

## Nitrification in Mono Lake, California: Activity and community composition during contrasting hydrological regimes

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### Abstract

Rates of nitrification, geochemical variables, and the associated ammonia oxidizer microbial community were investigated in the water column of Mono Lake, California, between August 2002 and August 2003. Ammonia oxidation rates were measured using a <sup>15</sup>N isotope tracer technique. 16S ribosomal deoxyribonucleic acid, functional gene, and fluorescence in situ hybridization (FISH) analyses were used to characterize the ammonia oxidizer population. Peak ammonia oxidation activity occurred consistently between 12 and 14 m; the maximum integrated rate was observed in November 2002. The ammonia-oxidizing bacterial (AOB) community exhibited sequences most closely related to halo and/or alkaline tolerant *Nitrosomonas*-like sequences. The observed phylogeny represented a significant shift from previously documented AOB community composition and was coincident with Mono Lake's transition from monomixis to meromixis. Samples were also analyzed for ammonia-oxidizing archaea (AOA). FISH analysis revealed a substantial population of Crenarchaeota, the phylum encompassing all known AOA; however, no archaeal ammonia monooxygenase (*amoA*) sequences were detected. Unrealistic AOB cell-specific nitrification rates strongly indicate the possibility of a missing nitrification source, and correlations between nitrification rates, geochemical variables, and crenarchaeal and AOB abundance also indicate a significant AOA contribution to nitrification. However, the lack of verifiable archaeal *amoA* genes leaves open the question of whether AOA contribute to nitrification in Mono Lake.

Mono Lake is an alkaline (pH 9.8), saline (68–79 g kg<sup>-1</sup>) lake located just east of the Sierra Nevada range in northern California (38°N, 119°W). Like many closed-basin lakes, Mono Lake's hydrological regime alternates between periods of meromixis and monomixis depending on interannual variations of freshwater inflow (Melack and Jellison 1998). The prevailing hydrological mixing regime affects water column fluxes and resulting mixolimnetic ammonia (NH<sub>3</sub>) concentrations. During monomictic periods, fall turnover replenishes the mixolimnion with nutrient-rich deep water. During meromictic periods, the development of a steep salinity gradient (chemocline) isolates a portion of bottom water (monimolimnion) and prevents seasonal holomixis (Romero et al. 1998). The development of a persistent chemocline in 1995 produced an extended period of meromixis and resulted in the accumulation of high concentrations of NH<sub>3</sub> in the monimolimnion and chronic nitrogen (N) limitation in the

mixolimnion (Melack and Jellison 1998). Ammonia concentrations exert a major control on nitrification rates (Ward 1986), and, thus, changes in mixing regimes may affect nitrification activity.

Nitrification, an aerobic, chemolithotrophic process that converts ammonia (NH<sub>3</sub>) to nitrate (NO<sub>3</sub><sup>-</sup>) via nitrite (NO<sub>2</sub><sup>-</sup>), plays a key role in N cycling in aquatic environments (Ward 1986; Hastings et al. 1998; Joye et al. 1999). The products of nitrification (e.g., NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>) may serve as substrates for denitrifiers or anaerobic ammonia oxidizers, thus removing bioavailable N from the system through N gas evolution (Jenkins and Kemp 1984; Codispoti and Christensen 1985; Mulder et al. 1998). In alkaline ecosystems, however, nitrification may curtail the loss of fixed N via NH<sub>3</sub> volatilization by converting NH<sub>3</sub> to NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> (Joye et al. 1999). Ammonia volatilization is a critical aspect of the N cycle in Mono Lake as a result of the lake's low external N inputs, high internal nutrient recycling rates (Jellison et al. 1993), and strong N limitation of phytoplankton primary production (Jellison and Melack 1993).

Mono Lake nitrification was investigated just prior to and again shortly after the transition from monomixis to meromixis in 1995–1996. Significant ammonia oxidation rates and a viable ammonia-oxidizing bacterial (AOB) population were documented throughout the oxic water column in April and July 1995 (Joye et al. 1999; Ward et al. 2000). Subsequent phylogenetic analyses during early meromixis (August 1997 and April 1998) did not detect any AOB sequences using the original Ward et al. (2000) protocols or with a wide variety of general and nitrifier-specific 16S ribosomal deoxyribonucleic acid (rDNA) and functional gene primers, denaturing gradient gel electrophoresis (DGGE), and clone library analyses (Hovanec 1998). The AOB population was presumed to be below

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detection limits, and, thus nitrification was assumed to be insignificant (Hovanec 1998). The results presented here were obtained after an extended period of meromixis; the lake had not experienced holomixis for 8 yr, resulting in chronic N limitation in the mixolimnion (Melack and Jellison 1998).

We hypothesized that the dramatic physical and geochemical perturbations inherent in the 1995–1996 transition from monomixis to meromixis disrupted the AOB population and their nitrification activity. In this study, we examined whether the relatively stable geochemical and hydrological conditions characteristic of persistent meromixis facilitated a revival in nitrification activity. We also examined the effect of persistent meromixis on the nitrifier community composition.

## Methods

**Sampling**—Field sampling was conducted during an extended period of meromixis across seasons between August and November 2002 and May and August 2003. Depth profiles for each sampling time were obtained near a permanently moored buoy in the central basin of Mono Lake (Sta. 6; 37°57.822'N, 119°01.305'W). The entire water column was sampled for nutrients, dissolved gasses, and microbial community composition. Sample collection depths for nitrification measurements encompassed the epilimnion from the surface layer through the bottom of the oxycline.

**Limnology and geochemistry**—Temperature (T) and conductivity profiles were obtained using a SeaBird SeaCat CTD profiler. Oxygen concentrations were determined using an Orion® (model 842) dissolved oxygen meter fitted with a galvanic dissolved oxygen probe (Orion, model 084260). Samples were collected from discrete depths using a 5-liter Niskin water sampler and were stored appropriately (*see below*) for ensuing analyses.

Dissolved gas samples (10 mL) were transferred from the Niskin sampler into 20-mL helium-purged headspace vials via gas-tight syringe. The vials contained a sodium hydroxide (NaOH) pellet to arrest biological activity. Dissolved nitrous oxide (N<sub>2</sub>O) concentrations were measured using a headspace extraction–gas chromatographic technique (Joye and Paerl 1994).

Samples for nutrient analysis were filtered (Millipore Acrodisc, 0.2 μm) and stored at 4°C without headspace to avoid volatilization of NH<sub>3</sub> in these high-pH (~9.8) samples. Total ammonium (NH<sub>4</sub><sup>+</sup> + NH<sub>3</sub>) concentrations were determined at the field laboratory within 6 h using the phenol–hypochlorite method of Solarazano (1969); we use NH<sub>3</sub> to refer to the NH<sub>4</sub><sup>+</sup> + NH<sub>3</sub> pool. Nitrite and nitrate concentrations were quantified at the University of Georgia lab approximately 7–14 d later. Nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite (NO<sub>2</sub><sup>-</sup>) were measured cumulatively as NO<sub>x</sub> using an Antek®745 Nitrate and Nitrite Reducer (vanadium reduction assembly) inline with an Antek®7050 chemiluminescent nitric oxide detector (Álvarez-Salgado and Miller 1998). Reduction efficiency of the vanadium solution was checked after every 10 samples. Nitrite concentrations were

determined by spectrophotometry (Bendschneider and Robinson 1952) modified for Mono Lake water pH.

**Nitrification rates**—Water for nitrification rate measurements was transferred from the Niskin sampler to 500-mL rectangular plastic media bottles (Nalgene®), and the bottles were sealed without headspace. Nitrification rates were measured using a <sup>15</sup>N tracer technique (adapted from Ward 1987). Water samples from each depth (*n* = 3) were amended with a <sup>15</sup>NH<sub>4</sub>Cl solution (Cambridge Isotope 99.9 atom % <sup>15</sup>N) to achieve a final tracer concentration equal to 10% of the in situ NH<sub>4</sub><sup>+</sup> concentration. Samples were incubated for 24 h at in situ temperature in the dark, after which the sample was filter sterilized to halt biological activity.

The atom % enrichment of the NO<sub>x</sub> pools resulting from <sup>15</sup>NH<sub>3</sub> oxidation was determined using a modified NH<sub>3</sub> diffusion technique (Sigman et al. 1997). Following incubation, residual <sup>15</sup>NH<sub>3</sub> tracer was removed during sample reduction by incubation at 65°C, the sample NO<sub>x</sub> pool was reduced to NH<sub>3</sub> using Devarda's alloy, and this NH<sub>3</sub> was trapped on an acidified filter (as NH<sub>4</sub><sup>+</sup>; Sigman et al. 1997). Unamended controls were run as samples to correct for <sup>15</sup>N associated with dissolved organic nitrogen and Devarda's alloy (Sigman et al. 1997). Filters were subsequently analyzed using a Carlo Erba CHN (Model NA 1500) Combustion Analyzer coupled to a Finnigan Delta C Isotope Ratio Mass Spectrometer via a Finnigan ConFlo II interface (University of Georgia Ecology Analytical Laboratory).

Nitrification (NTR) rates were calculated by determination of the atom % enrichment of the NO<sub>x</sub> pool in each sample. The differences in atom % between initial and final <sup>15</sup>NO<sub>x</sub> pools in each sample were converted to nitrification rates (nmol NO<sub>3</sub><sup>-</sup> L<sup>-1</sup> per unit time; Eq. 1) and corrected for <sup>15</sup>NH<sub>3</sub> isotope tracer dilution (Eq. 2).

$$\text{nmol } ^{15}\text{NO}_3^- \text{ L}^{-1} \text{ d}^{-1} = \left( \left[ \text{atom } \% ^{15}\text{NO}_3^- \right]_{(f)} - \text{atom } \% ^{15}\text{NO}_3^- \right) \times \text{NO}_3^- / (1 \text{ d}) \quad (1)$$

In Eq. 1, <sup>15</sup>NO<sub>3</sub><sup>-</sup><sub>(f)</sub> and <sup>15</sup>NO<sub>3</sub><sup>-</sup><sub>(i)</sub> are the final and initial concentrations of <sup>15</sup>NO<sub>3</sub><sup>-</sup> in the sample, respectively, and NO<sub>3</sub><sup>-</sup><sub>(f)</sub> is the final NO<sub>3</sub><sup>-</sup> concentration in the sample.

$$\text{corrected rate} = \text{nmol NO}_3^- \text{ L}^{-1} \text{ d}^{-1} / \alpha \quad (2)$$

In Eq. 2, the rate from Eq. 1 is divided by α, the % <sup>15</sup>NH<sub>3</sub> in the NH<sub>3</sub> pool. The value of α is 10% (or 0.1), as the <sup>15</sup>NH<sub>3</sub> tracer accounted for 10% of the NH<sub>3</sub> pool.

Cell-specific rates of NH<sub>3</sub> oxidation were calculated by dividing the moles of NH<sub>3</sub> oxidized L<sup>-1</sup> by the total number of AOB or Crenarchaeota (Cren) cells L<sup>-1</sup>, as determined by whole cell hybridization (*see below*) at each depth.

**Molecular analyses**—Water samples for molecular analyses were transferred into 1–4-liter acid-cleaned, sample-rinsed containers and stored at 4°C until processing. Ten-milliliter subsamples for fluorescence in situ hybridization

(FISH) analysis were fixed with formalin (final concentration of 4%) for 30 min at the field laboratory and then stored frozen at  $-20^{\circ}\text{C}$  until analysis (modified from Pernthaler et al. 2001). The remaining water from each depth was passed through individual Millipore Sterivex filter cartridges ( $0.2\ \mu\text{m}$ ) to collect microbial biomass for subsequent DNA extraction. The final volume of sample remaining in the cartridge was expelled through the filter with air, replaced with an extraction buffer ( $1.5\ \text{mol L}^{-1}$  NaCl,  $5\ \text{mmol L}^{-1}$   $\text{MgCl}_2$ ,  $100\ \text{mmol L}^{-1}$  Tris HCl [pH 7],  $100\ \text{mmol L}^{-1}$  ethylenediamine tetraacetic acid [pH 8], and  $100\ \text{mmol L}^{-1}$   $\text{Na}_2\text{HPO}_4$  [pH 8]), and the cartridge was stored at  $-80^{\circ}\text{C}$  until processing (Ferrari and Hollibaugh 1999).

DNA was extracted from Sterivex filters using a technique modified from Murray et al. (1996) and Ferrari and Hollibaugh (1999). Filter cartridges containing extraction buffer were thawed at room temperature. Forty microliters of lysozyme ( $50\ \text{mg mL}^{-1}$ ) was added and the cartridges were incubated at  $37^{\circ}\text{C}$  for 30 min on an orbital shaker (25 rpm). Lysate was withdrawn from the cartridge and then the cartridge was rinsed with 1 mL extraction buffer, which was then withdrawn and added to the original lysate to ensure maximum yield. DNA was further liberated from the lysate using bead-mill homogenization (adapted from the MO BIO<sup>®</sup> Ultraclean Soil DNA kit protocol).

Previously characterized oligonucleotide primers and appropriate positive and negative controls were chosen for polymerase chain reaction (PCR) analysis of ammonia-oxidizing bacteria (16S rDNA gene and bacterial *amoA*), general archaea (16S rRNA gene), and ammonia-oxidizing archaea (archaeal *amoA* gene). Samples were screened for  $\beta$ -Proteobacteria ammonia-oxidizers using a nested approach: the EUB1 and EUB2 primer pair (Liesack et al. 1991) was used to amplify total bacterial 16S rDNA from raw extract, and that product was subjected to a second amplification using the Nit-A and Nit-B primer pair (Voytek and Ward 1995; Ward et al. 2000). An identical nested approach was used with the NOC1 and NOC2 primer pair to screen for members of the  $\gamma$ -Proteobacteria ammonia-oxidizers (Voytek 1996). The bacterial *amoA* gene was screened for using the primer set *amoA*-1F and *amoA*-2R (Rotthauwe et al. 1997). DNA extract was screened for the presence of ammonia-oxidizing archaea (AOA) using the primer sets Cren-*amo*1F and Cren-*amo*1R (Könneke et al. 2005) and Arch-*amo*AF and Arch-*amo*AR (Francis et al. 2005), which target the putative archaeal ammonia monooxygenase (*amoA*) gene. The positions of the two *amoA* primer sets were not suitable for a nested approach, as neither set nor combinations thereof can be internal to an initial PCR reaction. Universal archaeal primer sets Arch 21F and 1492R (DeLong 1992), Arch 344F and 915R (Stahl and Amann 1991), and Arch 344F and 517R (Raskin et al. 1994) were used to screen for general archaeal populations. Though no visible amplicons were produced using the Universal 21f and 1492r primer set, internal primer sets (Stahl and Amann 1991; Raskin et al. 1994) and various combinations thereof were nested using gel extract from the position where product from the

universal primer set PCR reactions should have been as template.

PCR products were isolated by agarose gel electrophoresis, extracted from the agarose (QIAquick<sup>®</sup> Gel Extraction Kit; Qiagen), inserted into the pCR<sup>®</sup>4-TOPO<sup>®</sup> plasmid vector, and transformed into chemically competent One Shot<sup>®</sup>TOP10 *Escherichia coli* cells, per the manufacturer's instructions (Invitrogen Corp). Transformed cells were plated on Luria-Bertani (LB) plates containing ampicillin ( $100\ \text{mg mL}^{-1}$ ) and incubated overnight at  $37^{\circ}\text{C}$ . Colonies were chosen at random from replicate plates and cultured overnight at  $37^{\circ}\text{C}$  in 1.2 mL of LB medium containing ampicillin ( $100\ \text{mg mL}^{-1}$ ).

*Clone library construction, screening, and sequence analysis*—Our clone library was constructed from pooled PCR extract from four discrete samplings that had produced nearly identical DGGE banding patterns (data not shown). Clones (192) were screened based on their ampicillin resistance and agarose gel analysis of clonal insert PCR products. DGGE was used to identify clones that produced multiple bands with identical electrophoretic mobility. By sequencing representative samples (approximately 50% of clones that produced >15 identical bands), sequence identity of the bands ( $\leq 1\%$  difference) was confirmed while reducing the number of clones required for sampling the population adequately.

Frozen, glycerol-preserved whole-cell clone cultures were sequenced (primer M13F) by SeqWright and yielded 92 robust sequence fragments of 690–800 base pairs. Sequences were analyzed for chimeric characteristics using the Ribosomal Database Project II's Chimera Check, version rdp8 (Maidak et al. 1999). A rarefaction curve was constructed using the Analytic Rarefaction program 1.3 with the formulations of Tipper (1979), provided by Steven Holland (Department of Geology, University of Georgia). Sequences were then compared with published sequences using BLAST (National Center for Biotechnology Information). Sequences from the data library exhibiting the highest similarities (closest relatives) were aligned with clone sequences using the Genetics Computer Group package. A phylogenetic tree was constructed using the neighbor-joining method and Jukes-Cantor distances (PHYLIP package version 3.5). The AOB nucleotide sequences generated in this study were deposited in GenBank and assigned accession numbers EU128521–EU128525.

*Whole cell hybridization*—FISH was used to determine the abundance of AOB and Crenarchaeota, a proxy for AOA (Könneke et al. 2005; Wüchter et al. 2006). FISH probes specific for AOA have not yet been developed, but all sequences recovered from putative AOA have been associated with the Crenarchaeota (Venter et al. 2004; Francis et al. 2005; Leininger et al. 2006). Although it is almost certain that not all crenarchaeotes oxidize  $\text{NH}_3$ , their enumeration does provide valuable information about the potential involvement of AOA in nitrification.

Ammonia-oxidizing bacteria were enumerated using previously described oligonucleotide probes NEU,

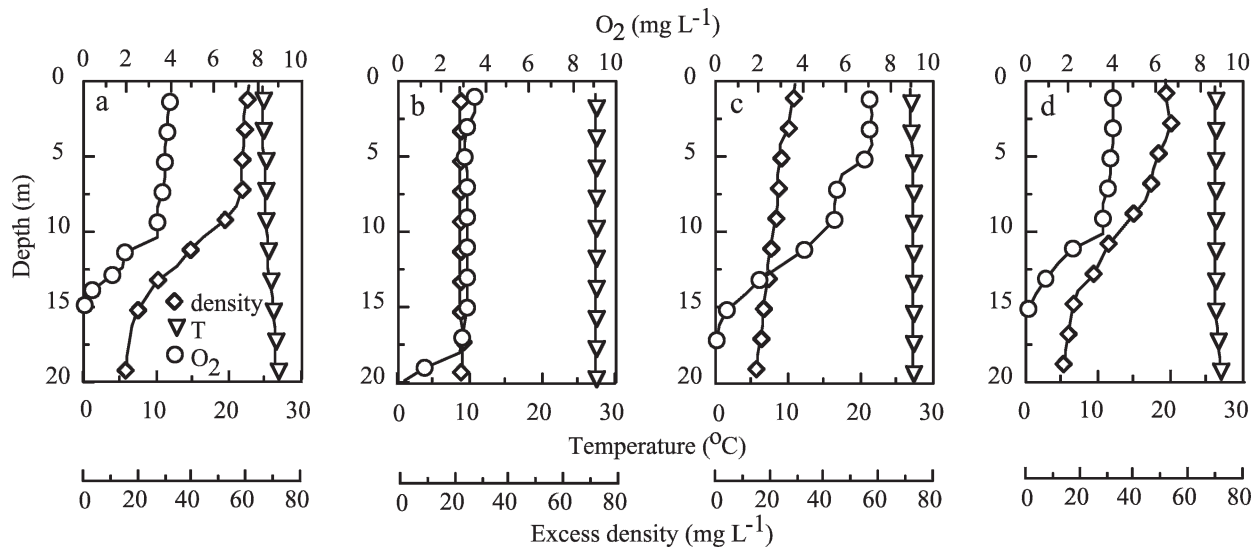


Fig. 1. Vertical depth profiles of temperature, oxygen, and excess density in Mono Lake from (a) August 2002, (b) November 2002, (c) May 2003, and (d) August 2003.

Nso190, and Nsm156, designed to detect halophilic and halotolerant *Nitrosomonas* spp., ammonia-oxidizing  $\beta$ -Proteobacteria, and some *Nitrosococcus* spp., respectively (Mobarry et al. 1996). The probes were synthesized by MWG Biotech and labeled with Texas Red. Formalin-preserved samples were thawed and mixed well. Then 500  $\mu$ L of sample was added to a sterile centrifuge tube containing 15 mL of phosphate-buffered saline (PBS) solution, mixed well, and then filtered through a 0.2- $\mu$ m polycarbonate membrane filter (Osmonics). The tube was then rinsed with PBS ( $2 \times 15$  mL), and this material was also passed through the filter to assure that all cells were transferred to the filter. The filter was washed with 15 mL of sterile water ( $2 \times$ ) and allowed to air dry in a Petri dish. Sections of individual filters were separated for 4',6-diamidino-2-phenylindole (DAPI)-only staining as well as hybridization with specific probes and DAPI counterstaining. AOB hybridization reactions were performed in succession on single filter sections to accommodate optimum formamide concentrations for each of the three probes (Mobarry et al. 1996). Filter sections were then washed, rinsed in milli-Q water, air dried, and counterstained with DAPI for 3 min (50  $\mu$ L of 2 mg mL<sup>-1</sup> solution). Enumeration of Crenarchaeota was carried out in the same manner, using probe Cren537, as described by Herndl et al. (2005).

Total microbial and hybridized nitrifier and Crenarchaeota cells were counted using epifluorescence microscopy by enumerating cells in each of 40 squares in 50 randomly selected fields for each sample ( $n = 2000$  grid-squares filter section<sup>-1</sup>). Cells per milliliter in each sample were calculated by taking the mean number of cells counted in the 50 selected fields per filter section multiplied by a conversion factor derived from a combination of the counting grid area, the percentage of the grid utilized, the surface area of the filter, and the volume of water filtered. Total cell counts from the DAPI-only stained filter sections and DAPI counts from counterstained, hybridized filters

were compared to controls for loss of cells from the filters that may have occurred during hybridization.

**Statistical analysis**—Linear regression ( $R^2$ ) or Pearson's Correlation Coefficient ( $r$ ) analyses were used to evaluate correlations between nitrification rates, pertinent geochemical variables, and microbial group-specific abundances. Spatial and temporal differences in  $\text{NH}_3$  oxidation rates were compared using paired  $t$ -tests ( $n = 3$ ). Differences between the spatial and temporal AOB and Crenarchaeota cell numbers derived from FISH data were compared using a combination of ANOVA and post hoc Tukey's analyses.

## Results

**Limnological and geochemical profiles**—Oxygen and T profiles displayed distinct seasonal patterns, while excess density, calculated from conductivity data (Jellison et al. 1999), remained relatively constant throughout the study period. The lake remained isohaline in the mixolimnion, with excess density averaging 69.4 mg L<sup>-1</sup> (Fig. 1). Temperature and O<sub>2</sub> profiles in August 2002 and August 2003 were strongly correlated ( $r = 0.99$  and 0.98, respectively) and demonstrated seasonal stratification with discrete oxyclines between 9 and 15 m (Fig. 1). The deterioration of thermal stratification was evident in November 2002 as O<sub>2</sub> concentrations and temperatures became relatively homogeneous to a depth of 20 m (Fig. 1b), while the onset of seasonal stratification became evident in the May 2003 profile (Fig. 1c).

Ammonia concentrations in the Mono Lake water column varied over depth during each of the sample dates. During August 2002, May 2003, and August 2003,  $\text{NH}_3$  concentrations were generally low ( $<1 \mu\text{mol L}^{-1}$ ) in the mixolimnion but increased dramatically (up to 20-fold) through the oxycline (Fig. 2a). In contrast, the November 2002 profile showed elevated  $\text{NH}_3$  concentrations (5–11  $\mu\text{mol L}^{-1}$ ) distributed more consistently throughout

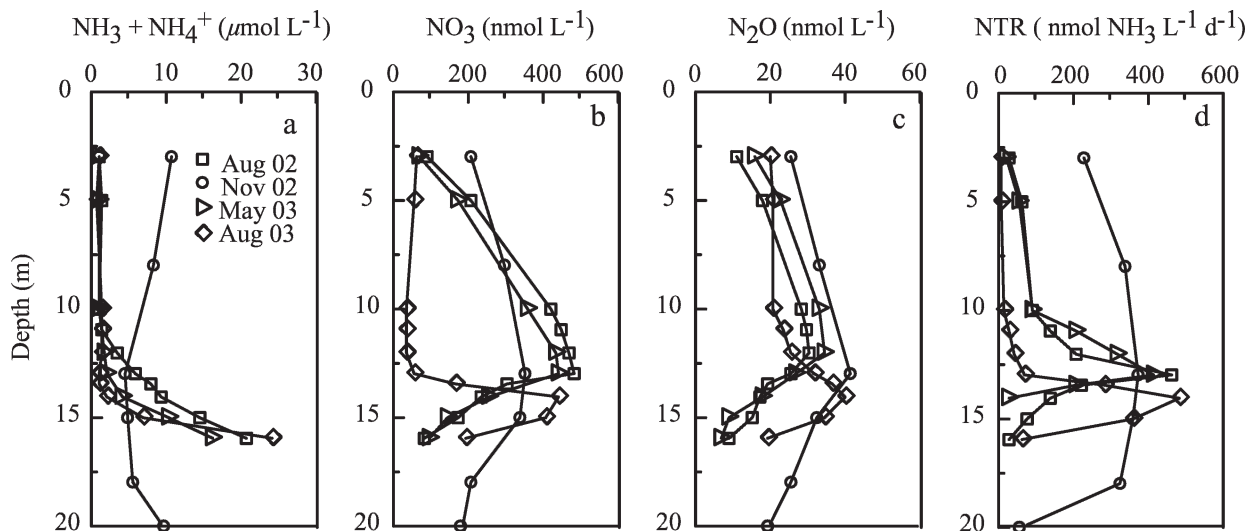


Fig. 2. Vertical depth profiles of nitrification rates and biogeochemical parameters in Mono Lake from August 2002, November 2002, May 2003, and August 2003: (a)  $\text{NH}_3$  concentration, (b)  $\text{NO}_x$  concentration, (c)  $\text{N}_2\text{O}$  concentration, and (d) nitrification rate.

the mixolimnion (Fig 2). All  $\text{NH}_3$  profiles displayed a concave up distribution between 11 and 15 m (Fig. 2a).

$\text{NO}_x$  concentrations during August 2002 and May 2003 were low ( $66\text{--}89\text{ nmol L}^{-1}$ ) in the surface water, slowly increased to peak levels at about 13 m, and then decreased to  $\sim 90\text{ nmol L}^{-1}$  at 16 m (Fig. 2b). The highest upper-water column  $\text{NO}_x$  concentration ( $204\text{ nmol L}^{-1}$ ) occurred in November 2002. November  $\text{NO}_x$  concentrations displayed a different profile, with significantly higher concentrations in the surface water and at the bottom of the oxycline (Fig. 2b). In August 2003 concentrations of  $\text{NO}_x$  remained relatively low ( $<60\text{ nmol L}^{-1}$ ) throughout the upper water column (Fig. 2b), and a sharp peak ( $441\text{ nmol L}^{-1}$ ) developed between 13 and 14 m, before the concentration decreased to  $190\text{ nmol L}^{-1}$  at 16 m (Fig. 2b). Nitrous oxide concentrations ranged from 10 to  $40\text{ nmol L}^{-1}$ , and profiles followed the same general spatial patterns as  $\text{NO}_x$  profiles (Fig. 2c). The concentration of  $\text{N}_2\text{O}$  in Mono Lake water was supersaturated with respect to equilibrium with atmospheric  $\text{N}_2\text{O}$  concentrations (expected to be between  $0.3$  and  $0.8\text{ nmol L}^{-1}$ ; calculated from the Bunsen solubility coefficients from Weiss and Price 1980). The  $\text{NH}_3$  and  $\text{NO}_x$  concentrations observed during this study were similar to those presented by Hovanec (1998; Fig. 3).

**Nitrification rates**—Nitrification activity was observed throughout the oxic portion of the water column on all four sampling dates. Profiles in August 2002, May 2003, and August 2003 exhibited relatively low activity ( $<60\text{ nmol L}^{-1}\text{ d}^{-1}$ ) in both the upper water column and proximate to the bottom of the oxycline, while activity peaks ( $420\text{--}480\text{ nmol L}^{-1}\text{ d}^{-1}$ ) occurred consistently between 13 and 14 m (Fig. 2d). Rates measured in November 2002 displayed a very different profile: activity in the upper water column was approximately four times higher ( $230\text{--}335\text{ nmol L}^{-1}\text{ d}^{-1}$ ) than during the other sampling dates,

and higher rates were more evenly distributed throughout the mixolimnion (Fig. 2d).

**Molecular analysis**—Water collected during each sampling and at every depth sampled tested positive with the NitAB and the bacterial *amoA* primer sets. Results of PCR using the NOC primer set were negative for all samples, showing a lack of detectable  $\gamma$ -Proteobacteria ammonia oxidizers in Mono Lake. Based on DGGE screening, five clones dominated the bacterial ammonia-oxidizing community at each sampling point. However, because of the nested

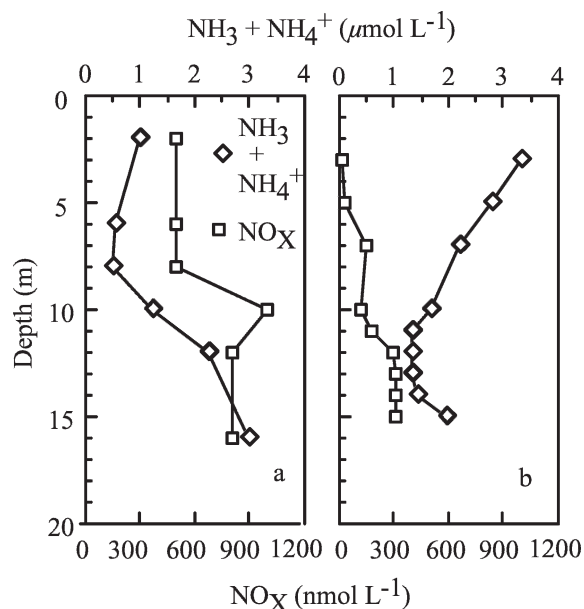


Fig. 3. Vertical depth profiles of  $\text{NH}_3$  and  $\text{NO}_x$  from (a) August 1997 and (b) April 1998 from the surface to the bottom of the oxycline (adapted from Hovanec 1998).

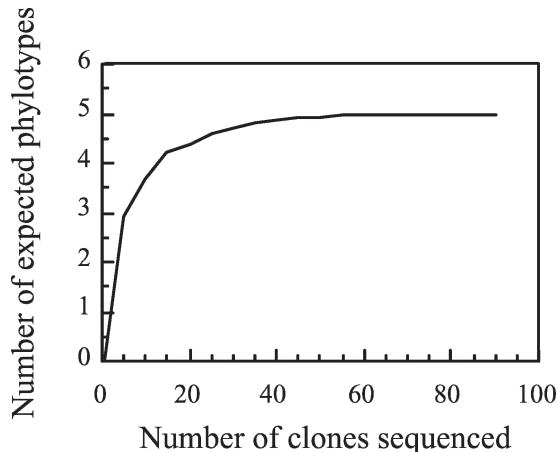


Fig. 4. Rarefaction curve generated for nitrifier 16S rDNA from clone sequences collected during this study.

protocol employed and the possibility of increased PCR bias, some microdiversity may have been obscured. All recovered sequences were affiliated with AOB from the  $\beta$ -Proteobacteria. Approximately 48% of the clone library was represented by a single *Nitrosomonas* sequence (ML\_AOB\_A2) that was most closely related (98%) to an uncultured *Nitrosomonas* sp. recovered from a Greenland alkaline tufa column (accession No. AJ431351). The remaining four unique sequences each comprised between 3% and 15% of the total AOB population. The associated rarefaction curve indicated that these five unique clones represented the dominant AOB species in Mono Lake during this study (Fig. 4). Sequences from clones ML\_AOB\_D4 and ML\_AOB\_E1 clustered with the dominant ML\_AOB\_A2 sequence and were most closely related (97–99%) to AOB clone ANs5 from a Mongolian soda lake (accession No. AY026317). The sequence from clone ML\_AOB\_A3 also clustered with sequences from clones ML\_AOB\_D4 and ML\_AOB\_E1 and the dominant ML\_AOB\_A2 but was most closely related (92%) to a *Nitrosomonas* sp. recovered from a Chinese saline lake (accession No. AY940537). ML\_AOB\_E4 was slightly more divergent but still most closely related to AOB clone ANs5 (Fig. 5).

All water samples screened during the course of the study tested negative for putative archaeal *amoA* genes despite consistent amplification of both the positive archaeal *amoA* PCR control and raw Mono Lake extract spiked with the positive control. In fact, no archaeal sequences were obtained using any of the general archaeal primer sets or combinations thereof. However, FISH analysis using probe Cren537 indicated the presence of Crenarchaeota, the phylum that encompasses the known archaeal ammonia oxidizers. Crenarchaeotes were present in all samples examined and their abundance varied over space and time (Fig. 6). Absolute Crenarchaeota cell numbers ranged from 1 to  $9 \times 10^5$  cells mL<sup>-1</sup> (Table 1) and comprised between 2% and 10% of total cells, as determined by DAPI enumeration. Crenarchaeote abundance profiles for all sampling dates were similar, with significant peaks between 13 and 14 m (Fig. 6). Crenarchaeote cell numbers correlated with NTR rates (regression  $p$

< 0.05 for August 2002 and May 2003 and  $p < 0.01$  for the sum of all data points, excluding November 2002; Table 2).

Ammonia-oxidizing bacteria comprised between 0.4% and 1.1% of the total bacterial population. Abundance varied significantly between sampling dates (ANOVA,  $p < 0.05$ ), but cell numbers did not vary significantly (ANOVA,  $p > 0.05$ ) over depth during any one sampling period (Fig. 6). In August 2002 AOB cell numbers were  $5.6 \times 10^4$  cells mL<sup>-1</sup> and decreased in November 2002 to  $3 \times 10^4$  cells mL<sup>-1</sup> (Fig. 6). Abundance of AOB steadily increased through May to a maximum observed during this study of  $6.3 \times 10^4$  cells mL<sup>-1</sup> in August 2003 (Fig. 6). Mono Lake AOB abundance did not correlate with nitrification rates (regression  $p > 0.05$  for each time point and the sum of all data points; Table 2). Estimates of AOB cell-specific nitrification rates ranged from 2.83 fmol NH<sub>3</sub> cell<sup>-1</sup> h<sup>-1</sup> in the surface water in August 2003 to 483.9 fmol NH<sub>3</sub> cell<sup>-1</sup> h<sup>-1</sup> in November 2002 (Table 2).

## Discussion

*Geochemical profiles and patterns of nitrification activity*—Ammonia, NO<sub>x</sub>, and N<sub>2</sub>O profile contours were consistent with the distribution of nitrification activity throughout the water column on all sampling dates (Fig. 1). Ammonia, the primary substrate for the first step in nitrification (Prosser 1989), regularly displayed profiles indicating consumption (Martens and Berner 1977); minimum NH<sub>3</sub> concentrations were consistently associated with the depths of maximum nitrification rates (Fig. 2). Concentrations of NO<sub>x</sub> and N<sub>2</sub>O correlated with NTR activity (regression  $R^2 > 0.86$ ;  $p < 0.01$ ; Table 1). Mono Lake NH<sub>3</sub> profiles from April and July 1995 also indicated consumption associated with measured NH<sub>3</sub> oxidation rates (Joye et al. 1999). These data indicate that geochemical profiles of these species are reasonable indicators of nitrification activity.

Ammonia and NO<sub>x</sub> profiles were measured in Mono Lake during August 1997 and April 1998. Although nitrification rates were not measured, NH<sub>3</sub> concentrations displayed profiles indicating consumption, while NO<sub>x</sub> concentrations increased with depth and peak NO<sub>x</sub> concentrations generally coincided with NH<sub>3</sub> minima (Fig. 3; adapted from Hovanec 1998). Available data indicate that nitrification occurred in 1995 (Joye et al. 1999) and 2002–2003 (this study). While no detectable AOB population was present (*see below*) during early meromixis (Hovanec 1998), available geochemical data indicate that nitrification was indeed occurring at that time as well.

During this study, subsurface peaks of nitrification occurred regularly (Fig. 2d), similar to patterns observed at oceanic sites such as the Southern California Bight and Station ALOHA. At these sites, subsurface rate maxima were commonly observed and attributed to factors including oxygen concentration, competition with phytoplankton for substrate, and photoinhibition (Olson 1981a; Ward 1987; Dore and Karl 1996). Maximum Mono Lake nitrification rates were generally 5–10 times higher than rates reported in estuarine habitats (Enoksson 1986; Bernounsky and Nixon 1993) and 50–200 times higher

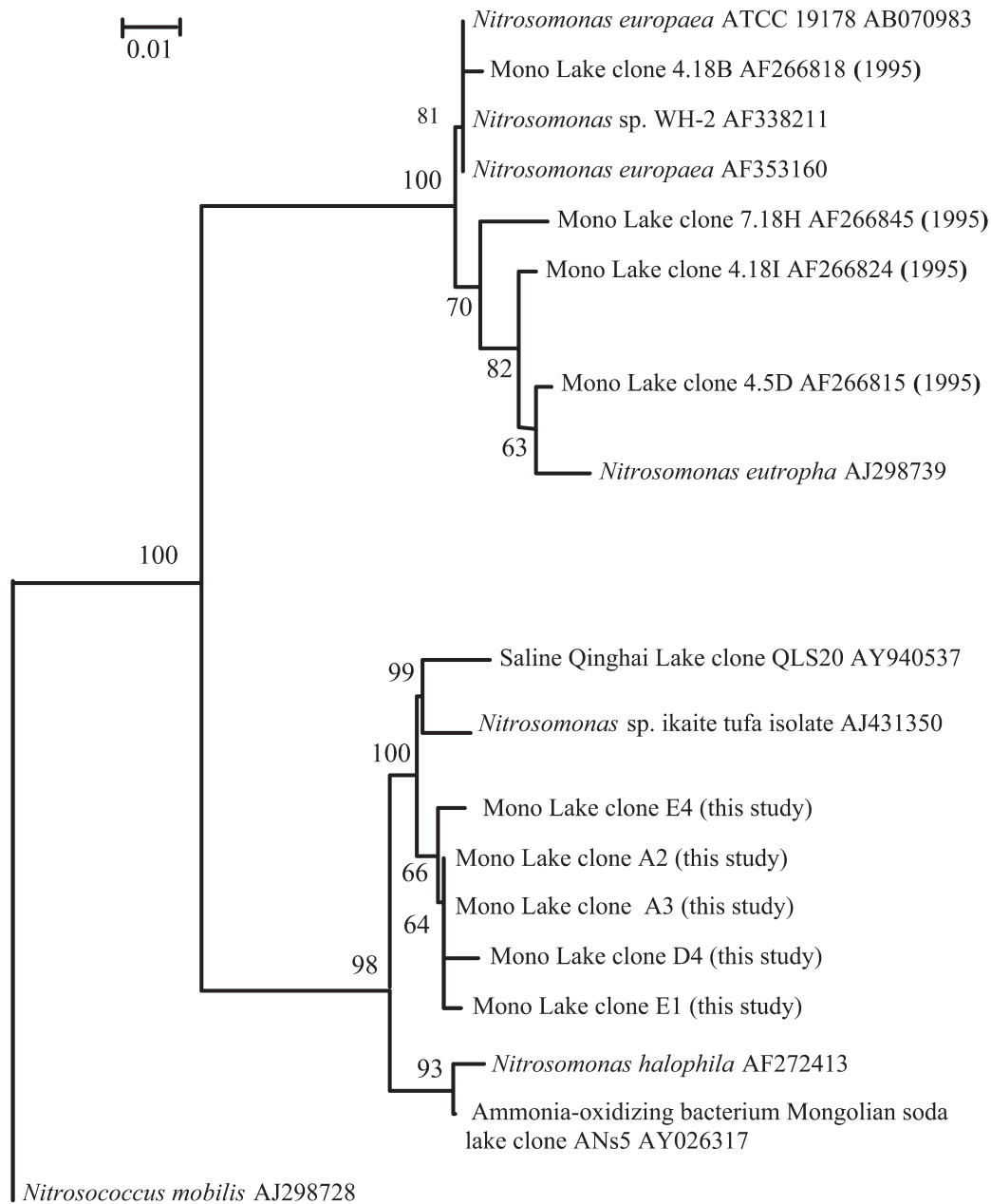


Fig. 5. Phylogenetic relationship of ammonia-oxidizing bacterial clone sequences from the Mono Lake water column. The tree was constructed using the neighbor-joining method and Jukes–Cantor distances. Bootstrap values > 50% (100 iterations) are indicated at the appropriate nodes. The scale bar represents 1% sequence divergence.

than those observed in Washington coastal waters (Ward et al. 1984), the Southern California Bight (Olson 1981b; Ward 1987), and at Station ALOHA (Dore and Karl 1996).

Integrated water column nitrification rates revealed that maximum Mono Lake nitrification activity occurred in November 2002 (Table 1), during which high rates of activity were distributed throughout the mixolimnion (Fig. 2d). The degradation of seasonal stratification deepened the mixolimnion (Fig. 1), and the entrainment of  $\text{NH}_3$ -rich water from the seasonally anoxic zone increased  $\text{NH}_3$  concentrations (Fig. 2a) and fueled nitrification. Higher mixolimnetic  $\text{NH}_3$  concentrations during fall

mixing likely lessened substrate competition between nitrifiers and phytoplankton. Further, lower light penetration due to increased algal productivity (Melack and Jellison 1998) meant less potential for photoinhibition of nitrifiers.

Calculated AOB cell-specific  $\text{NH}_3$  oxidation rates in zones of high nitrification activity ranged from 2 to over 400  $\text{fmol NH}_3 \text{ h}^{-1}$ ; many values were up to 20-fold higher than reported cell-specific rates for cultured nitrifying bacteria (Ward 2002). Even if Mono Lake AOB exhibit substantially higher cell-specific rates than available cultured AOB (Ward 2002), such high cell-specific rates and

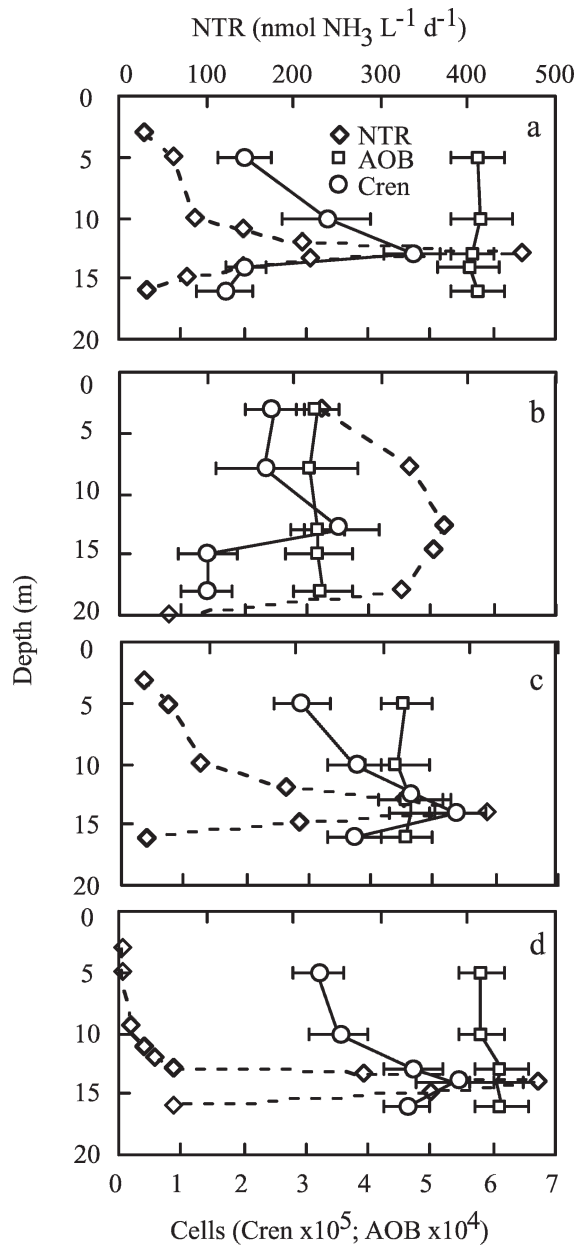


Fig. 6. Vertical profiles of  $\text{NH}_3$ -oxidizing bacterial (AOB) and Crenarchaeota (Cren) abundance and associated nitrification rate profiles from (a) August 2002, (b) November 2002, (c) May 2003, and (d) August 2003. Abundance error bars are standard deviations around the mean.

the likely occurrence of nitrification in the absence of detectable AOB, as implied by the Hovanec (1998) data (Fig. 3), indicate that a more parsimonious explanation is that AOA account for a substantial portion of the nitrification in Mono Lake. If both AOA and AOB contribute to nitrification, the calculated cell-specific rates fall within a more believable range ( $\leq 20 \text{ fmol NH}_3 \text{ h}^{-1}$ ; Table 2).

*AOB phylogenetic transition*—*Nitrosomonas* sequences with very high affinity to *Nitrosomonas europaea* and

*Nitrosomonas europaea*, species rarely encountered outside of freshwater or soil environments, dominated the Mono Lake AOB population in 1995 (Ward et al. 2000). Thirty-four unique sequences derived from NitAB amplifications were most closely related to *N. europaea* ( $>95\%$  similarity), and five were not differentiable ( $<2.5\%$  different) from the sequence of *N. europaea* (Stackebrandt and Goebel 1994; Ward et al. 2000). No  $\text{NH}_3$  oxidizers from the  $\gamma$ -Proteobacteria were detected. Subsequent molecular analyses in August 1997 and April 1998 were negative for all nitrifying bacteria (Hovanec 1998), indicating a significant decline in the ammonia-oxidizing bacterial community at that time.

Sequence data from the present study demonstrated a significant shift in AOB community composition at the species level accompanied by a decrease in diversity relative to the 1995 population. While the population was still dominated by *Nitrosomonas* species during 2002–2003 (Fig. 5), none of the sequences were most closely related to *N. europaea* strains (7–11% difference), and only five unique clone sequences were retrieved. Reexamination of the phylogenetic affiliations of the 1995 sequences confirmed the original results of Ward et al. (2000) and ensured that new additions to the sequence database were not responsible for the observed differences noted between the two studies. Pairwise sequence alignments also established that sequences retrieved during the current study were distinct from 1995 sequences.

Although the phylogenetic data from this study and the data presented in Ward et al. (2000) were collected from different sampling sites, general cross-site phylogenetic comparisons between these two studies have revealed very similar microbial population distributions (Hollibaugh et al. 2001; Humayoun et al. 2003). The upper water column of Mono Lake appears to be fairly homogeneous on this sampling scale. The combination of geochemical profiles, nitrification rate measurements, and phylogenetic information indicate a discontinuity in AOB nitrifier populations between 1995–1996 and 2002–2003 and a potential for AOA nitrification activity. We propose that the contrasting hydrological mixing regimes resulted in differential  $\text{NH}_3$  availability in the mixolimnion and drove the observed community composition shift of the AOB. The 1995 study was conducted at the end of an extended period of monomixis, during which mixolimnetic  $\text{NH}_3$  concentrations were significantly higher ( $>3 \mu\text{mol L}^{-1}$  for up to 60% of the year), and additional  $\text{NH}_3$  that had been compartmentalized during stratified periods was regularly redistributed by annual holomixis (Melack and Jellison 1998).

The transition to a meromictic regime in 1996 changed the nitrogen dynamics in the mixolimnion considerably. Ammonia that accumulated in the monimolimnion remained confined below a strong pycnocline. Mixolimnetic  $\text{NH}_3$  concentrations were limited by diffusion across the pycnocline, which drastically reduced  $\text{NH}_3$  availability to the upper water column. Ammonia concentrations became chronically low, and by 1997 mixolimnetic concentrations were  $<1 \mu\text{mol L}^{-1}$  throughout most of the year (Melack and Jellison 1998). Bacterial nitrifier populations became undetectable in the first few years (1997–1998) following

Table 1. Depths sampled for each time point and corresponding nitrification (NTR) rates and nitrogenous species concentrations. Summary rows for each sampling date include integrated NTR rates ( $\Sigma$  in  $\mu\text{mol m}^{-2} \text{d}^{-1}$ ) and regression analysis  $R^2$  and significance levels for NTR rates plotted against nitrogenous species concentration.

Sample	Depth (m)	Rate ( $\text{nmol L}^{-1} \text{NH}_3 \text{d}^{-1}$ )		$\text{NH}_3$ ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_3^-$ ( $\text{nmol L}^{-1}$ )	$\text{N}_2\text{O}$ ( $\text{nmol L}^{-1}$ )
Aug 2002	3	26.6		1.1	88.8	10.9
	5	62.7		1.3	208.8	17.7
	10	86.8		1.0	421.8	28.0
	11	141.3		1.5	448.0	29.5
	12	208.1		3.4	471.7	30.2
	13	459.2		5.9	482.7	25.5
	13.5	219.3		7.9	302.2	19.6
	14	140.9		9.5	237.2	17.5
	15	76.0		14.6	175.7	14.9
	16	30.3		20.6	82.8	9.1
		$\Sigma=1.41$	$R^2$	0.02	0.49*	0.28
Nov 2002	3	228.9		10.9	204.0	25.2
	8	336.6		8.2	300.0	32.8
	13	370.6		4.5	350.0	41.3
	15	357.2		4.8	340.0	32.7
	18	322.8		5.4	210.0	25.5
	20	55.5		9.8	180.0	19.0
		$\Sigma=5.31$	$R^2$	0.55	0.61	0.69*
May 2003	3	20.8		1.1	66.0	15.8
	5	54.8		1.1	174.2	22.8
	10	90.9		1.2	356.5	33.0
	12	204.0		1.6	431.2	34.8
	13	320.7		2.1	439.1	27.0
	14	417.3		4.2	257.0	17.8
	15	205.6		10.5	146.0	9.3
	16	29.6		16.1	94.2	7.2
		$\Sigma=1.79$	$R^2$	0.02	0.29	0.03
Aug 2003	3	4.57		1.0	59.2	20.3
	5	3.95		0.8	57.0	20.7
	10	14.71		1.5	37.9	20.4
	11	28.53		1.5	36.2	23.3
	12	44.02		1.4	36.2	25.8
	13	70.93		1.2	58.6	31.7
	13.5	279.57		1.2	164.0	36.4
	14	479.98		2.2	441.4	39.7
	15	356.92		6.8	406.9	34.8
	16	62.83		24.0	190.9	19.2
		$\Sigma=1.29$	$R^2$	0	0.86**	0.80**
Total			$R^2$	0.004	0.48**	0.32**

\*  $p < 0.05$ .

\*\*  $p < 0.01$ .

the transition to meromixis (Hovanec 1998). It is possible that the previous population, dominated by *N. europaea*-like sequences, was unable to adapt to meromictic conditions and the resulting change in mixolimnetic  $\text{NH}_3$  availability. Ammonia-oxidizing bacteria are notoriously slow growing (Prosser 1989), and recovery from environmental perturbation may proceed gradually. The observed shift in nitrifier phylogeny from 1995 to 2002–2003 may reflect the development of an AOB nitrifier population more adapted to meromictic conditions and the associated decrease in  $\text{NH}_3$  availability.

*Potential importance of archaeal vs. bacterial ammonia oxidizers*—Presumptive evidence for potential AOA con-

tribution to environmental ammonia oxidation (Francis et al. 2005; Könneke et al. 2005; Wüchter et al. 2006) has been corroborated by quantifying active expression of the putative archaeal *amoA* gene in the nitrite maxima of the Black Sea (Lam et al. 2007). Mono Lake possesses many of the presumptive indicators of AOA activity, including strong correlations between nitrification, geochemical variables, and crenarchaeal abundance, and a potentially large “missing” source of nitrification.

Mono Lake AOB cell-specific nitrification rates far exceed published rates (Ward 2002). However, measured cell-specific rates lie in a more reasonable range if Crenarchaeota are assumed to contribute to nitrification (Table 2). FISH analysis using probe Cren537 indicated

Table 2. Depths sampled for each time point and corresponding nitrification (NTR) rates, ammonia-oxidizing bacterial (AOB) community, Crenarchaeota (Cren) abundance, and cell-specific  $\text{NH}_3$  oxidation for AOB only and AOB + Cren. Summary rows for each sampling date include integrated NTR rates ( $\Sigma$  in  $\mu\text{mol m}^{-2} \text{d}^{-1}$ ) and regression analysis  $R^2$  and significance levels for NTR rates plotted against AOB, Crenarchaeota, and AOB + Cren abundance.

Sample	Depth (m)	Rate $\text{NH}_3$ ( $\text{nmol}^{-1} \text{d}^{-1}$ )	AOB ( $10^4 \text{ ml}^{-1}$ )	Cren ( $10^5 \text{ ml}^{-1}$ )	$\text{fmol NH}_3 \text{ AOB}^{-1} \text{ h}^{-1}$	$\text{fmol NH}_3 \text{ h}^{-1} (\text{AOB} + \text{Cren})^{-1}$
Aug 2002	3	26.6				
	5	62.7	5.8	3.6	45.40	6.33
	10	86.8	5.8	5.8	62.28	5.67
	11	141.3				
	12	208.1				
	13	459.2	5.7	8.2	337.84	21.83
	13.5	219.3				
	14	140.9	5.6	3.6	104.73	14.28
	15	76.0				
	16	30.3	5.8	3.0	21.96	3.58
		$R^2$	0.25	0.76*		
Nov 2002	3	228.9	3.2	4.4	298.82	20.42
	8	336.6	3.0	4.0	460.17	32.58
	13	370.6	3.2	6.2	483.90	23.69
	15	357.2	3.2	2.8	466.40	48.50
	18	322.8	3.3	2.5	410.40	48.42
	20	55.5				
		$R^2$	0.01	0.0		
May 2003	3	20.8				
	5	54.8	4.6	5.1	49.97	4.15
	10	90.9	4.4	6.5	85.34	5.46
	12	204.0				
	13	320.7	4.6	8.5	288.68	14.91
	14	417.3	4.7	9.4	373.08	17.71
	15	205.6				
	16	29.6	4.6	6.5	27.02	1.78
		$R^2$	0.48	0.89*		
Aug 2003	3	4.57				
	5	3.95	5.8	5.6	2.83	0.27
	10	14.71	5.8	6.1	10.56	0.92
	11	28.53				
	12	44.02				
	13	70.93	6.2	8.2	48.04	3.35
	13.5	279.57				
	14	479.98	6.1	9.4	329.68	20.09
	15	356.92				
16	62.83	6.2	8.1	42.55	3.02	
Total			$R^2$	0.13	0.6	
			$R^2$	0.18	0.06	
Total without November				0.00	0.45**	

\*  $p < 0.05$ .

\*\*  $p < 0.01$ .

that crenarchaeotes were present in all samples, and their numbers exhibited a strong correlation with NTR rates (Table 2); furthermore, Mono Lake AOB abundance did not correlate with nitrification rates (Table 2). An enrichment culture of Crenarchaeota from North Sea water demonstrated that Crenarchaeota abundance, but not that of AOB, correlated with NTR (Wüchter et al. 2006). The North Sea and Black Sea (Lam et al. 2007) data provides robust evidence that Crenarchaeota provide a good proxy for AOA. Although an AOA contribution to nitrification in Mono Lake seems likely, the correlative nature of the data and the lack of archaeal *amoA* amplification make it

impossible to assess the relative contribution to nitrification of archaeal and bacterial ammonia oxidation in Mono Lake at this time.

All water samples screened during the course of the study tested negative for putative archaeal *amoA* genes. These results were somewhat surprising in light of the ubiquity of AOA in many diverse environments (Francis et al. 2005; Leininger et al. 2006; Park et al. 2006). The lack of any recovered archaeal sequences, despite evidence for active Mono Lake archaeal populations, deserves consideration and comment. Measurable rates of water column anaerobic methane oxidation (Joye et al. 1999), a

process mediated by archaea, water column sample whole cell hybridization to archaeal-specific FISH probes (S. B. Joye unpubl.), and identification of archaeal-specific biomarkers (Turic et al. 2007) verify the presence of archaea in Mono Lake. It seems likely that Mono Lake archaea in general and perhaps archaeal *amoA* sequences in particular are not detected by current archaeal primer sets and that further investigation and methods development are needed to address the occurrence of archaea in Mono Lake.

Geochemical profiles and  $^{15}\text{N}$  tracer experiments demonstrated that significant nitrification activity was occurring in Mono Lake during this study. Biogeochemical and phylogenetic data indicated that nitrification was also occurring in Mono Lake in 1995. However, data from 1997 to 1998 indicate a discontinuity in the Mono Lake AOB population. Contrasting hydrological mixing regimes resulting in differential  $\text{NH}_3$  availability in the mixolimnion may have contributed to the observed shift in the AOB population. Though AOB abundance and nitrification activity were not correlated, crenarchaeota cell numbers exhibited a strong correlation with NTR rate measurements. Thus, we suspect AOA account for the "missing" nitrification source. The lack of recoverable archaeal *amoA* genes and of Mono Lake archaeal sequences in general indicates that existing PCR primers may not detect Mono Lake archaeal sequences and, by extension, a fraction of the archaeal population in other extreme environments.

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