, above the OMZ (O₂ ≥ 10 μM), the rates of NH₄⁺-derived N incorporation into N₂O (0.01–0.02 nM/day) were similar to what was observed in Lake Lugano, although the yield (as defined in this paper) was substantially higher (80 × 10⁻⁵; Ji et al., 2015). In the North Pacific Gyre, Wilson et al. (2014) observed no changes in δ¹⁵N-N₂O during incubations with either 1 μM NA NH₄⁺ + 1 μM ¹⁵NO₂⁻ or 1 μM ¹⁵NH₄⁺ + 1 μM NA NO₂⁻. Critically, however, when they reduced the NA NH₄⁺ addition to 100 nM and added 1 μM ¹⁵NO₂⁻, the δ¹⁵N-N₂O increased significantly over the course of the incubation. This suggests that the rate of ammonia oxidation influences the degree to which N derived from exogenous NO₂⁻ can be incorporated into N₂O, with higher rates of ammonia oxidation perhaps flooding the intracellular NO₂⁻ pool and preventing N derived from exogenous NO₂⁻ from being incorporated into N₂O.

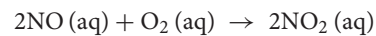
It is difficult at this point to determine whether the same hybrid N₂O reaction mechanism(s) can explain the results of both the Lake Lugano incubations, which were numerically dominated by AOB, and the Namibian Upwelling incubations, which were dominated by AOA. To date, it is not known whether AOA enzymes also catalyze the reaction between NH₂OH and NO₂⁻ (Figure 1, pathway 2) as observed for AOB by Hooper (1968). The original AOB periplasmic enzyme complex purified in that study included HAO as well as other enzyme components. No homologs of *hao* have been identified in AOA genomes, though alternatives have been proposed (Stahl and de la Torre, 2012). Furthermore, if a periplasmic reservoir of NO₂⁻ plays a role in the incorporation of NO₂⁻-derived N into N₂O, as we hypothesize here, then differences in the NO₂⁻ permeability of the outer membranes/cell walls of AOB vs. AOA could contribute to differences in the degree to which N derived from NH₄⁺ vs. exogenous NO₂⁻ contribute to N₂O formation. Unlike AOB, almost all archaea tested have only a single cell membrane bounding the cytoplasm (Albers and Meyer, 2011). Rather than having an outer membrane, AOA have an S-layer protein cell wall separating a pseudo-periplasm from the surrounding environment (Stieglmeier et al., 2014a). Model predictions of AMO protein structure in the soil AOA *Candidatus Nitrosotalea devanterra* suggest that the membrane-bound enzyme faces outward into the pseudoperiplasm (Lehtovirta-Morley et al., 2016). For future reference during ¹⁵N tracer studies of N₂O production, it would be helpful to confirm that NH₂OH and NO₂⁻ both form in the pseudoperiplasm of AOA, and investigate what controls the rates at which exogenous NO₂⁻ enters, and periplasmic NO₂⁻ exits, this compartment.

A Putative Link to O₂

During both the Lake Lugano and Namibian Upwelling incubations, more ¹⁵N-N₂O was produced at the reduced-O₂ concentrations (O₂ = 70 and 20 μM, respectively) than at the untreated-O₂ concentrations (O₂ = 290 and

220 μM, respectively). It is well known that N₂O yields by AOB increase during growth at suboxic O₂ concentrations (Goreau et al., 1980), and previous work on the mechanisms causing this increase implicated induction of the nitrifier denitrification pathway at very low O₂ concentrations (e.g., Frame and Casciotti, 2010). Reducing O₂ from 290 to 70 μM in the Lake Lugano incubations nearly tripled the yield of ⁴⁵N₂O and ⁴⁶N₂O during the ¹⁵NH₄⁺ incubations. However, the reason for this increase was probably not increased nitrifier denitrification, since there was no ⁴⁶N₂O production during any of the incubations with ¹⁵NO₂⁻ (Figure 5C).

The mechanism conferring this O₂ sensitivity may not necessarily involve direct regulation of enzyme activity. In particular, NO removal by O₂ is a possible abiotic NO-sink that would become more important at higher O₂ and NO concentrations:



$$k = 1.8 \times 10^{11} \text{M}^{-2} \text{day}^{-1} (\text{Awad and Stanbury, 1993}) \quad (13)$$

Once NO₂ is formed, in aqueous solutions it tends to react with water to form NO₃⁻ and NO₂⁻ (Park and Lee, 1988), or react with NO to form N₂O₃ (Grätzel et al., 1970). Martens-Habbena et al. (2015) measured NO concentrations of ~50–80 nM during oxic incubations of *N. maritimus* with 10 μM NH₄⁺. If similar NO concentrations were produced during incubations in the present study, liquid-phase reactions between NO and O₂ may deplete NO concentrations significantly, with the rate of depletion increasing in proportion to [O₂] as well as [NO]². Thus, abiotic reaction with O₂ may compete for NO with N₂O-forming reactions that also consume NO, particularly when incubation O₂ concentrations are high.

Like O₂, NO tends to partition into the gas phase over the aqueous phase (Schwartz and White, 1981). If it is NO (aq), rather than NO₂⁻, that participates in biological hybrid N₂O formation, an implication is that inclusion of a headspace during incubations of ammonia oxidizers suspended in water will reduce aqueous NO concentrations, and therefore slow down liquid-phase NO-dependent reactions (such as the reaction of NO with NH₂OH to form N₂O). Differences in aqueous NO concentrations might contribute to the discrepancy in the literature over whether reduced-O₂ growth conditions increase the yields of N₂O produced by AOA (e.g., Löscher et al., 2012; Stieglmeier et al., 2014b), particularly if there is variation in aeration procedures and ratios of headspace to liquid volumes.

CONCLUSIONS

Previous studies have shown that decreases in pH can increase N₂O production by AOB cultures (e.g., Jiang and Bakken, 1999a) but did not separate the effect of pH-dependent NH₃ limitation from the influence of pH on the N₂O production mechanisms. Here we have shown that acidification enhances the N₂O yields of ammonia oxidizers even when it does not substantially change the ammonia oxidation rates. We have demonstrated

that hybrid N₂O formation (i.e., the combination of NH₄⁺- and NO₂⁻-derived N) occurs among the *Nitrosospira*-dominated ammonia oxidizer community in the shallow hypolimnion of Lake Lugano and that this mechanism contributes to the increased yield of N₂O under acidified conditions. The NH₄⁺-derived reactant in this hybrid N₂O production pathway is probably NH₂OH, while the NO₂⁻-derived reactant could be one of several inter-convertible nitrogen oxides (NO₂⁻/HNO₂, NO, N₂O₃). Our results suggest that nitrifier denitrification was not an important source of N₂O in this environment. While N derived from exogenous NO₂⁻ contributed significantly to N₂O formation under acidified conditions, N derived from NH₄⁺ was always a more important contributor to N₂O. Finally, we report preliminary isotopic evidence that hybrid N₂O formation also occurs among the subsurface AOA-dominated nitrifier community present in the Namibian Upwelling zone.

Our results are not necessarily predictive of the long-term influence of acidification on N₂O production by ammonia oxidizers, since acidification may also change ammonia oxidizer community composition (Bowen et al., 2013) and pH decreases may have cascading chemical and biological effects in lake and ocean ecosystems. However, our results are applicable to environments that experience rapid changes in pH such as stratified lakes that undergo episodic mixing or rapid influx of acidified precipitation, and ocean upwelling zones where CO₂-rich, low-pH deeper water may enhance N₂O production when it comes in contact with shallower ammonia-oxidizing communities.

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AUTHOR CONTRIBUTIONS

CF conceived of and performed experiments. EL and EN analyzed and interpreted genetic sequence data. TG provided instrumentation support and sample analysis. CF and ML performed chemical data analysis and interpretation. All authors contributed to writing this paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.02104/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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