A BIOGEOCHEMICAL AND MOLECULAR ECOLOGICAL STUDY OF AEROBIC METHANE OXIDATION AND NITRIFICATION IN MONO LAKE CALIFORNIA, USA

by

STEPHEN A. CARINI

(Under the Direction of Samantha B. Joye)

ABSTRACT

Methane (CH_4) and ammonia (NH_3) oxidation play key roles in the biogeochemical cycles of carbon (C) and nitrogen (N). In this dissertation, biogeochemical and molecular analyses were combined to elucidate environmental and microbiological controls on these processes and link specific activity with microbial community composition in Mono Lake, CA, a hypersaline, alkaline lake located just east of the Sierra Nevada range in northern California. Aerobic CH_4 oxidation varied spatially and temporally coincident with a shifting microaerophilic zone associated with seasonal stratification. Sequence analysis indicated slight shifts in methanotroph community composition and stable absolute cell numbers. Variable CH_4 oxidation rates in the presence of a relatively stable methanotroph population suggested that zones of high CH_4 oxidation resulted from an increase in activity of a sub-set of the existing methanotroph population. Nitrification activity and NH_3 oxidizer community composition were also assessed. Geochemical profiles and rate measurements indicated nitrifying activity. Bacterial NH_3 oxidizer abundance varied seasonally but absolute cell numbers remained consistent over depth for each sample date while Crenarchaeota abundance was strongly correlated between with rate measurements. However, the lack of verifiable archaeal *amo*A gene copies and the presence of sufficient AOB cell numbers to physiologically account for all measured nitrification preclude

specific assignment of nitrifying activity to Mono Lake Crenarchaeota, although a potential AOA contribution to nitrification in Mono Lake cannot be ruled out.

Enrichment experiments identified interactions between methane oxidation and nitrification. The highest CH_4 oxidation rates occurred in the NH_4^++CH_4 enrichment, inferring that methanotrophs benefited from nitrogen (N) addition. FISH and DGGE analysis demonstrated that the methanotrophs in the CH_4-only enrichment were able to take advantage of elevated CH_4 through community composition adaptation. Crenarchaeota abundance correlated with NO_X accumulation and nitrification activity while AOB abundance decreased, suggesting that ammonia-oxidizing archaea (AOA) may have contributed to nitrification in the NH_4^+-only enrichment. In contrast, there was a decrease in crenarchaeal abundance and an increase in AOB abundance in the CH_4+NH_4^+ enrichment that correlated with increased nitrification activity. The reversal in the correlation between AOB and nitrification relative to that observed in the NH_4^+-only enrichment plus the significant decline in crenarchaeal abundance in the CH_4+NH_4^+ enrichment suggests that AOB were likely more active in nitrification in the presence of CH_4 and/or methanotrophs.

INDEX WORDS: Biogeochemistry, Mono Lake, Methane oxidation, Type I methanotrophs, Type II methanotrophs, Nitrification, Ammonia oxidation, Ammonia-oxidizing bacteria, Archaeal oxidizers of ammonia, Molecular, Phylogeny, Abundance, PCR, DGGE, FISH

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DEDICATION

If not for the devotion and commitment of my family, especially my wonderful parents Paul and Judy, I would not be in a position to offer this work, which epitomizes what has been and what is still to come, as a small tribute to their boundless love and support.

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And, of course, here's to my dissertation committee: Mandy Joye, Tim Hollibaugh, Mary Ann Moran, Ming-Yi Sun, and Barny Whitman who have been so patient and supportive, especially Mandy - who'd have ever thought I wouldn't have the words…I would be remiss if I didn't also thank all the people in the Marine Science Department who I have pestered over the years. There are too many of you to mention but special medals should be awarded to Wendy and Jennifer from the Moran lab and an encore hats off to Nasreen, Hopefully your nightmares are beginning to fade.

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CHAPTER 1

INTRODUCTION

Methane- and ammonia-oxidizing bacteria play key roles in the biogeochemical cycles of carbon and nitrogen (Cicerone and Oremland, 1988). Methane-oxidizing bacteria (MOB) convert methane (CH_4) to carbon dioxide (CO_2) providing a significant link between the reduced and oxidative phases of the carbon (C) cycle (equation 1.1). Similarly, ammonia-oxidizing bacteria (AOB) convert ammonia (NH_3) to nitrite (NO_2^-), a central component linking the reduced and oxidized constituents of the nitrogen (N) cycle (equation 1.2).

(1.1) $CH_4 + O_2 \xrightarrow{MMO} CH_3OH \xrightarrow{MDH} HCHO \xrightarrow{FADH} HCOOH \xrightarrow{FDH} CO_2$

Methane — Methanol — Formaldehyde — Formate

(abbreviations above the reaction arrows indicate the catalytic enzymes methane monooxygenase (MMO), methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FADH), and formate dehydrogenase (FDH))

(1.2) $NH_3 + H_2O \xrightarrow{AMO} NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + H_2O + H^+$

Ammonia — Hydroxylamine — Nitrate

(abbreviations above the reaction arrows indicate the catalysts ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO)).

A recent isolate has demonstrated that Crenarchaeota also are capable of ammonia oxidation (Könneke et al., 2005) and a large body of presumptive data (Francis et al., 2005; Wütcher et al., 2006; Lenninger et al., 2006) suggests that ammonia-oxidizing archaea (AOA) likely play a significant role in the global N cycle. The primary mechanism of action of these groups of microorganisms, specifically the oxidation of reduced substrates via molecular oxygen (O_2), compels interaction between these two important geochemical cycles (Fig. 1.1).

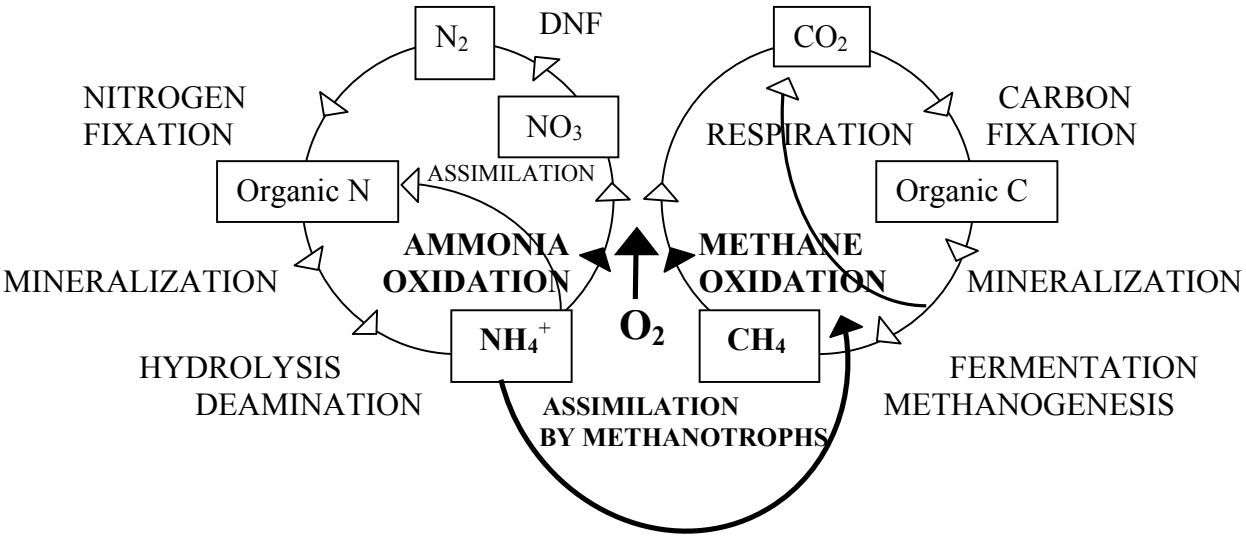

Figure 1.1. Simplified schematic representation of the nitrogen and carbon cycles with emphasis on processes of interest through which the cycles interact (DNF = denitrification).

Methane and ammonia oxidation are also important processes that regulate environmental conditions from ecosystem to local scales. Methane-oxidizing bacteria (MOB) consume up 90% of the methane produced in freshwater and marine soils and sediments (Reeburgh et al., 1993). Methanotrophic activity converts methane to biomass or carbon dioxide, decreasing the flux of methane, a radiative trace gas, to the atmosphere (Topp and Hanson, 1991). Ammonia oxidation to nitrite (NO_2^-) is the first step in the process of nitrification, which may also produce nitrate

(NO_3^-) via the coupled action of nitrite-oxidizing bacteria (Prosser, 1989). Ammonia oxidation provides critical links to other nitrogen cycling processes (e.g. denitrification and anammox) that regulate concentrations and bioavailability of N in specific systems.

The data presented here focus on the occurrence, magnitude, and controls on methane and ammonia oxidation. Biogeochemical methods were combined with molecular techniques to elucidate environmental and microbiological controls on these processes and link specific activity with microbial community composition in Mono Lake, CA. Mono Lake is a closed basin, alkaline salt lake located just east of the Sierra Nevada range in northern California (38° N, 119° W). Saline lakes are numerous, geographically widespread, and comprise a significant part of the world's inland aquatic ecosystems (Fig. 1.2). In fact, although the vast majority of the

Figure 1.2. Shaded areas indicate the global distribution of slat lakes (From Williams, W.D., 2002).

earth's water is contained in the oceans, about half of the accessible liquid water on the planet is contained in salt lakes (Table 1.1). Many salt lakes, such as Mono Lake, are terminal basins (with no outlets) and limnological conditions in Mono Lake undergo periodic changes in lake-wide mixing regimes due to climactic trends and the resulting hydrological balance between inflow and evaporation.

Table 1.1. Partitioning of water in the various inland aquatic compartments (Williams, W.D., 1996). Emphasis on comparable Freshwater and Saline lakes percentages added.

Compartment	Volume (1000 cu.km)	Per cent
Oceans	1,370,000	97.61
Glaciers, ice, snow	29,000	2.08
Subsurface	4,067	0.295
Freshwater lakes, rivers	126	0.009
Saline lakes	104	0.008
Atmosphere	14	0.001

Vertical mixing in Mono Lake is predominantly driven by a seasonal cycle of summer thermal stratification and winter turnover (once per year = monomixis). However, periodic freshening of the epilimnion can create a chemical density stratification that results in meromixis. During periods of meromixis, the development of a steep salinity gradient

(chemocline) isolates a portion of bottom water (monimolimnion) and prevents seasonal holomixis (Romero et al, 1998; MacIntyre and Romero, 2000). Meromictic periods may persist for years and dramatically change limnological and geochemical conditions in the lake. However, meromixis brings an accompanying stability inherent in a stratified system. Stratification creates layers that lead to gradients and fluxes. Gradients exert a powerful influence on microbial activity and community structure.

During this study, Mono Lake was meromictic and the monimolimnion had been isolated below the pycnocline since 1995 (Melack and Jellison, 1998). We selected station 6 (Fig. 1.3) a relatively deep, mid-lake site to examine patterns of methanotroph activity and associated community composition during the development of seasonal stratification in Mono Lake **(CHAPTER 2)**.

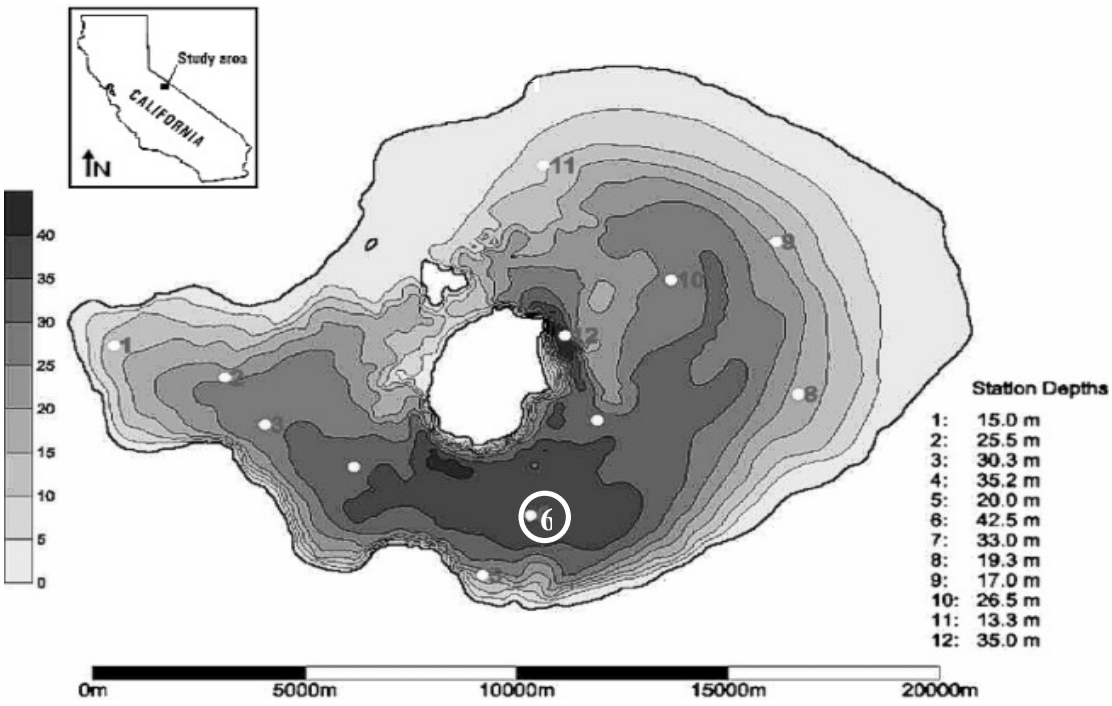

Figure 1.3. Mono Lake bathymetric map depicting the position of station 6.

Aerobic methane-oxidizing bacteria (methanotrophs) are distinguished by their ability to use methane (CH_4) as their sole source of metabolic energy and structural carbon. Biological CH_4 oxidation is the predominant sink mitigating the flux of CH_4, an important radiative trace gas, to the atmosphere (Topp and Hanson, 1991). Methanotrophic bacteria consume CH_4 produced in freshwater and marine environments and may be a significant CH_4 sink in saline and alkaline lakes (Iverson et al., 1987; Joye et al., 1999; Khmelenina et al., 2000). Culture independent molecular genetic techniques have become an important tool for quantifying the dominant organisms and diversity of natural microbial communities (Pace, 1997). Methanotroph populations and community structure in different environments have been examined using PCR (McDonald et al., 1995; Holmes et al., 1995; Henckel et al., 1999), PCR/DGGE (Wise et al., 1999), and PCR/TRFLP (Costello and Lidstrom, 1999; Horz et al., 2001) analyses.

Fluorescence *in situ* hybridization (FISH) permits the direct enumeration of individual cells in a sample and can provide quantitative information complimentary to DGGE and sequence data. Methanotroph family-specific probes have been used to identify and enumerate methanotroph cells in estuarine sediment slurry enrichment cultures (Bourne et al., 2000) and in rice paddy soils (Eller and Frenzel, 2001). The data gathered during this study support three major conclusions. CH_4 oxidation rate and geochemical measurements demonstrated that zones of peak CH_4 oxidation activity migrated through the water column during seasonal stratification and consistently occurred in the microaerophilic zone at the bottom of the oxycline. Methanotroph abundance determined by FISH analysis revealed a stable number of both type I and type II methanotrophs at all sample depths over the course of the study. The combination of rate measurements, FISH analysis, and DGGE community profiles suggest that changes in

activity and/or ratios of sub-sets of the existing methanotroph population, rather than a change in overall methanotroph numbers, drove the observed shifts in zones of elevated CH_4 oxidation.

Interannual patterns of nitrification and the relative contributions of AOB and AOA to nitrification were examined in **CHAPTER 3**. Ecological ramifications of nitrification include the production and efflux of a radiative trace gas (nitrous oxide, N_2O) to the atmosphere, alteration of the concentrations and distributions of biologically available N, and loss of fixed N via coupling to denitrification (Ward, 1986). Nitrification can be coupled with other N processes to remove fixed N from a system, e.g., the nitrate and nitrite produced by nitrification may serve as e^- acceptors for denitrifiers or anaerobic ammonium oxidizers. Denitrifying bacteria transform NO_3^- and NO_2^- to nitrogen gas (N_2); thus, coupled nitrification-denitrification can remove fixed N (Jenkins and Kemp, 1984; Codispoti and Christiansen, 1985). Nitrite produced by nitrification may also be used as an oxidant for anaerobic ammonium oxidation (anammox), which also results in N_2 loss (Mulder et al., 1998). In alkaline ecosystems, however, nitrification may reduce the loss of fixed N from a system. By converting NH_3 to NO_2^- and NO_3^-, N losses due to NH_3 volitalization are curtailed (Joye et al, 1999). These various biogeochemical transformations of N determine local concentrations and distributions of biologically available N and can have a profound impact on ecosystem primary production (Jellison and Melack, 1993).

Geochemical profiles reflected depletion or production of nitrification substrates and products that were indicative of nitrifying activity. Nitrification rate data demonstrated that peak activity occurred between 13 and 14 m throughout the year and depth-integrated rates were highest in November 2002. Phylogenetic analysis revealed a shift from a nitrifying population previously dominated by *N. europaea* and *N. eutropha* strains, to nitrosonomads predominantly affiliated with alkaline and saline environments. Bacterial ammonia oxidizer abundance varied

seasonally but absolute cell numbers remained consistent over depth for each sample date. Crenarchaeota abundance varied over time and depth and maximum cell numbers correlated with peaks in nitrification activity. Although correlations between nitrification and Crenarchaeota and AOB abundance followed a pattern similar to patterns demonstrated in other studies that have suggested significant AOA activity, the lack of verifiable archaeal *amo*A gene copies and the presence of sufficient AOB cell numbers to physiologically account for all measured nitrification in Mono Lake leaves open the question as to whether AOA contribute significantly to nitrification in Mono Lake.

Physiological, biochemical, and ecological similarities between aerobic methane- and ammonia-oxidizing bacteria promote interactions that may significantly affect methane and ammonia oxidation rates. These interactions were investigated in **CHAPTER 4** using aerobic enrichment cultures. The mono-oxygenase systems of methane and ammonia oxidizers are evolutionarily related (Holmes et al., 1995). Methane- and ammonia-oxidation co-occur in sediments near the oxic-anoxic boundary where both oxygen and reduced substrate are available. Both inhibition (King and Schnell 1994) and stimulation (Bodelier et al., 2000) of methane oxidation by ammonia availability have been documented. Similarly, methanotroph activity may enhance (Bodelier and Frenzel 1999) or retard nitrification (Megraw and Knowles 1987; Roy and Knowles 1994). Moreover, methanotrophic demand for N during periods of increased activity can actually suppress NH_3 oxidation activity and influence AOB community composition by sequestering available nitrogen. Galveston Bay, TX sediment enrichments that received supplemental CH_4+NH_4^+ had the highest pMOX rates observed, no detectable nitrification activity, and DIN concentrations similar to the control (e.g. very low NH_4^+ NO_2^- and

NO_3^-). Furthermore, DGGE analysis revealed a different nitrifier population in the CH_4+NH_4^+ enrichment than that observed in the NH_4^+ -only enrichment (Carini et al., 2003).

The Mono Lake enrichment experiments demonstrated that both CH_4 and NH_3 oxidation rates increased over time in enrichments amended with their primary substrate. Community composition data from our CH_4 enrichments suggest a combination of additional methanotroph abundance and increased activity of the initial *in situ* methanotroph population likely contributed to the higher CH_4 oxidation rates observed in those treatments. The rate of NH_3 oxidation increased in the NH_4^+-only enrichment relative to the control, demonstrating that nitrification was N limited. Significant changes in both crenarchaeal and AOB abundance in the NH_4^+-only enrichment provided stronger correlative data supporting potential AOA activity in Mono Lake relative to *in situ* observations. Crenarchaeota abundance increased significantly as the nitrification rate increased, while AOB abundance decreased. Clearly, members of the Crenarchaeota proliferated over the AOB population under N-replete conditions and were strongly correlated with nitrifying activity. Due to the significant decrease in AOB abundance, AOB cell specific nitrification rates in the NH_4^+-only enrichment were generally higher than cell specific rates reported for pure cultures (*Nitrosospira*) and amended soils were generally less than 10 fmol $cell^{-1}$ hr^{-1} indicating that the AOB population may not have had the physiological capacity to account for all the measured nitrification in the NH_4^+-only enrichment. Taken together, these findings, while still correlative in nature, are more suggestive of an AOA contribution to nitrification in the NH_4^+-only enrichment.

Methanotrophic activity and community composition reacted differently dependent on the presence or absence of additional NH_4^+. The methanotroph population of the CH_4 + NH_4^+ enrichment had a higher potential for CH_4 oxidation due to readily available NH_3. However, the

methanotroph population in the CH_4-only enrichment was also able to take advantage of higher CH_4 availability even without supplemental N through community composition adaptation. Nitrification activity was not significantly affected by supplemental CH_4; however, nitrifier community composition reacted differently dependent on the presence or absence of additional CH_4. Contrary to the response in the NH_4^+-only enrichment, Crenarchaeota abundance decreased and AOB increased in the CH_4 + NH_4^+ enrichment suggesting that AOB were likely more active in nitrification in the CH_4+NH_4^+ enrichment.

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CHAPTER 2

PATTERNS OF AEROBIC METHANE OXIDATION AND METHANOTROPH COMMUNITY COMPOSITION DURING SEASONAL STRATIFICATION IN MONO LAKE[1]

[1] Carini, S.A., N. Bano, G. LeCleir, and S.B. Joye. 2005 *Environmental Microbiology* 7 (8): 1127-1138.

ABSTRACT

Patterns of aerobic methane (CH_4) oxidation and associated methanotroph community composition were investigated during the development of seasonal stratification in Mono Lake, California (USA). CH_4 oxidation rates were measured using a tritiated CH_4 radiotracer technique. Fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and sequence analysis were used to characterize methanotroph community composition. A temporally shifting zone of elevated CH_4 oxidation (59-123 nM d^{-1}) was consistently associated with a sub-oxycline, microaerophilic zone that migrated upwards in the water column as stratification progressed. FISH analysis revealed stable temporal and spatial numbers of type I (4.1-9.3 x 10^5 cells ml^{-1}) and type II (1.4-3.4 x 10^5 cells ml^{-1}) methanotrophs. DGGE and Sequence analysis indicated slight shifts in methanotroph community composition despite stable absolute cell numbers. Variable CH_4 oxidation rates in the presence of a relatively stable methanotroph population suggested that zones of high CH_4 oxidation resulted from an increase in activity of a sub-set of the existing methanotroph population. These results challenge existing paradigms suggesting that zones of elevated CH_4 oxidation rates result from the accumulation of methanotrophic biomass and illustrate that type II methanotrophs may be an important component of the methanotroph population in saline and/or alkaline pelagic environments.

INTRODUCTION

Aerobic methane-oxidizing bacteria (methanotrophs) are distinguished by their ability to use methane (CH_4) as their sole source of metabolic energy and structural carbon. Methanotrophs inhabit a variety of terrestrial and aquatic habitats and play an important role in

global carbon, oxygen, and nitrogen cycling (Cicerone and Oremland, 1988). Biological CH_4 oxidation is the predominant sink mitigating the flux of CH_4, an important radiative trace gas, to the atmosphere (Topp and Hanson, 1991). Methanotrophic bacteria consume up to 80% of the CH_4 produced in freshwater and marine environments (Reeburgh *et al.,* 1993) and may be a significant CH_4 sink in saline and alkaline lakes (Iverson *et al*., 1987; Joye *et al.,* 1999; Khmelenina *et al.,* 2000).

Methanotrophs are grouped into two families based primarily on internal membrane arrangement and on the carbon assimilation pathway employed. Type I methanotrophs have disc-shaped membrane bundles distributed throughout the cytoplasm, assimilate carbon as formaldehyde via the RuMP pathway, and comprise a distinct cluster within the gamma subclass of the *Proteobacteria* (γ-*Proteobacteria*). Type II methanotrophs possess paired internal membrane structures aligned with the periphery of the cell, assimilate formaldehyde via the serine pathway, and form a distinct cluster in the α-*Proteobacteria* (Hanson and Hanson, 1996). Type I methanotrophs have been described as the dominant or exclusive family of methanotrophs observed in estuarine, marine, and hypersaline environments (Bourne *et al.,* 2000; Holmes *et al.,* 1996; Khmelenina *et al.,* 2000). Although type II methanotrophs are reported to be a significant component of the methanotroph populations in freshwater sediments (Costello and Lidstrom, 1999), peat bogs (Deydysh *et al.,* 2000), and rice paddy soils (Henckel *et al.,* 1999; Eller and Frenzel, 2001), these organisms have not been observed previously in saline and/or alkaline environments such as Mono Lake.

Saline lakes are numerous, geographically widespread, and comprise a significant part of the world's inland aquatic ecosystems (Williams, 2002). Mono Lake is an alkaline salt lake located just east of the Sierra Nevada range in northern California (38°N, 119°W). During this

study, the lake was meromictic and the monimolimnion (bottom water) had been isolated below the pycnocline since 1995. This isolation resulted in persistent anoxia and accumulation of high concentrations of dissolved CH_4 (50-100 μM). During winter, the lake was isothermal to the pycnocline. Increased solar heating in the spring and summer generated thermal stratification. As seasonal thermal stratification progressed, the oxycline migrated upward in the water column. Thus, over time, discrete depths were exposed to different concentrations of dissolved oxygen and fluxes of methane and nutrients. Seasonal stratification provided an ideal *in situ* environment for examining the effects of changing geochemical conditions on CH_4 oxidation rates and methanotroph community and population dynamics using biogeochemical and molecular techniques.

Culture independent molecular genetic techniques have become an important tool for quantifying the dominant organisms and diversity of natural microbial communities (Pace, 1997). Methanotroph populations and community structure in different environments have been examined using PCR (McDonald *et al.,* 1995; Holmes *et al.,* 1995; Henckel *et al.,* 1999), PCR/DGGE (Wise *et al.,* 1999), and PCR/TRFLP (Costello and Lidstrom, 1999; Horz *et al.,* 2001) analyses. Fluorescence *in situ* hybridization (FISH) permits the direct enumeration of individual cells in a sample and can provide quantitative information complimentary to DGGE and sequence data. Methanotroph family specific probes have been used to identify and enumerate methanotroph cells in estuarine sediment slurry enrichment cultures (Bourne *et al.,* 2000) and in rice paddy soils (Eller and Frenzel, 2001). Here, we report results of methanotroph specific FISH, DGGE, and sequence analysis in a pelagic system. Combining molecular approaches with rate assays may elucidate the relationship between methane oxidation activity

and methanotroph abundance and community structure (Auman *et al.,* 2000; Eller and Frenzel, 2001; Carini *et al.,* 2003).

METHODS

Sample Collection

Water samples were collected from 10-20 discrete depths near a permanently moored buoy in the central basin of Mono Lake (station 6; 37° 57.822' N, 119° 01.305' W). Water samples were obtained using a 5 L Niskin bottle and were stored appropriately (see below) for methane oxidation rate, diversity, abundance, dissolved gas, and chemical analyses.

Limnology and Geochemistry

Temperature (°C) and density profiles over depth were measured using a Seabird SeaCat profiler. Dissolved oxygen (O_2) concentration was determined using a YSI O_2 sensor. Ammonium (NH_4^+) concentrations were determined using a phenol, hypochlorite method (Solaranzo, 1969). Ammonium standards were prepared using filtered, helium (He) purged (to remove NH_3) Mono Lake water to account for matrix effects. *In situ* dissolved CH_4 concentrations were determined using a headspace extraction – gas chromatography technique (Oremland and Des Marais, 1983). Briefly, sub-samples (10 ml) from each Niskin bottle were transferred, via gas tight syringe, to a 20 ml He purged headspace vial containing a NaOH pellet. The base pellet served to arrest biological activity. To account for solubility effects, standards were prepared using He purged filtered Mono Lake water treated identically as the samples. Appropriate volumes of a certified standard (10% CH_4 in balance of ultra-pure He: Scott Specialty gas mix 875) were added to the standard bottles to achieve concentrations ranging from

1 to 80 µM. The CH_4 concentration in samples and standards was quantified using a Shimadzu 14-A gas chromatograph equipped with a Porpak® T column and a flame ionization detector.

Methane Oxidation Rates

Methane oxidation rates were measured using a tritiated (^3H) CH_4 radiotracer technique (Sandbeck and Reeburgh, 1989). Briefly, 10 ml gas tight syringes (n=3 live samples plus 1 filter-sterilized control per depth) were filled to overflowing at each depth with water from the Niskin bottle. The syringes were sealed by inserting a plunger into the barrel and closing the hub end with modified luer lock tips fitted with gas tight butyl rubber septa (Joye *et al.,* 1999). A C^3H_4 stock solution was prepared by equilibrating 38 ml of filtered, degassed, Mono Lake water with 1 ml C^3H_4 in a gas tight serum bottle. A 100 to 200 µl aliquot of C^3H_4 tracer solution was injected into each syringe through the butyl septa in the luer tip yielding a tracer activity of ~30,000 dpm/ml. Sample syringes were incubated at *in situ* temperature for 48 hours. Rates were linear for over 48 hours (checked during time courses; data not shown). Incubations were terminated by dispensing the contents of each syringe into a glass scintillation vial containing a NaOH pellet, which served to halt biological activity. Labeled C^3H_4 was expelled by purging the sample with water saturated CH_4 (5 min.), vortexing, and venting the vials on a shaker table (50 rpm for 30 min.). Scintillation cocktail (Scintiverse BD, Fisher Scientific) was added and samples were counted for ^3H_2O activity using a Beckman 6500 liquid scintillation counter. Methane oxidation rate constants (k) were calculated ($k = [\alpha(A_{3H2O} / A_{C3H4} + A_{3H2O})] / t$ where α = isotopic fractionation factor, A = activity, and t = time) and multiplied by *in situ* CH_4 concentrations to determine methane oxidation rates.

Fluorescence In Situ Hybridization (FISH)

Oligonucleotide probes Mγ84, Mγ705, and Mα450 were used to detect Type I and Type II methanotrophs, respectively (Eller *et al.,* 2001). Probes were synthesized by MWG Biotech (Ebersberg, Germany) and labeled with fluorochromes Oregon green (Mα450) and Texas Red (Mγ84 and Mγ705). Sub-samples (10 ml) from each Niskin cast were fixed at room temperature with 4% formaldehyde (in PBS) for 35 min then stored at -20°C until the hybridization reaction (Pernthaler *et al.,* 2001). Samples were thawed and mixed well, then 500 µl of sample was added to a sterile centrifuge tube containing 15 ml PBS. The resulting mixture was filtered through a 0.2 µm polycarbonate membrane filter (Osmonics Inc). Typical Mono Lake methanotrophs range in size from 0.5 – 3 um suggesting the retention of all methanotrophs in the sample. The tube was then rinsed with PBS (2 x 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 15-20 ml of sterile water and allowed to air dry in a petri dish. Sections of individual filters were separated for DAPI-only staining and dual hybridization with methanotroph probes and DAPI counter-staining. Hybridization reactions were performed based on the technique of Pernthaler et al. (2001) modified for optimum formamide concentrations (Eller and Frenzel, 2001). Filter sections were rinsed in DI, air dried, and counterstained with DAPI (50 µl of 2 mg ml^{-1} per filter) for 3 min. Total microbial and hybridized methanotroph cells were counted using epifluorescence microscopy by enumerating the cells in each of 40 squares in 50 randomly selected fields for each sample (n=2000 grid squares per sample filter section). Total cell counts from the DAPI-only stained filter sections and DAPI counts from counterstained, hybridized filters to verify that no loss of cells from the filters occurred during hybridization. Images were obtained using confocal laser scanning microscopy with a Leica TCS SP2 microscope, a 63x oil immersion lens,

and LCS software® capable of producing overlay images that illustrated simultaneous detection of both type I and type II methanotrophs.

Statistical Methods

Spatial and temporal differences in CH_4 oxidation rates were compared using two-sample t-Tests assuming unequal variances (n = 3). Differences between types of methanotroph and spatial and temporal methanotroph ell numbers derived from FISH data were compared using a combination of ANOVA and Tukey's analysis.

Cell Specific Methane Oxidation

Cell specific rates of CH_4 oxidation were calculated by dividing the moles of CH_4 oxidized per volume by the total number of methanotroph cells per volume as determined by FISH at each depth.

DNA Collection and Extraction

Water samples for DNA extraction (1 to 4 liters) were collected from each Niskin cast and stored in clean sample-rinsed polycarbonate bottles or LDPE cubitainers in a cooler (at 4°C) until processing (within 4 hrs). Microorganisms were collected on a Sterivex filter cartridge (0.22 μm; Millipore) and total community DNA was extracted from the filters (Ferrari and Hollibaugh 1999). Briefly, after addition of 40 μl of lysozyme (50 mg ml^{-1}), cartridges were incubated for 60 min at 37°C. Then, 50 μl of proteinase K (20 mg ml^{-1}) and 100 μl of SDS (20% wt/vol) were added and the cartridges were incubated at 55°C for 2 h. The lysate was transferred to 15 ml tubes and combined with 1 ml of lysis buffer that was used to rinse the

cartridge. DNA for community analysis was purified from 800 µl of lysate by sequential extraction with 800 µl of phenol-chloroform-isoamyl alcohol (25:24:1), chloroform-isoamyl alcohol (24:1) and finally *n*-butanol. The aqueous phase was removed, placed in a Centricon-100 concentrator (Amicon), mixed with 500 µl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and centrifuged at 1,000 x g for 10 min. Then, 500 µl of TE was added to the Centricon and it was centrifuged for another 10 min. Blanks were prepared from Sterivex cartridges through which no water had been filtered.

PCR Amplification

Previously characterized oligonucleotide primers were used to examine patterns of methanotroph community composition. Samples were screened to evaluate the presence or absence of methane oxidizing bacteria using primer pairs MethT1dF / MethT1bR (Wise *et al.,* 1999) and F27 / type2b (Costello and Lidstrom, 1999) which target regions of 16S rDNA specific for type I and type II methane-oxidizers, respectively. Methanotroph amplicons were isolated by agrose electrophoresis. Bands with the correct electrophoretic mobility (compared to bands generated using a 1 kb DNA ladder; Promega) were excised, purified (OIAquick Gel Extraction Kit, Quiagen), and used as template for subsequent PCRs to produce amplicons suitable for DGGE analysis. The primer pair 341f / 534r (Muyzer *et al.,* 1993) was modified with a GC clamp, labeled with fluoroscein, and used to amplify the variable 3 region of the methanotroph template DNA (Murray *et al.,* 1996). For all PCR reactions a "master mix" was prepared containing sterile water, 1x buffer (Promega), 2.5 mM MgCl, 2 µM forward and reverse primers, and 200 µM dNTPs. One hundred µl of the master mix was then dispensed into individual 0.2 ml reaction tubes. Template DNA (4-10 ng) was added and after 5 min at 95° C in

the thermocycler (MJ Research DNA Engine®), 2.5 units of Taq DNA polymerase (Promega) was added while the reaction tubes were held at 80° C. A touchdown thermal cycling program (Don *et al.,* 1991), optimized for initial and final temperature, was used for each of the primer pairs and each PCR was run for 30 cycles. All reactions were run with type I (*Methylococcus capsulatus* Bath, *Methylobacter leuteus* ACM 3304, *Methylomicrobium album* BG8, and *Methylomonas methanica* S1) or type II (*Methylosinus trichosporium* OP3b and *Methylocystis parvus* OBBP) culture extract as positive controls (obtained from The Savannah River Ecology Lab culture collection via Dr. Mark Wise). Negative controls consisted of opposing type primer sets and culture (i.e., type I primer pairs with type II culture template and vise versa). The PCR product was verified for amplification and fragment size by agrose gel electrophoresis.

DGGE Analysis

DGGE analysis was performed using a CBS Scientific (Del Mar, CA) system (Ferrari and Hollibaugh, 1999). Denaturing gels were 6.5% polyacrylamide with a denaturant gradient ranging from 40 to 70%. The denaturants in a 100% solution were 40% deionized formamide and 7M urea. PCR products were concentrated by ethanol precipitation and resuspended. Samples (250 ng DNA) were mixed 1:1 with neutral loading dye and loaded onto the gel. Gels were run at 80 V for 15 hrs in a 60° C, 1x TAE buffer and visualized on a Hitachi FMBIO III scanner equipped for fluorescein detection. Multiple DGGE gels were run from independent PCR reactions to confirm accurate band numbers and relative electrophoretic mobility for community fingerprint analysis and for clones chosen for sequencing. True replicate analysis from separate filters was not conducted. However, the samples collected from 3 discrete depths in February may be viewed as replicates by proxy due to the homogeneity of the mixolimnion

during this sampling. DGGE patterns for both families of methanotrophs were identical for all depths suggesting no sampling bias.

Cloning and Phylogenetic Analysis

Samples from Feb 2002 (13 m) and June 2002 (13 m) contained all the major bands observed in both Type I and Type II methanotroph DGGE analyses from bulk water samples and were chosen to generate clone libraries. PCR products from both MethT1dF / MethT1bR and F27 / type2b primer pairs were isolated by agarose gel electrophoresis, extracted from the agrose (QIAquick® Gel Extraction Kit; Qiagen), purified using Wizard® PCR preps (Promega), ligated into pCR®4-TOPO® plasmid vector (Invitrogen), and transformed into chemically competent One Shot® TOP10 *E. coli* cells (Invitrogen). Transformed cells were plated on Luria-Bertani (LB) plates (n=2 for each methanotroph type) containing ampicilin (100 $\mu g\ ml^{-1}$) and incubated overnight at 37°C. Forty colonies were chosen at random from each of the 4 plates, and cultured (overnight at 37°C) in 3 ml of LB medium containing ampicilin (100 $mg\ ml^{-1}$).

PCR / DGGE analysis was used to identify unique clones for sequencing. Bands from specific clones were cut out, re-amplified with the PCR, and re-run using DGGE. Cut out bands that did not have the same electrophoretic mobility as the original bands (based on multiple lanes of standard distributed across the gel) or re-PCRs that produced more than 1 DGGE band were discarded.

Six type I and 11 type II methanotroph clones had DGGE mobility that corresponded to bands of interest observed on DGGE gels from bulk water samples and these clones were chosen for sequencing. Plasmid DNA containing PCR products from both MethT1dF / MethT1bR and F27 / MethT2R primer pairs from selected clones was extracted and purified using a QIAprep®

spin Miniprep Kit (Quiagen). DNA sequencing was carried out by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia. Plasmids were sequenced using the primer M13 forward, which resulted in the retrieval of the entire 883 bp (Type I) or 902 bp (Type II) fragment sequence. Sequences were then compared to known 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 (GCG) package (Madison Wisc.) Phylogenetic trees were constructed using the neighbor-joining method and Jukes-Cantor distances (PHYLIP package v. 3.5).

RESULTS

Three depths, 13, 15, and 23 m, were selected for detailed analysis based on geochemical and CH_4 oxidation activity data. These depths represent discrete zones of aerobic methanotrophy are associated with changing geochemical regimes due to seasonal stratification. Three time points were selected: February, April, and June 2002. These time points marked the temporal transition from well mixed to stratified conditions.

Limnology and Geochemistry

In February the Mono Lake water column was divided into two distinct layers. Anoxic bottom water was separated from the oxygenated upper water column by a sharp pycnocline at 26 m. The upper layer was well mixed and dissolved O_2 concentrations > 5 mg l^{-1} persisted to the pycnocline (Fig. 2.1). CH_4 and NH_4^+ concentrations were generally less than 1 μM in the mixed layer but increased sharply at the pycnocline.

The development of thermal stratification was evident in April (Fig. 2.1). The oxycline separated and migrated upwards from the pycnocline creating a microaerophilic zone between 15-16 m. Dissolved O_2 decreased to zero between 16 and 17 m creating a seasonal anoxic zone that extended to the pycnocline (Fig. 2.1). The CH_4 concentrations were twice as high in the upper water column and oxycline regions compared to February. In the seasonally anoxic zone, CH_4 concentrations increased 4 to 6 fold and concentrations remained high (~60 μM) below the pycnocline. NH_4^+ concentrations remained low in the upper water column but increased in the seasonally anoxic zone (Fig. 2.1).

Stratification progressed into June with the oxycline becoming sharper and more defined at 12 m. The microaerophilic zone broadened, shifted up slightly in the water column, and encompassed an area between 12 and 15 m. June CH_4 and NH_4^+ concentrations in the upper water column were similar to concentrations measured in April. Concentrations in the seasonally anoxic and microaerophilic zones continued to increase suggesting greater flux of these reduced species across the gradient created at the oxycline (Fig. 2.1).

Methane Oxidation Activity

CH_4 oxidation activity was observed throughout the Mono Lake water column during the entire course of the study (anaerobic CH_4 oxidation rates are presented elsewhere; Joye et al. in preparation). However, methanotroph activity at discrete depths varied significantly over time (paired t-Tests; with the exception of 13 and 15m in February all $p < 0.01$). In February, low rates (1-2 nmol l^{-1} day^{-1}; hereafter denoted as nM day^{-1}) of CH_4 oxidation were uniformly distributed throughout the mixolimnion with the exception of a zone of higher activity (3-5 nM day^{-1}) between 22 and 24 m (Fig. 2.2). By April CH_4 oxidation rates increased at 13 m and 15 m

(from 1 to 5 nM day^{-1} and 2 to 123 nM day^{-1}, respectively). Aerobic CH_4 oxidation ceased below 20 m due to a lack of molecular O_2 (Fig. 2.2). As stratification progressed into June, the zone of peak activity migrated upwards in the water column coincident with the microaerophilic zone at the base of the oxycline. The highest rate of CH_4 oxidation remained at 15 m (87 nM day^{-1}) but also increased at 13 m by a factor of 6 compared to April rates (Fig. 2.2).

Methanotroph Abundance and Distribution

Probes Mγ84, Mγ705, and Mα450 are specific and detect all known type I and type II methanotrophs (Eller *et al.,* 2001). Due to the unique chemistry of Mono Lake water, a variety of tests were conducted using various methanotroph cultures added to bulk water samples to confirm the performance and specificity of the probes and to optimize dual hybridization conditions. The combination of these probes demonstrated simultaneous identification of both type I and type II methanotrophs (data not shown). The fluorescence intensity of bound probes allowed methanotroph cells to be easily distinguished from background- and auto-fluorescence in Mono Lake samples. Hybridization with non-sense probe NON338 indicated no non-specific probe binding under optimized hybridization conditions (data not shown). Both type I and type II specific probes hybridized with cells collected from Mono Lake bulk water samples.

FISH analysis revealed relatively stable numbers of both populations of methanotrophs at all sample depths. Type I methanotrophs were more abundant (ANOVA, $p < 0.05$) in almost all (>95%) samples examined (Fig. 2.3). Type I cell counts ranged from 5-9 x 10^5 cells ml^{-1} and they accounted for 3 to 5% of the total microbial community. Type II methanotrophs were less abundant throughout the water column with cell numbers ranging between 1.5-3.5 x 10^5 cells

ml^{-1}, accounting for about 2 % of the microbial community (Fig. 2.3). Absolute methanotroph cell numbers did not vary significantly between depths over time (ANOVA, $p > 0.05$; Table 1).

Cell Specific Methane Oxidation

Rates of CH_4 oxidation calculated on a per cell basis were extremely low compared to rates calculated in culture and various environments. In February cell specific rates were 9-25 x 10^{-5} fmol CH_4 $cell^{-1}$ hr^{-1}. In zones of high activity in April (15 m) and June (13 and 15 m) cell specific oxidation ranged from 0.5-6.0 x 10^{-3} fmol CH_4 $cell^{-1}$ hr^{-1} (Table 2.1).

Methanotroph Community Analysis and Phylogeny

PCR amplification of 16S rDNA with type I and type II methanotroph specific primers consistently yielded products in all samples (data not shown) confirming that both families of methanotrophs were present at all sample depths. Subsequent DGGE analyses revealed diverse communities of both type I and type II methanotrophs.

In February, bulk water samples produced identical banding patterns at all depths consistent with a well-mixed water column (Figs. 2.4 and 2.5). The major bands obtained from amplifications using the type I primer set (TI-317 and TI-34) were related to sequences from *Methylococcus* and *Methylomicrobium* spp. respectively (Fig. 2.4; accession nos. X72771 and D89279). Minor bands were closely related to major bands displaying similar electrophoretic mobility with the exception of band T1-316 that was most closely related to an uncultured γ-*Proteobacteria* and *Dechloromarinus chlorophilius* (Fig. 2.6). DGGE banding patterns generated from April and June samples suggested changes in type I methanotroph community structure over time and between depths. The banding pattern in samples from 23 m remained

relatively constant over time while a general trend of decreasing diversity was apparent at 13 and 15 m (Fig. 2.4). Band T1-316 became more prominent at 15 m in April and at 13 m in June while bands T1-38 and T1-712 were less evident in April and absent in June.

Banding patterns generated using the type II primer set also demonstrated changes in community structure over time. In February, identical banding patterns occurred at all depths in the mixolimnion (Fig. 2.5). Major bands T2-38 and T2-39 were most closely related to a *Methylobacterium* spp. (accession no. D32236) while T2-32 and T2-36 were most closely related to *Methylosinus* spp. (accession nos. AJ458474 and Y18947 respectively). Minor bands were most closely related to *Methylosinus* spp. with the exception bands T2-29 and T2-25 that were related to sequences from *Methylobacterium* and *Rhodobaca* spp. respectively (Fig. 6; accession nos. D32236 and AF384205). The number of bands decreased at all depths in April and at 15 and 23 m in June. These changes in banding patterns consisted mainly of the disappearance of band T2-25 and T2-31. However, diversity appeared to increase at 13 m in June with the appearance of bands T2-38, T2-29, T2-34, and the reappearance of band T2-31 (Fig. 2.5). Our sequence data have been submitted to the GenBank database under accession numbers AY682725-AY682741.

DISCUSSION

The data gathered during this study support three major conclusions:

1. CH_4 oxidation rate and geochemical measurements demonstrated that zones of peak CH_4 oxidation activity migrated through the water column during seasonal stratification and consistently occurred in the microaerophilic zone at the bottom of the oxycline.

2. Methanotroph abundance determined by FISH analysis revealed a stable number of both type I and type II methanotrophs at all sample depths over the course of the study.
3. The combination of rate measurements, FISH analysis, and DGGE community profiles suggest that changes in activity and/or ratios of sub-sets of the existing methanotroph population, rather than a change in overall methanotroph numbers, drove the observed shifts in zones of elevated CH_4 oxidation.

Patterns of CH_4 Oxidation Activity

Aerobic CH_4 oxidation activity in Mono Lake exhibited spatial and temporal variation consistent with previous results from Mono Lake (Joye *et al.,* 1999) and other alkaline and/or hypersaline lakes (Iverson *et al.,* 1987; Khmelenina *et al.,* 2000). Rates of aerobic CH_4 oxidation ranged from 2-123 nM day $^{-1}$ (Fig. 2.2). These rates were greater than rates observed in marine environments such as the Black Sea (Reeburgh *et al.,* 1991), the Southern California Bight (Ward and Kilpatrick, 1993), and the Eel River Basin (Valentine *et al.,* 2001) which are generally < 1nM day^{-1}. Maximum aerobic CH_4 oxidation rates in seasonally stratified freshwater lakes have been observed to be 10-100 fold higher (Rudd *et al.,* 1974; Harrits and Hanson, 1980). Patterns of CH_4 oxidation in Mono Lake demonstrated zones of high methanotrophic activity in sub-oxycline microaerophilic zones, similar to findings in stratified lake water columns (Rudd *et al.,* 1974; Rudd and Hamilton, 1978; Harrits and Hanson, 1980; Joye *et al.,* 1999). The microaerophilic zones had O_2 concentrations $\leq$ 2 mg l^{-1} and were coincident with a distinct CH_4 gradient (Rudd *et al.,* 1974; Rudd and Hamilton, 1978; Harrits and Hanson, 1980). The migration of the microaerophilic zone through the Mono Lake water column during seasonal stratification exposed discrete depths to comparable O_2 concentrations ($\leq$ 2 mg l^{-1}) and gradients

of CH_4. Elevated rates of CH_4 oxidation in Mono Lake were associated with these geochemical conditions (Fig. 2.2).

Zones of increased CH_4 oxidation in the water column of lakes during summerlake may result from accumulation of methanotrophic bacterial biomass at a specific depth (Rudd *et al.,* 1974; Harrits and Hanson, 1980). Increased methanotroph abundance has correlated with higher CH_4 oxidation rates in soils and freshwater sediments (Bender and Conrad, 1994; Henckel et al., 2000). However, the molecular data presented here suggests that absolute methanotroph abundance in Mono Lake is stable over time. Therefore, the changes in activity that were observed are hypothesized to have resulted from increased activity of a sub-set of the existing methanotroph population and that higher CH_4 oxidation rates were induced by changes in the local geochemical regime. Unfortunately, we cannot determine which fraction of the population increased their activity but it is clear from FISH and DGGE data that the bulk response stemmed from a sub-set of the population rather than an overall increase in methanotroph biomass.

Stability of Methanotroph Cell Numbers and Cell Specific CH_4 Oxidation

The FISH data demonstrated that methanotroph numbers in Mono Lake did not change significantly over time (Table 2.1). The spatial and temporal stability of methanotroph cell numbers observed throughout the mixolimnion would require that Mono Lake methanotrophs tolerate variable and dynamic geochemical conditions. Methanotroph populations at discrete depths were exposed to a wide range of CH_4 (< 1-10 μM) and O_2 (0-8 mg l^{-1}) concentrations as well as temperature (2-20°C). In February, the mixolimnion exhibited the lowest CH_4 oxidation rates (Fig. 2.2). Low CH_4 activity resulted from high dissolved O_2 concentrations, low temperatures, and low CH_4 concentrations (Hanson and Hanson, 1996; Fig. 2.1). Low CH_4

concentrations (< 5 μM) resulting in negligible CH_4 oxidation rates may decrease methanotroph numbers via energy limitation (Harrits and Hanson, 1980).

However, stable numbers of methanotroph cells were observed throughout the Mono Lake water column regardless of local geochemical conditions or CH_4 oxidation activity suggesting that methanotroph populations were maintained even under the adverse conditions typical of winter in Mono Lake (Fig. 2.3). Methanotrophs adapted to low *in situ* CH_4 concentrations are found in many natural environments (Bender and Conrad, 1994), suggesting that they may persist even when CH_4 is limiting. Such persistence assures that the methanotrophic population is subsequently able to take advantage of favorable geochemical regimes when such conditions occur locally during stratification.

A stable population of methanotrophs throughout the Mono Lake water column also requires aerobic methanotrophs to survive periods of seasonal anoxia at depth in the mixolimnion. Methanotrophs can tolerate periods of anoxia. Anoxic peat and anoxic sediment subsequently incubated under oxic conditions displayed immediate and substantial potential for aerobic CH_4 oxidation (King *et al.,* 1990). The lack of O_2 and associated oxygen radicals and peroxides may make exposure to anaerobic conditions less stressful to aerobic methanotrophs than starvation (King, 1996). Constant methanotroph cell numbers at 23 m during anoxic conditions in April and June demonstrated that aerobic methanotrophs may persist in seasonally anoxic zones in Mono Lake. Constant numbers of methanotroph cells persisted in anoxic rice paddy soils where no aerobic methanotrophic growth or activity was possible leading the authors to hypothesize that methanotrophs persisted for extended periods as vegetative cells in a state of anaerobic dormancy (Eller and Frenzel, 2001).

Methanotroph cell numbers also remained constant in the upper water column even in zones of increased CH_4 oxidation rates demonstrating that cell numbers were not driven by increased activity (or vise versa). Based on our observations and those cited above, we hypothesize that stable numbers of methanotrophs are maintained throughout the mixolimnion of Mono Lake via physiological adaptation to chronically low CH_4 concentrations in the upper water column and the ability to survive periods of anoxia in deeper, seasonally anoxic waters.

Cell specific CH_4 oxidation rates calculated for the Mono Lake methanotroph population provide a compelling reason for methanotrophs to adopt a strategy of maintaining a stable population that persists through time rather than growing in under favorable conditions. In culture, reports of methanotroph cell specific CH_4 oxidation averaged ~0.2 fmol cell^{-1} hr^{-1} (Hanson and Hanson, 1996). In soils and freshwater sediments, cell specific rates calculated for viable cells agreed well with rates obtained in cultures ranging from 0.2 to 0.3 fmol cell^{-1} hr^{-1} (Bender and Conrad, 1994). Cell specific rates in Mono Lake were extremely low (2-3 orders of magnitude lower than rates reported for cultures; Table 1). Given relatively slow chemoautotrophic growth rates and the observed cell specific CH_4 oxidation rates cited above, methanotroph populations in Mono Lake would be unable to "grow in" in time to take advantage of favorable geochemical conditions that appear on a time scale of days - weeks during seasonal stratification.

Methanotroph Distribution and Community Analysis

Surprisingly, both type I and type II families contributed to the methanotroph abundance observed in Mono Lake (Fig. 2.3). Type I methanotrophs have been described as the exclusive or dominant methanotrophs observed in marine, estuarine, and hypersaline environments

(Holmes *et al.,* 1996; Bourne *et al.,* 2000; Khmelenina *et al.,* 2000). Although type II methanotrophs are reported to be a significant component of the methanotroph populations in freshwater sediments (Costello and Lidstrom, 1999), peat bogs (Deydysh *et al.,* 2000), and rice paddy soils (Henckel *et al.,* 1999; Eller and Frenzel, 2001), these organisms have not been observed in saline environments. The FISH data presented here demonstrates that type II methanotrophs comprise a substantial fraction of the total methanotroph population in the Mono Lake water column, illustrating that ecotypes of type II methanotrophs may be adapted to saline habitats. Presumptive evidence that type II methanotrophs were present in a marine enrichment cultures has been reported recently (Rockne and Strand, 2003). Those results, along with our observations, suggest that type II methanotrophs may be more common in saline habitats than previously thought.

Community profiles from DGGE analysis indicated a dynamic population of type I and type II methanotrophs. A general trend of decreasing diversity in type I methanotrophs occurred at certain sample depths during the course of stratification. A *Methyloccocus*-like sp. became a more dominant component of the population in zones of high CH_4 oxidation activity (Figs. 2.2 and 2.4). This suggests that *Methylococcus*-like species may have out competed other species under geochemical conditions associated with increased methanotrophic activity such as low O_2 tension and increased CH_4 flux. Type II methanotrophs also demonstrated changes in diversity. However, no general trend was apparent and changes in diversity were not associated with changes in activity (Figs. 2.2 and 2.5). Similar results were obtained in rice paddy soils where a change in type I methanotroph community structure was associated with changes in CH_4 oxidation while type II community composition did not correlate directly with *in situ* activity (Eller and Frenzel, 2001).

The species-level shifts in type I methanotroph community structure in response to the changing environmental conditions observed were associated with increased CH_4 oxidation rates. *Methylococcus*-like species appeared to be better adapted to take advantage of favorable geochemical conditions. Whether the increased dominance of the Type-I methanotroph community by *Methylococcus*-like species resulted from their ability to out-compete other methanotrophs for substrates (e.g., O_2 or CH_4) or because they are more efficient energetically and hence, grow faster, is presently unknown. Laboratory experiments are underway to evaluate how geochemical factors influence the community composition of aerobic methanotrophs in Mono Lake.

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Table 2.1. Percentage of total DAPI stained cells hybridized with methanotroph probes and total methanotroph cell numbers detected using FISH. CH_4 oxidation rates and total methanotroph cell numbers were used to calculate cell specific CH_4 oxidation rates.

sample	% DAPI stained cells hybridized with methanotroph probes	Total methanotroph cells L^{-1} (10^8)	nM CH_4 oxidized day^{-1}	fmol CH_4 oxidized $cell^{-1}$ hr^{-1}
Feb. 13 m	3.6	8.8	1.7	0.00008
Feb. 15 m	3.6	8.8	1.7	0.00008
Feb. 23 m	4.3	11	6.7	0.00025
April 13 m	6.5	9.2	10.6	0.00048
April 15 m	5.2	8.6	123.1	0.00596
April 23 m	4.3	8.6	0	--
June 13 m	5.0	5.2	19.5	0.00156
June 15 m	5.3	6.3	79.3	0.00524
June 23 m	4.0	8.8	0	--

FIGURE LEGENDS

Figure 2.1. Vertical profiles of CH_4, O_2, NH_4^+, and temperature in Mono Lake for (A) February, (B) April, and (C) June 2002.

Figure 2.2. Vertical profiles of CH_4, O_2, and CH_4 oxidation rates (nM day-1) in Mono Lake for (A) February, (B) April, and (C) June 2002.

Figure 2.3. Cell counts of total DAPI stained cells, type I methanotroph cells, and type II methanotroph cells in Mono Lake for (A) February, (B) April, and (C) June 2002. Error bars represent the SD of the mean.

Figure 2.4. Representative DGGE banding pattern obtained from Mono Lake bulk water samples using type I methanotroph specific primers. Bands that corresponded to DGGE bands produced by individual clones that were subsequently sequenced are indicated.

Figure 2.5. Representative DGGE banding pattern obtained from Mono Lake bulk water samples using type II methanotroph specific primers. Bands that corresponded to DGGE bands produced by individual clones that were subsequently sequenced are indicated.

Figure 2.6. Phylogenetic relationship of type I and type II methanotroph 16S 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 5% sequence divergence

Figure 2.1.

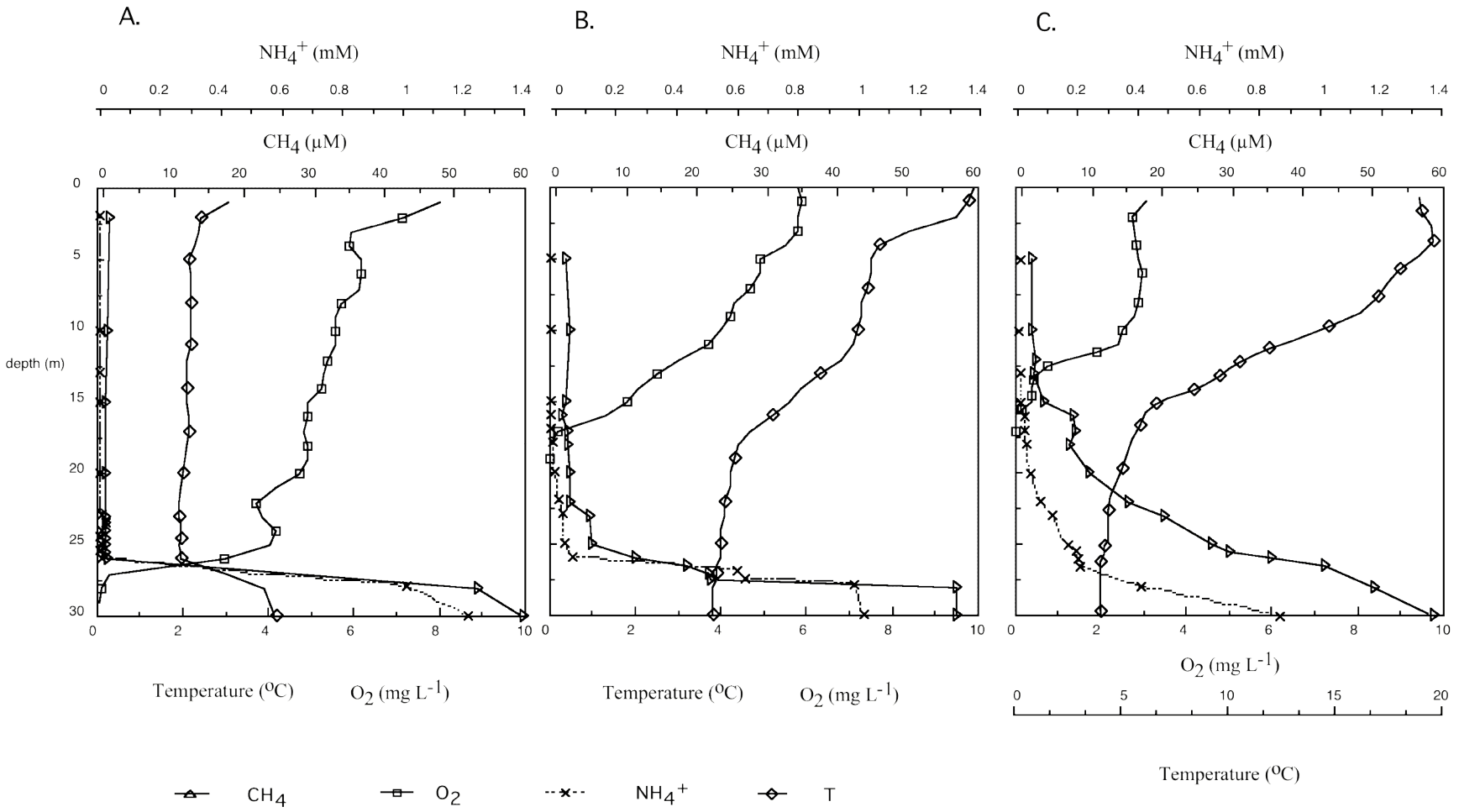

A.
B.
C.
NH_4^+ (mM)
0 0.2 0.4 0.6 0.8 1 1.2 1.4
CH_4 (μM)
0 10 20 30 40 50 60
depth (m)
0 5 10 15 20 25 30
Temperature (°C)
O_2 (mg L^{-1})
0 2 4 6 8 10
0 5 10 15 20
CH_4
O_2
NH_4^+
T

Figure 2.2.

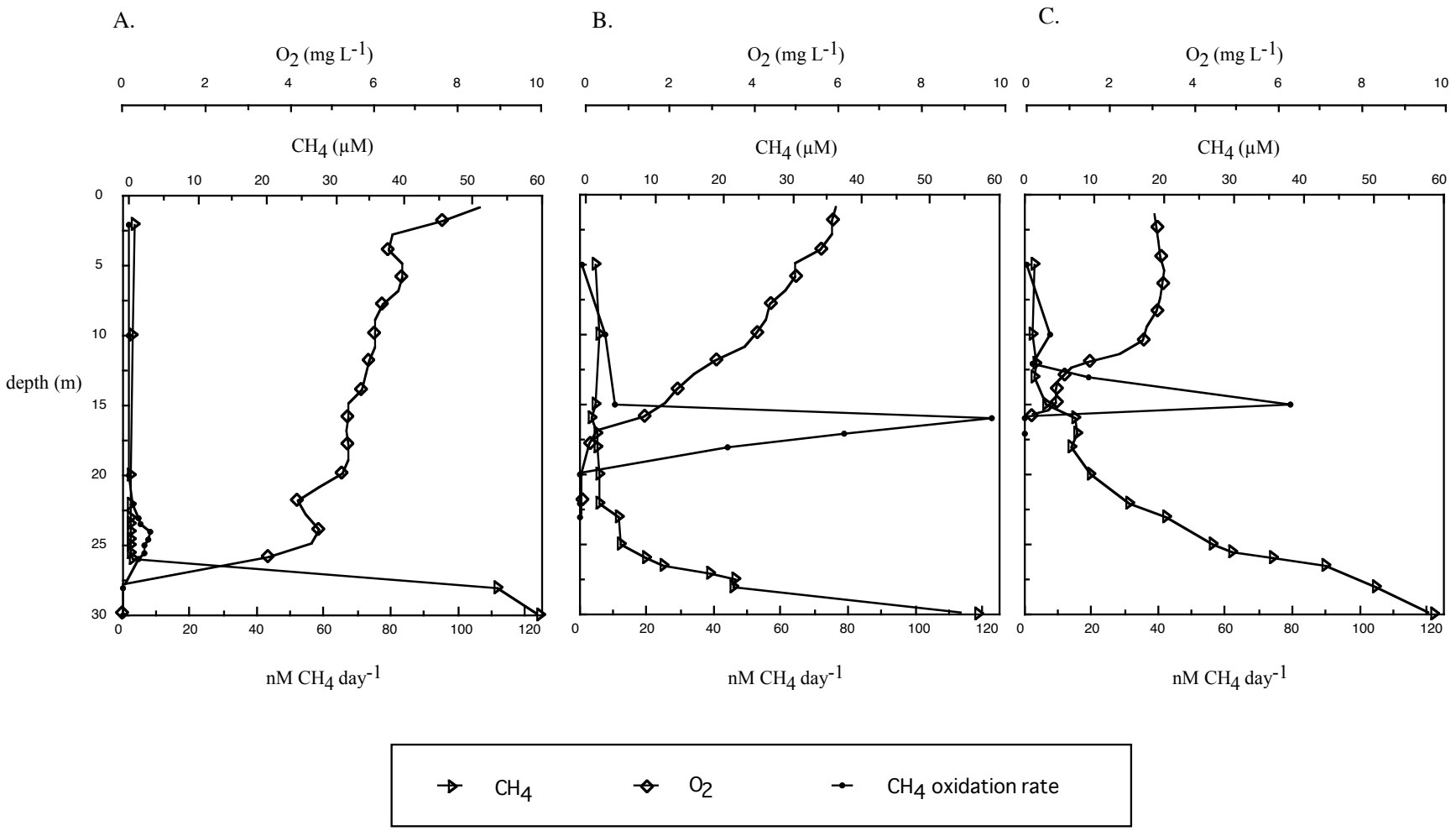

A.
B.
C.
O2 (mg L-1)
0 2 4 6 8 10
CH4 (μM)
0 10 20 30 40 50 60
depth (m)
0 5 10 15 20 25 30
0 20 40 60 80 100 120
nM CH4 day-1
CH4
O2
CH4 oxidation rate

Figure 2.3

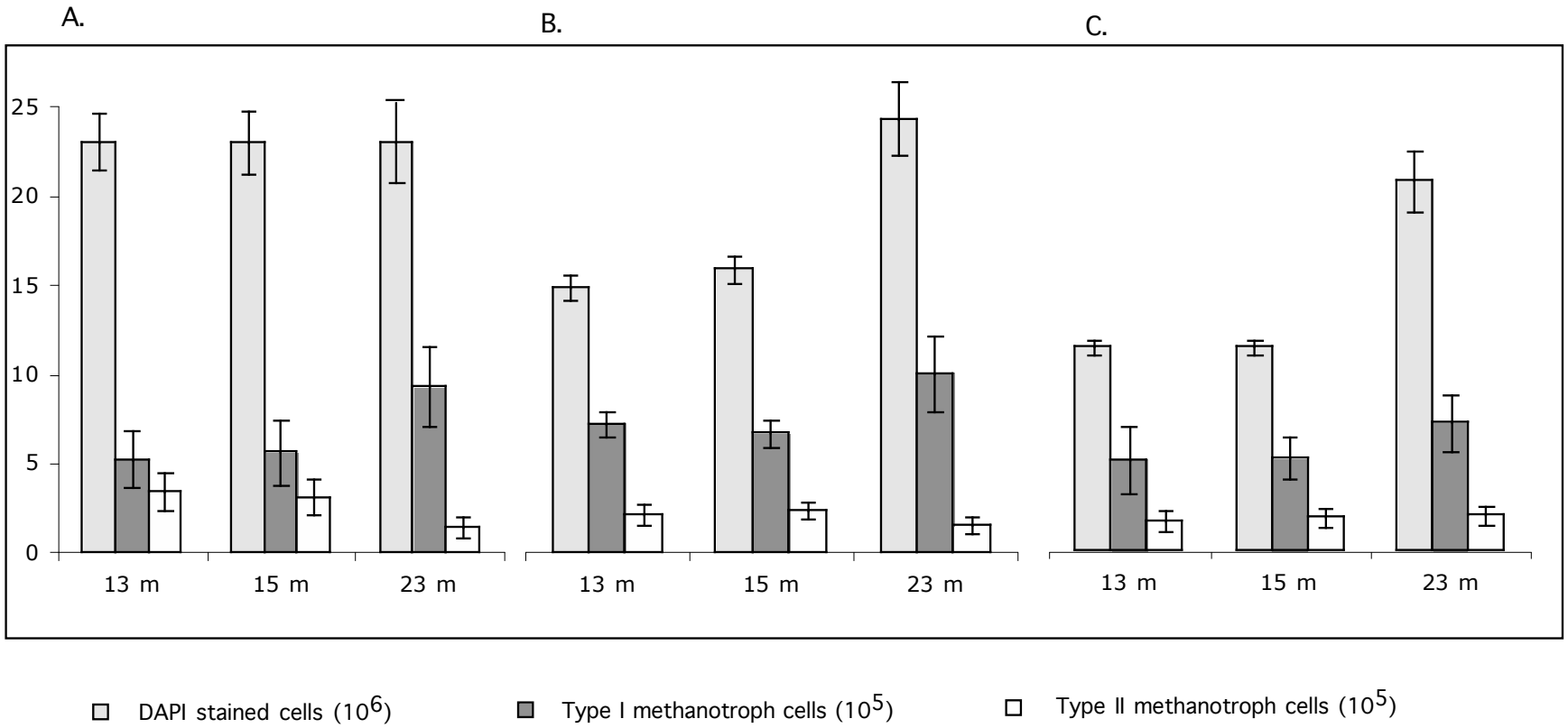

A.
B.
C.
25
20
15
10
5
0
13 m
15 m
23 m
13 m
15 m
23 m
13 m
15 m
23 m
DAPI stained cells (10^6)
Type I methanotroph cells (10^5)
Type II methanotroph cells (10^5)

Figure 2.4.

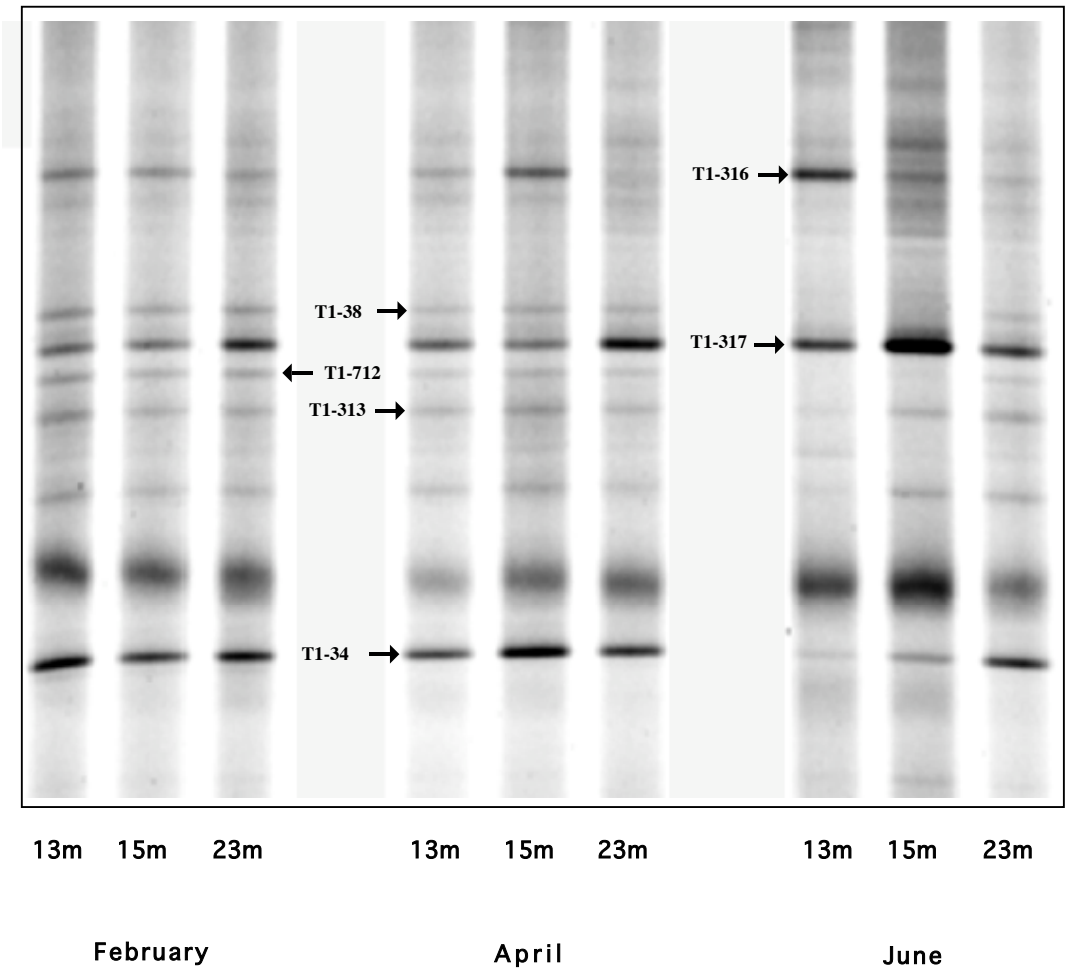

T1-316
T1-38
T1-317
T1-712
T1-313
T1-34
13m
15m
23m
13m
15m
23m
13m
15m
23m
February
April
June

Figure 2.5.

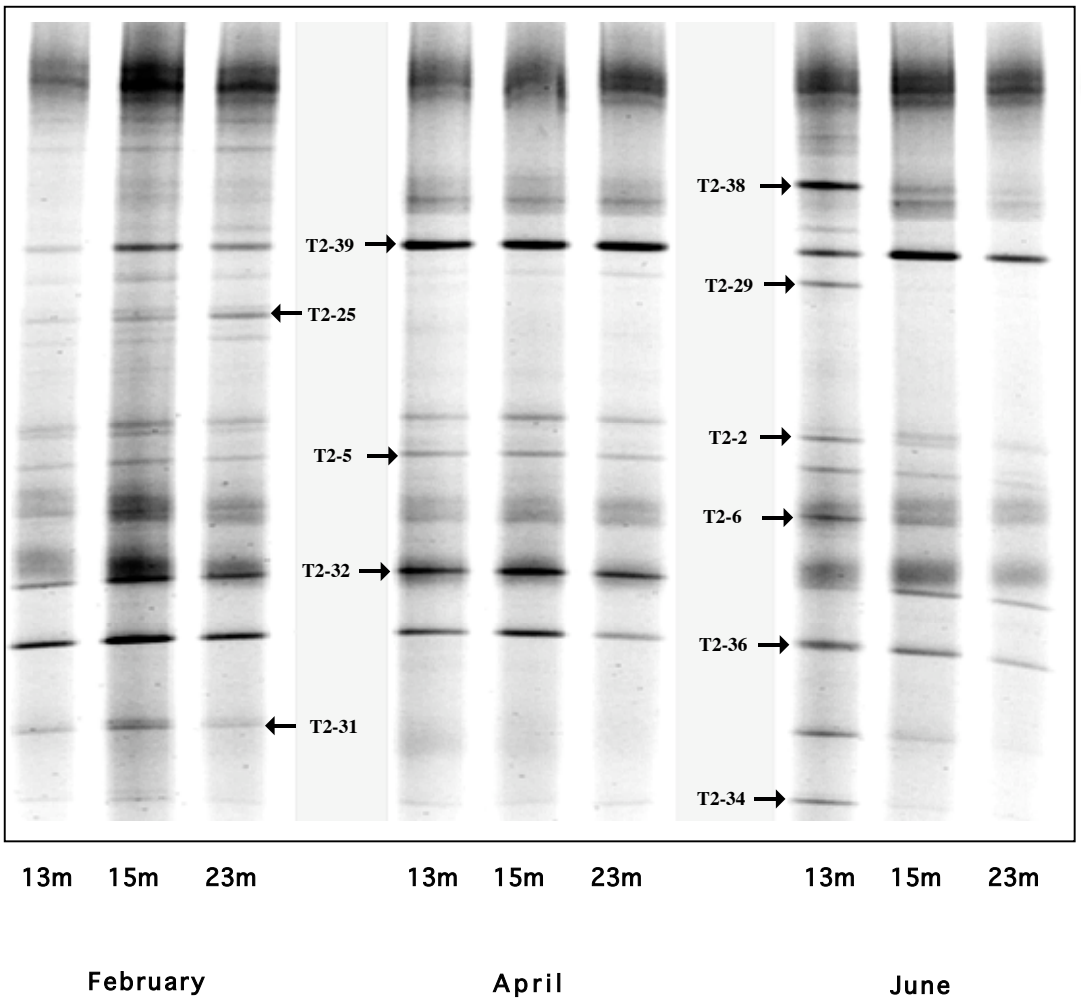
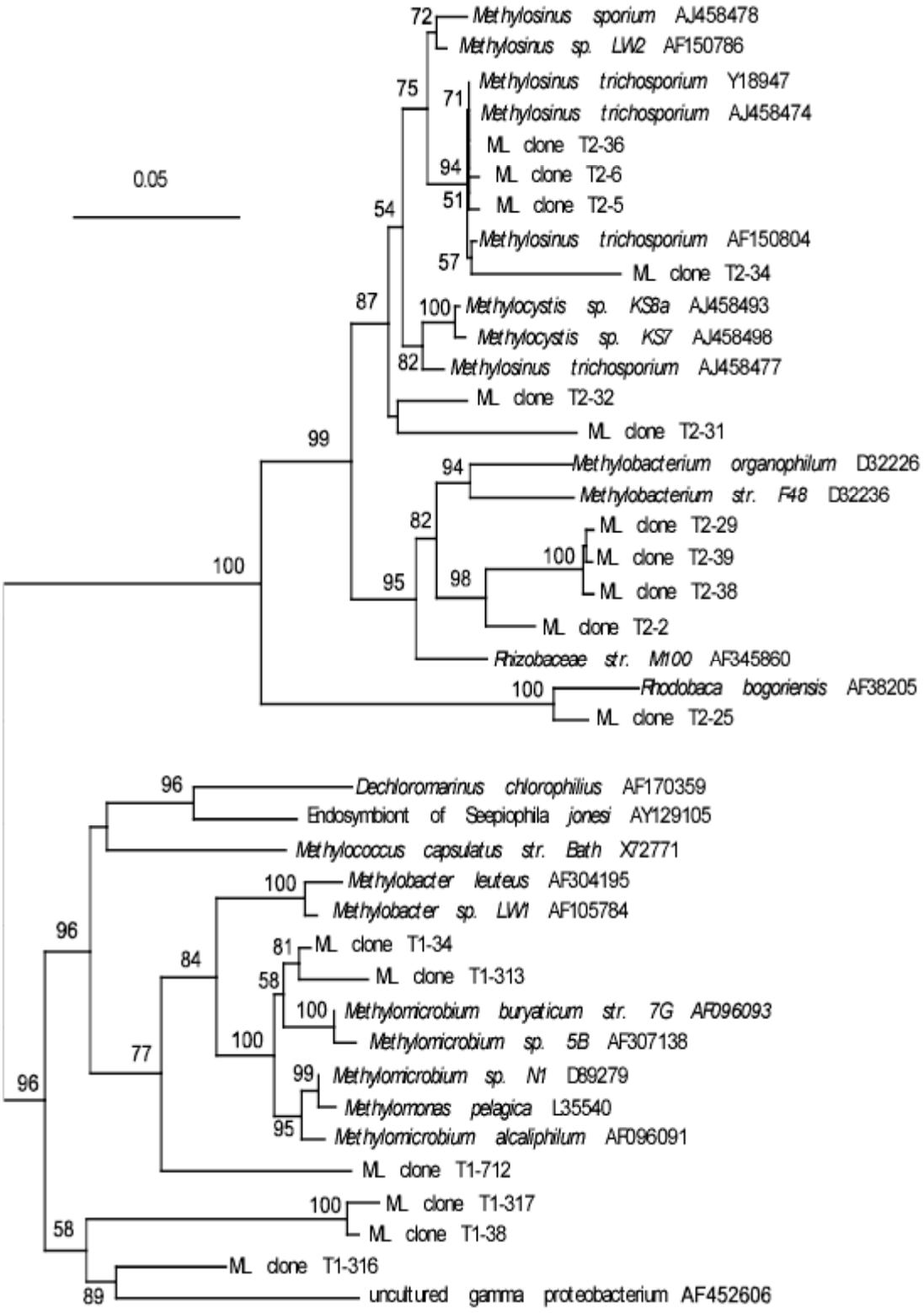

T2-38
T2-39
T2-29
T2-25
T2-2
T2-5
T2-6
T2-32
T2-36
T2-31
T2-34
13m
15m
23m
13m
15m
23m
13m
15m
23m
February
April
June

Figure 2.6.


0.05
Methylosinus sporium AJ458478
Methylosinus sp. LW2 AF150786
Methylosinus trichosporium Y18947
Methylosinus trichosporium AJ458474
ML clone T2-36
ML clone T2-6
ML clone T2-5
Methylosinus trichosporium AF150804
ML clone T2-34
Methylocystis sp. KS8a AJ458493
Methylocystis sp. KS7 AJ458498
Methylosinus trichosporium AJ458477
ML clone T2-32
ML clone T2-31
Methylobacterium organophilum D32226
Methylobacterium str. F48 D32236
ML clone T2-29
ML clone T2-39
ML clone T2-38
ML clone T2-2
Rhizobaceae str. M100 AF345860
Rhodobaca bogoriensis AF38205
ML clone T2-25
Dechloromarinus chlorophilius AF170359
Endosymbiont of Seepiophila jonesi AY129105
Methylococcus capsulatus str. Bath X72771
Methylobacter leuteus AF304195
Methylobacter sp. LW1 AF105784
ML clone T1-34
ML clone T1-313
Methylomicrobium buryaticum str. 7G AF096093
Methylomicrobium sp. 5B AF307138
Methylomicrobium sp. N1 D89279
Methylomonas pelagica L35540
Methylomicrobium alcaliphilum AF096091
ML clone T1-712
ML clone T1-317
ML clone T1-38
ML clone T1-316
uncultured gamma proteobacterium AF452606

CHAPTER 3

NITRIFICATION IN MONO LAKE, CA: INTERANNUAL VARIATIONS IN RATES AND A POSSIBLE ROLE FOR ARCHAEAL AMMONIA OXIDIZERS[1]

[1] Carini, S.A. and S.B. Joye. Prepared for submission to *Applied and Environmental Microbiology*

ABSTRACT

Patterns of geochemical variables, nitrification rates, and the associated ammonia oxidizer microbial community were investigated between August 2002 and August 2003 in Mono Lake, California (USA). Ammonia (NH_3) oxidation rates were measured at four time points using a ^{15}N stable isotope tracer technique. 16S rDNA as well as functional gene analysis, and fluorescence *in situ* hybridization (FISH) were used to characterize the ammonia oxidizer population. Ammonia oxidation activity was observed throughout the oxic portion of the water column at each time point and was correlated with profiles of nitrogenous geochemical species (e.g., nitrate and nitrous oxide). Maximum activity occurred consistently between 12 and 14 m. Depth integrated nitrification rates were highest in November 2002. 16S sequence analysis identified an ammonia-oxidizing bacterial (AOB) community that was dominated by nitrosomonads, with sequences most closely related to *Nitrosomonas*-like sequences retrieved from a Greenland alkaline tufa column, Mongolian soda lakes, and a Chinese salt lake. The observed AOB phylogeny was significantly different from a previously documented population and the transition between AOB populations coincided with a shift in Mono Lake mixing regimes. Samples were also analyzed for ammonia-oxidizing archaea (AOA) using previously described PCR primers specific for archaeal ammonia monooxygenase (*amo*A). Despite FISH analysis that revealed a substantial population of Crenarchaeota (the phylum that encompasses all known AOA), no archaeal *amo*A sequences were obtained. In fact, no archaeal sequences of any type were obtained even using general 16S archaeal PCR primers. We hypothesize that the lack of archaeal DNA recovered from water column samples is likely due to sequence divergence of archaea in Mono Lake and other similar exotic environments. Although correlations

between nitrification and Crenarchaeota and AOB abundance followed a pattern similar to patterns demonstrated in other studies that have suggested significant AOA activity, the lack of verifiable archaeal *amo*A gene copies and the presence of sufficient AOB cell numbers to physiologically account for all measured nitrification in Mono Lake leaves open the question as to whether AOA contribute significantly to nitrification in Mono Lake.

INTRODUCTION

Nitrification plays an important role in the nitrogen (N) cycle in freshwater and marine aquatic environments, as well as in soils and sediments (Hastings et al. 1998; Ward 1986; McDonald 1979). Ecological ramifications of nitrification include the production and efflux of a radiative trace gas (nitrous oxide, N_2O) to the atmosphere, alteration of the concentrations and distributions of biologically available N, and loss of fixed N via coupling to denitrification (Ward, 1986). Nitrous oxide, a byproduct of nitrification (Ritchie and Nicholas, 1972), is a potent greenhouse gas that contributes to radiative forcing and stratospheric ozone destruction (Crutzen, 1970). Nitrification can be coupled with other N processes to remove fixed N from a system, e.g., the nitrate (NO_3^-) and nitrite (NO_2^-) produced by nitrification may serve as substrates for denitrifiers or anaerobic ammonium oxidizers. Denitrifying bacteria transform NO_3^- and NO_2^- to nitrogen gas (N_2); thus, coupled nitrification-denitrification can remove fixed N from an ecosystem (Jenkins and Kemp, 1984; Codispoti and Christensen, 1985). Nitrite produced by nitrification may also be used as an oxidant for anaerobic ammonium oxidation (anammox), which also results in N_2 loss (Mulder et al., 1998). In alkaline ecosystems, however, nitrification may reduce the loss of fixed N from a system. By converting NH_3 to NO_2^- (and ultimately NO_3^-), N losses due to NH_3 volatilization are curtailed (Joye et al., 1999). These various biogeochemical N transformations determine local concentrations and distributions of

biologically available N and can have a profound impact on ecosystem primary production (Jellison and Melack, 1993).

Nitrification is an obligate aerobic chemolithotrophic process by which ammonia is converted to nitrate (Prosser, 1989). Ammonia oxidation, the first step in the nitrification process, is catalyzed by ammonia monooxygenase (*amo*). Ammonia-oxidizing bacteria (AOB) fix inorganic carbon via the Calvin Benson cycle (Watson et al., 1989) using energy derived from the oxidation of ammonia (NH_3) to nitrite (NO_2^-). Ammonia oxidizers are grouped in the γ- and β- subdivisions of the Proteobacteria and consist primarily of *Nitrosococcus* and *Nitrosomonas* species, respectively (Head et al., 1997). Nitrite-oxidizing bacteria (NOB) convert NO_2^- to nitrate (NO_3^-) thereby completing the nitrification of NH_3. Nitrite oxidation is carried out primarily by *Nitrobacter* species that belong to the α subdivision of the Proteobacteria (Prosser, 1989).

Ammonia-oxidizing bacteria and, to a minor extent, the NH_3 co-metabolizing methanotrophs were long considered the sole contributors to aerobic NH_3 oxidation (Bock and Wagner 2001). However, recent evidence indicates that certain members of the domain Archaea are also chemoautotrophic nitrifiers (hereafter, Ammonia Oxidizing Archaea or AOA). Sequences affiliated with marine Crenarchaeota retrieved from a genomic diversity survey in the Sargasso Sea revealed a gene encoding for a unique ammonia monooxygenase sub-unit (*amo*A) that suggested the potential for archaeal oxidation of ammonia (Venter et al., 2004). Discovery of a Sargasso Sea-like *amo*A homolog in soil Crenarchaeota indicated that archaea from diverse environments might nitrify (Schleper et al., 2005). Confirmation of a direct link between archaeal *amo*A and nitrification was provided by the isolation of a pure culture (SCM1) from the marine 1 group of the Crenarchaeota; the growth curve of SCM1 correlated with near

stoichiometric conversion of NH_3 to NO_2^- (Konneke et al., 2005). Unique PCR primers based on alignments of archaeal *amo*A genes and deduced amino acid sequences from the Sargasso Sea and German soil Crenarchaeota amplified *amo*A sequences in diverse areas of the ocean including the base of the euphotic zone, suboxic water columns, and in estuarine and coastal sediments (Francis et al., 2005). New studies continue to demonstrate the ubiquitous distribution of AOA (Leinineger et al., 2006; Park et al., 2006), yet data from hyper-saline and/or alkaline environments are lacking.

Mono Lake is an alkaline (pH 9.8), saline (68-79 g kg^{-1}) lake located just east of the Sierra Nevada range in northern California (38°N, 119°W). Like many closed-basin saline lakes, Mono Lake's hydrological regime alternates between periods of mero- and monomixis depending on inter-annual variations in freshwater inflow (Melack and Jellison, 1998). During monomictic conditions, the lake undergoes a seasonal cycle of thermal stratification followed by overturn and complete lake-wide mixing (holomixis). During summer, temperature differences between warmer surface water (epilimnion) and the cooler water below create a thermal density barrier (thermocline) that isolates the lower water column (hypolimnion) from the effects of wind driven mixing. Biological processes (e.g., respiration) in the isolated hypolimnion create a seasonally anoxic zone where reduced geochemical species (e.g., nutrients and redox metabolites) accumulate. During this time mixing only occurs in the epi- or mixolimnion and biological activity in this zone quickly depletes the nutrient supply. When the surface water cools sufficiently in the fall, the thermal density gradient degenerates and the lake undergoes holomixis. Nutrients accumulated in the hypolimnion are entrained throughout the water column, and O_2 from the mixolimnion aerates the entire lake.

During meromictic periods, freshwater inflows create a chemical density barrier (pycnocline). The hypolimnion (referred to as the monimolimnion during periods of meromixis) is permanently isolated below the pycnocline and reduced geochemical species accumulate to high concentrations (Miller et al., 1993). Mono Lake also experiences seasonal thermal stratification during meromictic periods. Fall mixing is not strong enough to penetrate the pycnocline, and the reduced species and regenerated nutrients remain primarily in the monimolimnion, reaching the mixolimnion only via diffusion. This combination of factors makes Mono Lake an ideal habitat for examining the distribution and activity of microbes in a seasonal series of varying geochemical regimes or between contrasting periods of whole lake mixing patterns.

The understanding of nitrogen dynamics in Mono Lake (Jellison et al., 1993; Miller et al., 1993; MacIntyre et al., 1999) derives mainly from studies on NH_3 concentration and flux and the effect of N availability on primary production (PP). Due to low external N inputs and thermal or chemical stratification, dissolved inorganic nitrogen (DIN) concentrations in the upper water column are low (but periodically replenished due to turnover) during monomictic cycles or chronically low throughout periods of meromixis. Dissolved inorganic phosphorous (DIP) concentrations are high (> 400 μM; Jellison et al., 1993) resulting in extremely low DIN to DIP ratios (<< 0.5), indicating chronic nitrogen limitation in the mixolimnion (Joye et al., 1999).

The activity and phylogeny of ammonia-oxidizing bacteria (AOB) in Mono Lake have been examined (Joye et al., 1999; Ward et al., 2000). Although ammonia-oxidizing bacteria are generally ubiquitous in freshwater and marine environments, they account for a very small proportion of the total bacterial population in natural environments (Voytek and Ward 1995; Phillips et al., 2000). However, nitrifiers may contribute significantly to total primary production

(30 – 80%) in saline and/or alkaline environments with similar nutrient and/or stratification regimes (Indrebø et al., 1979; Cloern et al., 1983). In Mono Lake, the AOB community accounted for a smaller fraction (1–7%) of PP but was still estimated to be an important link to other N cycling processes (Joye et al., 1999).

Previous activity and phylogenetic analyses related to nitrification in Mono Lake were performed at the end of an extended period of monomixis (1988-1995). The bacterial ammonia-oxidizing community in 1995 was dominated by sequences with a very close affinity to *N. europaea* and to a lesser extent, *N. eutropha*. Primers specific for γ-Proteobacteria did not produce amplicons from any samples; only sequences associated with the β-Proteobacteria were identified (Ward et al., 2000). Subsequent phylogenetic analyses were undertaken following the transition to meromixis that occurred in 1996. Samples from August 1997 and April 1998 were screened for ammonia- and nitrite-oxidizing bacterial (NOB) sequences using a wide variety of general and nitrifier specific16S and functional gene primers, molecular probing, DGGE and clone library analyses (Hovanec, 1998). Results were negative for all analyses except for one ambiguous positive result for a *Nitrosospira*-like NOB. The author concluded that the AOB and NOB populations were below detection limits and that NH_3 consumption was more likely due to phytoplankton uptake than nitrification (Hovanec, 1998). AOB are notoriously slow growing and recovery from environmental perturbations (such as a transition from mono- to meromixis) may proceed slowly. The current study was conducted from August 2002 to August 2003 after an extended period of persistent meromixis. The lake had not experienced holomixis for 8 years, with the monimolimnion continually isolated below the pycnocline.

We employed a combination of biogeochemical and molecular ecological methods to investigate nitrification activity and ammonia oxidizer community composition and abundance

and to compare the results with previous data obtained during monomixis and early meromixis (Joye et al., 1999; Ward et al., 2000; Hovanec, 1998). We hypothesized that prolonged meromixis created a new set of relatively stabile geochemical and hydrological regimes that may have facilitated a revival of nitrification. Subsequent to the discovery of AOA in culture and other environments, this study also provided the opportunity to investigate possible AOA populations and their potential contribution to nitrification in Mono Lake.

METHODS

Study site

Field sampling was conducted over the course of a year and encompassed seasonal time points of Aug. 2002, Nov. 2002, May 2003, and Aug. 2003. Depth profiles for each time point were obtained near a permanently moored buoy in the central basin of Mono Lake (station 6; 37°57.822' N, 119°01.305' W). The entire water column was sampled for nutrients, dissolved gasses, and molecular composition. Sample collection depths for nitrification measurements spanned the epilimnion from the upper oxic layer through the bottom of the oxycline. The data presented here corresponds to the depths sampled for nitrification rate assays.

Sample Collection and Geochemical Analyses

Geochemistry

Temperature (T) and conductivity profiles were obtained using a SeaBird SeaCat CTD profiler. Oxygen concentrations were determined using a polarographic (YSI) oxygen sensor equipped with a Clark-type electrode. Samples were collected from discrete depths using a 5 L 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 (He) purged headspace vials via gas tight syringe. The vials contained a NaOH pellet to arrest biological activity. After introduction of the sample, the vial was closed with a stopper and crimp sealed. Dissolved N_2O concentrations were measured using a headspace extraction-gas chromatographic technique (Joye and Pearl, 1994). The sealed headspace vials contained 10 ml of aqueous sample and 10 ml of "headspace" (He plus dissolved gasses stripped from the sample). Eight ml of headspace from each sample was collected into a gas tight syringe by injecting an equal volume of NaCl saturated brine solution into the headspace vial. The gas sample was injected subsequently into a Shimadzu GC-8 gas chromatograph equipped with electron capture detection (ECD). Multiple aliqouts (n=3) of a single point standard (Scott specialty gas mix 447: N_2O 100 ppm in a balance of He) were injected into the ECD at the beginning and end of each set of samples (Joye and Paerl 1994).

Samples for nutrient analysis were filtered (Millipore Acrodisc, 0.2 μm) and stored at 4°C without headspace, which was necessary to avoid volatilization of NH_3 in these high pH (~9.8) samples. Ammonium samples were analyzed at the field laboratory within 6 hrs. Ammonium concentrations were determined using the phenol-hypochlorite method (Solarzano, 1969). To account for matrix effects, standards were prepared using He purged Mono Lake surface water and internal standards were run. Concentrations in samples were calculated from linear regressions of standard data (5 concentrations).

Nitrite and nitrate concentrations were quantified at the UGA lab ~7 to 14 days later. Nitrate (NO_3^-) and nitrite (NO_2^-) were measured cumulatively as NO_x using an Antek®745 Nitrate/ Nitrite Reducer (vanadium reduction assembly) inline with an Antek ®7050 chemiluminescent nitric oxide detector (Álvarez-Salgado & Miller 1998). Reduction efficiency

checks of the vanadium solution were run after every 10 samples. Nitrite concentrations were determined by spectrophotometry (Bendschneider and Robinson, 1952) modified for Mono Lake water chemistry. Standards for these analyses were made using artificial Mono Lake Water that reproduced *in situ* ionic strength and pH conditions to account for matrix effects (Oremland et al., 2002). Samples and standards were adjusted to ~pH 7 with concentrated HCl before addition of reagents.

Nitrification Rates

Water for nitrification rate measurements was transferred to 500 ml rectangular PETG plastic media bottles (Nalgene®), and the bottles were sealed without headspace. Nitrification rates were measured using a $^{15}NH_3$ tracer technique (adapted from Ward, 1987). Water samples from each depth (n= 3) were amended with a $^{15}NH_4^+$ solution (Cambridge Isotope 99.9 atom % ^{15}N) for a final tracer concentration equal to 10% of the *in situ* NH_4^+ concentration. Ammonia (NH_3) is the actual substrate for nitrification and is also the dominant form of the NH_4^+/ NH_3 equilibrium pair in alkaline environments such as Mono Lake. Therefore the NH_3 nomenclature will be used subsequently except when the species referred to is specifically NH_4^+ (such as the initial tracer salt). The bottles were incubated for 24 hrs at *in situ* temperature in the dark after which the sample was filter sterilized (GF/F) to halt biological activity and remove biologically incorporated label.

The atom % enrichment of the NO_X pools resulting from oxidation of $^{15}NH_3$ was determined using a modified ammonia diffusion technique (Sigman et al., 1997). The sample volume was first reduced by 66% in a 65 °C incubator (Sigman et al., 1997). Elevated temperature facilitated sample volume reduction and the decomposition of labile dissolved

organic nitrogen (DON). The high pH of Mono Lake water combined with decreased gas solubility due to salt concentration and increased temperature drove off the remaining $^{15}NH_3$ / $^{15}NH_4^+$ label. Filter sterilized Mono Lake water samples, with and without $^{15}NH_4^+$ additions just prior to the sample volume reduction step, were treated in the same manner as the samples to evaluate the potential for residual ^{15}N tracer; tracer free additions permitted us to determine initial (natural abundance) $^{15}NO_X$ values.

Diffusion packets consisting of acidified (25 µl 5N H_2SO_4) pre-combusted (400 °C) Whatman GF/D filters (1 cm diameter) sandwiched between two 25 mm Teflon membranes (Millipore) were assembled, and 1 packet was floated in each sample bottle. Three hundred mg of Devarda's alloy was then added to each sample and the bottles were immediately sealed. Samples were incubated at 65°C for 4 days to facilitate NO_X reduction to NH_3 catalyzed by Devarda's alloy. The samples were then shaken at room temperature (10 days @75 rpm) to trap converted NH_3 as NH_4^+ on the filter packets (Sigman et al., 1997). Filters were subsequently removed from the packet, dried, and analyzed using a Carlo Erba CHN (Model NA 1500) Combustion Analyzer (Milan, Italy) coupled to a Finnigan Delta C Isotope Ratio Mass Spectrometer (Bremen, Germany) via a Finnigan Conflo II interface (UGA Ecology Analytical Laboratory).

Nitrification (NTR) rates were calculated by determination of the atom % enrichment of the NO_X pool in each sample. Sample blanks (described above) indicated that initial $^{15}NO_X$ atom % was at natural abundance levels (0.0036) and that there was no $^{15}NH_4^+$ tracer carry-over. Final $^{15}NO_X$ atom % was determined by EA-irMS analysis of each filter. Controls were run to correct for N blanks associated with DON and Devarda's alloy (Sigman et al., 1997). The differences in atom % between initial and final $^{15}NO_X$ pools in each sample were converted to nitrification rates

(nmol NO_3^- l^{-1} per unit time; equation 3.1) and corrected for $^{15}NH_3$ isotope tracer dilution (equation 3.2).

(3.1) nmol $^{15}NO_3^-$ $l^{-1}day^{-1}$ = ([atom%$^{15}NO_3^-{}_{(f)}$-atom%$^{15}NO_3^-{}_{(i)}$] *$NO_3^-{}_{(f)}$] / 1 day])

Where $^{15}NO_3^-{}_{(f)}$ and $^{15}NO_3^-{}_{(i)}$ are the final and initial concentrations of $^{15}NO_3^-$ in the sample, respectively and $NO_3^-{}_{(f)}$ is the final NO_3^- concentration in the sample.

(3.2) Corrected Rate = nmol $^{15}NO_3^-$ day^{-1} / (α*0.1)

Where α is the %$^{15}NH_3$ in the $^{15}NH_4^+$ tracer pool (equilibrium determined by sample pH and T). Since the $^{15}NH_4^+$ tracer addition equaled 10% of *in situ* NH_3 concentrations, the total nitrification rate must be corrected by a factor of 10.

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. Within 4-6 hours back at the field laboratory, a 10 ml sub-sample for FISH analysis was taken, fixed with formalin (final concentration of 4%) for 30 minutes and then stored frozen at –20°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 μm) to collect microbial biomass for subsequent DNA extraction. Excess water in the filter cartridge was replaced with an extraction buffer and stored at -70°C until processed (Ferrari and Hollibaugh, 1999).

DNA was extracted from Sterivex filters using a modified technique of Murray et al. (1996) and Ferrari and Hollibaugh (1999). Filter cartridges containing extraction buffer (1.5 M NaCl, 5 mM $MgCl_2$, 100 mM Tris HCl - pH 7, 100 mM EDTA - pH 8, 100 mM Na_2HPO_4 - pH 8) were thawed at room temperature. Forty µl of lysozyme (50 mg/ml) was added and the cartridges were incubated at 37°C for 30 min on an orbital shaker (25 rpm). Lysate was expelled from the cartridge and then the cartridge was rinsed with extraction buffer to ensure maximum yield.

DNA was further liberated from the lysate using bead-mill homogenization (method adapted from the MO BIO® Ultraclean Soil DNA kit protocol). Seven hundred µL of raw extract combined with glass beads (100 µl), 100 µl proteinase K (10 mg/ml), and 100 µl SDS (20%) was vortexed at high speed for 10 min. After centrifugation (12,000 x g for 5 min), the supernatant was treated with 250 µl potassium acetate (4 M, pH 5) and centrifuged (12,000 x g) for 5 more min. The supernatant was then added to 750 µL of 6 M guanadine thiocyanate and 1 mg sterile diatomaceous earth and mixed by inversion. The diatomaceous earth-bound DNA was trapped on a GF/F filter, washed twice with 0.5 ml guanadine thiocyanate (6M), twice with 0.5 ml isopropanol solution (25 % isopropanol, 25 % ethanol, 100 mM NaCl, and 10 mM Tris HCl, pH 8), and once with 0.5 ml ethanol (95%). Washing steps were all performed by adding the appropriate solution to the spin column and centrifuging at 12,000xg for 1 min. DNA was eluted from the filter with 60 µl sterile water (pH 8). The DNA obtained was suitable for the PCR and no subsequent purification was necessary.

Previously characterized oligonucleotide primers were chosen to analyze for ammonia-oxidizing bacteria (16S), general archaea (16S), and ammonia-oxidizing archaea (*amo*A gene). Samples were screened for β-Proteobacteria ammonia-oxidizers using a nested approach: the

EUB1/EUB2 primer pair (Liesack et al. 1991) was used to amplify total bacterial rDNA, and that product was subjected to a second amplification using the Nit-A/Nit-B primer pair (NitAB; Voytek and Ward 1995; Ward et al., 2000). This nested approach was used because previous work has shown this to be more sensitive for detecting less abundant sequences (Voytek and Ward, 1995). A nested approach was also used with the NOC1 and NOC2 primer pair to screen for members of the γ-Proteobacteria ammonia-oxidizers (Voytek, 1996). DNA extract was screened for the presence of ammonia-oxidizing archaea using the primer sets Cren-amo1F / Cren-amo1R (Konneke et al., 2005) and Arch-amoAF / Arch-amoAR (Francis et al., 2005), which targets the putative archaeal ammonia monooxygenase (*amo*A) gene. The universal archaeal primer set Arch 21F and 1492R was also used to screen for general archaeal populations (Delong, 1992). Because of the unique environment of Mono Lake several different modifications were made to the standard, published archaeal PCR protocols to try and optimize results. These included adjustments of template, primer, and magnesium concentrations as well as several different annealing temperature gradients.

PCR products were isolated by agarose gel electrophoresis, extracted from the agarose (QIAquick® Gel Extraction Kit; Qiagen), inserted into the pCR®4-TOPO® plasmid vector and transformed into chemically competent One Shot®TOP10 *Escherichia coli* cells as per the manufacturer's instructions (Invitrogen Corp). Transformed cells were plated on Luria–Bertani (LB) plates containing ampicilin (100 mg ml $^{-1}$) and incubated overnight at 37 °C. Colonies were chosen at random from replicate plates and cultured overnight at 37 °C in 1.2 ml of LB medium containing ampicilin (100 mg ml^{-1}). PCR/DGGE analysis was used to identify unique clones for sequencing. Bands suggestive of unique clones were cut out, re-amplified with the PCR and re-run using DGGE. Clones that produced bands which did not have the same electrophoretic

mobility as the original bands (based on multiple lanes of standard distributed across the gel) or that produced more than one DGGE band were discarded. Frozen, glycerol preserved whole -cell clone cultures (2x96 well micro-titer plates) were sequenced (primer M13F) by SeqWright (Houston, TX) and yielded robust sequence fragments of 550-800 bp. The obtained 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 (GCG) package (Madison, WI). A phylogenetic tree was constructed using the neighbor-joining method and Jukes-Cantor distances (PHYLIP package v. 3.5).

Fluorescence *in situ* hybridization (FISH) was used to determine the abundance of AOB and Crenarcheota, a proxy for AOA (Konneke et al., 2005; Wuchter 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; Schleper et al., 2004; Konneke et al., 2005; Francis et al., 2005; Wuchter et al., 2006; Leininger et al., 2006; Hallum et al., 2006). Although it is almost certain that not all crenarchaeotes oxidize 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, Nso190, and Nsm156, designed to detect halophilic/halotolerant *Nitosomonas* spp., ammonia-oxidizing β-Proteobacteria, and some *Nitosococcus* spp. respectively (Mobarry et al, 1996). The probes were synthesized by MWG Biotech (Ebersberg, Germany) and labeled with Texas Red. Formalin-preserved samples were thawed and mixed well. Then 500 µl 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 µm

polycarbonate membrane filter (Osmonics). The tube was then rinsed with PBS (2 x 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 (2x) and allowed to air dry in a Petri dish. Sections of individual filters were separated for 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 3 probes (Mobarry et al., 1996). Filter sections were then washed, rinsed in milliQ water, air dried and counterstained with DAPI for 3 min (50 µl of 2 mg ml^{-1} 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 per sample filter section). Cells ml^{-1} 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 (GA), the percentage of the grid utilized (% Grid), the surface area of the filter (SA_F), and the volume of water filtered (vol.; equation 3.3).

(3.3) Cells ml^{-1} = (mean counted cells*[SA_F/ (GA*%Grid)]) / vol.

Total-cell counts from the DAPI-only stained filter sections and DAPI counts from counterstained, hybridized filters were compared to control for loss of cells from the filters that may have occurred during hybridization.

AOB Cell Specific Nitrification

Cell specific rates of NH_3 oxidation were calculated by dividing the moles of NH_3 oxidized per volume by the total number of AOB cells per volume as determined by FISH at each depth.

Statistics

Linear regression and/or Pearson's Correlation Coefficient (r) analyses were used to evaluate correlations between nitrification rates, pertinent geochemical variables, and group-specific abundances. Spatial and temporal differences in NH_3 oxidation rates were compared using paired t-tests assuming unequal variances (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 Tukey's analyses.

RESULTS

Geochemical Profiles

Oxygen and temperature (T) profiles displayed distinct seasonal patterns while excess density, calculated from conductivity measurements (Jellison et al., 1999), remained relatively constant throughout the study period. The lake remained isohaline (averaging 69.4 mg l^{-1}) in the mixolimnion with spatial and temporal variation of less then 5 % (Fig. 3.1). Temperature (2-20 °C) and O_2 (<1 –7 mg l^{-1}) profiles in Aug. 2002, May 2003, and Aug. 2003 were strongly correlated (r = 0.99, 0.95, and 0.98, respectively) and demonstrated seasonal stratification with discrete oxyclines between 9 and 15 m (Fig. 3.1). Stratification was not well defined in

November as O_2 concentrations became less variable and penetrated to 20 m and temperatures remained constant, at about 14 °C, through the mixolimnion (Fig. 3.1).

Ammonia concentrations in the Mono Lake water column varied over depth during each of the sample dates. During Aug. 2002, May 2003, and Aug. 2003, NH_3 concentration in the mixolimnion were generally low (< 1μM). However, NH_3 increased dramatically (up to 20-fold) through the oxycline (Fig. 3.2). In contrast, the November 2002 profile showed elevated NH_3 concentrations (9 to 11 μM) in the surface water (Fig 3.2) and less of an increase through the oxycline. All NH_3 profiles displayed a concave distribution between 11 and 15 m (Fig. 3.2b).

Similar NO_X concentrations and profile shapes were observed during August 2002 and May 2003. Low concentrations (66 to 89 nM) in the surface water slowly increased to peak levels of 490 and 400 nM, respectively, at about 13 m. Concentrations then decreased on each sample date to ~90 nM by16 m (Fig. 3.2). The highest upper water column NO_X concentration (204 nM) occurred in November 2002. November NO_X concentrations peaked (350 nM) at 13 m and then gradually decreased with depth to 180 nM at 20 m (Fig. 3.2). The August 2003 sampling produced a quite different profile. Concentrations of NO_X remained relatively low (< 60 nM) throughout the upper water column (Fig. 3.2). A sharp peak (441 nM) developed between 13 and 14 m before the concentration decreased to 190 nM at 16 m (Fig. 3.2).

Nitrous oxide profiles followed the same general spatial patterns as NO_X profiles. August 2002 and May 2003 had relatively low concentrations in the surface water (15 and 11 nM, respectively) and gradually increased to peak levels of 30 and 35 nM N_2O respectively at 13 m (Fig. 3.2). Concentrations for both profiles then decreased to about 8 nM. The November 2002 N_2O profile had the highest concentrations throughout, ranging from 25 nM in the upper water column to 41 nM (at 13 m). The N_2O concentration then gradually decreased to 20 nM at 20 m

(Fig. 3.2d). August 2003 N_2O concentrations remained constant at 20 nM between 3 and 10 m. Below 10 m the concentration increased to a peak level (40 nM) at 14 m and then returned to 20 nM at 16m (Fig. 3.2). The concentration of N_2O in Mono Lake water was extremely saturated with respect to equilibrium with atmospheric N_2O concentrations (expected to be between 0.3and 0.8 nM; calculated using the Bunsen solubility coefficients from Weiss and Price, 1980).

Nitrification Rates

Nitrification activity was observed throughout the oxic portion of water column on all 4 sampling dates. Profiles in August 2002, May 2003, and August 2003 exhibited relatively low activity (< 60 nM day^{-1}) in both the upper water column and proximate to the bottom of the oxycline (O_2 vs. NTR is depicted in Fig. 3.4) while distinct peaks of activity (350 to 480 nM day^{-1}) occurred consistently between 13 and 14 m (Fig. 3.2). Rates measured in November 2002 displayed a much different profile. Activity in the upper water column was ~4 times higher (230 to 335 nM day^{-1}) than during the other sampling dates. Peak activity (370 nM day^{-1}) coincided spatially with the other samples but exhibited a much broader profile (Fig. 3.2).

Molecular analysis

A total of 92 AOB sequences were obtained from Mono Lake water samples. Eight clones dominated the bacterial ammonia-oxidizing community. All recovered sequences were affiliated with AOB from the β-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 (Stougaard et al., 2002). The remaining 7 unique sequences each comprised between 3and 15 % of the total AOB population. Sequences from clones ML_AOB_H1, ML_AOB H3,

MLAOB_G4, and ML_AOB_A3 clustered together (Fig. 3.3) and were most closely related (86 – 89 %) to a *Nitrosomonas* sp. recovered from a Chinese saline lake (Dong et al., 2006). 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 (Sorokin et al., 2001, direct submission). ML_AOB_E4 was slightly more divergent but still most closely related to AOB clone ANs5 (Fig. 3.3).

Results of PCR with the NOC primer set were negative and suggested a lack of detectable AOB in Mono Lake affiliated with the γ-Proteobacteria. It is possible that the nested amplification procedure might have missed *N. oceanus* if it were rare and if it were selected against by the EUB primers in the initial amplification (Ward et al., 2000). In addition, our FISH analysis was designed to enumerate total AOB abundance rather than to distinguish between individual clades of AOB. Therefore, we cannot rule out completely the presence of *N. oceanus* in Mono Lake at some low level.

All water samples screened during the course of the study tested negative for putative *amo*A genes. In fact, no archaeal sequences were obtained using the general archaeal 21F / 1492R primer set. 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. Crenarchaeal abundance varied over space and time (Fig. 3.4). Absolute Crenarchaeota cell numbers ranged from 1 to $9x10^5$ cells ml^{-1} (Table 3.1) and comprised between 2 and 10 % of total cells (determined by DAPI enumeration). Abundance profiles for all sampling dates were similar with significant peaks between 13 and 14 m (Fig. 3.4).

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. 3.4). In August 2002 AOB cell numbers were $6x10^4$ ml^{-1} and decreased in November 2002 to $3x10^4$ cells ml^{-1} (Fig. 3.4). Abundance steadily increased through May to a maximum observed during this study of $10x10^4$ cells ml^{-1} in August 2003 (Fig. 3.4). AOB cell specific oxidation rates ranged from near 0 in the surface water in Aug. 2003 to almost 5 fmol $cell^{-1}$ hr^{-1} in November 2002 (Table 3.1).

DISCUSSION

Significant nitrification occurred in Mono Lake during this study and rates correlated with nitrogen-species geochemical profiles. Phylogenetic analysis revealed a shift from a previously documented nitrifying population dominated by *N. europaea* and *N. eutropha* strains, to *Nitrosomonas*-like spp. affiliated with alkaline and/or saline environments. Despite a significant population of Crenarchaeotes, neither archaeal *amo*A nor archaeal sequences in general were amplified suggesting that existing archaeal primers may do not detect archaea from Mono Lake. Correlations between nitrification rates and Crenarchaeota and AOB abundance in Mono Lake followed a pattern similar to patterns demonstrated in other studies that have suggested significant AOA activity. However, the lack of verifiable archaeal *amo*A gene copies and the presence of sufficient AOB cell numbers to physiologically account for all measured nitrification in Mono Lake preclude specific assignment of nitrifying activity to Mono Lake Crenarchaeota, but a potential AOA contribution to nitrification in Mono Lake cannot be ruled out.

Geochemical Profiles and Patterns of Nitrification Activity

Ammonium, NO_X, and N_2O profile contours were consistent with the distribution of nitrification activity throughout the water column on all sampling dates. Ammonium, the primary substrate for the first step in nitrification (Prosser, 1989), regularly displayed profiles indicating consumption (Martens and Berner, 1977); the minimum NH_3 concentrations were associated with the maximal nitrification rates (Fig. 3.2). Production of NO_X and N_2O also correlated with NTR activity ($p < 0.01$). These data suggest that geochemical profiles of these species are a reasonable indicator of the actively nitrifying zone. Mono Lake ammonia profiles from April and July 1995 also indicated consumption associated with measured ammonia oxidation rates (Joye et al., 1999).

In a study that sampled Mono Lake during August 1997 and April 1998, no AOB or NOB sequences retrieved and geochemical data were not suggestive of nitrification (Hovanec, 1998). Ammonia concentrations were consistently low (1-2 μM) in the epilimnion, and it was difficult to discern a zone that may have indicated consumption associated with nitrification. Nitrate + nitrite concentrations were relatively low (< 200 nM), variable, and profiles did not demonstrate a peak consistent with nitrification-associated production in the oxic portion of the water column (Hovanec, 1998). The data from these previous studies indicates that nitrification was occurring in 1995 (Joye et al., 1999; Ward et al., 2000) and 2002-2003 (this study), but suggests that activity may have ceased in the period following the transition from mono- to meromixis in 1996 (Hovanec, 1998).

Profiles of ammonia oxidation rates were distinctly different between this study and that from 1995. Although the biogeochemical data from the present study and that of Joye et al.

(1999) was obtained from different sampling sites (St. 6 versus St. 3, respectively), a proposed two-dimensional mixing model for Mono Lake indicated a significant lateral advection component in lake-wide mixing (MacIntyre et al., 1999), suggesting some degree of homogeneity in the mixolimnion throughout the lake.

In most cases, nitrification activity during this study was negligible or relatively low in the surface water and near the bottom of the oxycline. Sub-surface peaks of activity regularly occurred in a zone between 13 and 14 m (Fig. 3.2a). In contrast, peak activity during April and July of 1995 occurred in the surface waters (5-7 m) and decreased with depth (Joye et al., 1999). Rate profile characteristics measured at several oceanic stations in the Southern California Bight and Station ALOHA were similar to profiles generated in the current study. Sub-surface rate maxima were commonly observed and attributed to photoinhibition of nitrifiers in the upper euphotic zone (Olsen, 1981; Ward 1987; Dore and Karl 1996). Joye et al. (1999) accounted for photoinhibition when calculating nitrification rates (rates were determined in dark incubations). Possibilities for the observed rate maxima in the upper water column of Mono Lake include temporal adaptation (e.g. maximum nitrification activity occurring during the night) or a shift to a different population of nitrifiers by 2002-2003 (discussed below). Different nitrifying bacteria have demonstrated differential susceptibility to photo-inhibition (Olson, 1981b).

Peak nitrification activity from the two Mono Lake studies generally agreed, with differences (up to a factor of 5) that could easily be accounted for by the different rate measurement methodologies employed. The measurements made in1995 used a ^{14}C bicarbonate dark fixation technique (modified from Billen, 1976) and calculated values can vary depending on the conversion factor used to translate C fixed to NH_3 oxidized (Billen, 1976; 1978). It is possible that nitrification rates were indeed different in1995 than in 2002-2003 but Mono Lake

rates, in both cases (0-2,800 and 5-480 nM day^{-1}, respectively), were comparable to rates reported in estuarine habitats (Enoksson, 1986; Bernounsky and Nixon, 1993) and up to an order of magnitude higher than those reported in pelagic oceanic nitrification studies. Rates in Washington coastal waters (Ward et al., 1984), the Southern California Bight (Olsen, 1981a; Ward, 1987), and at station ALOHA (Dore and Karl, 1996) ranged between 2 and 137 nM day^{-1}.

Integration of the nitrification rate profiles revealed an unexpected winter maximum in Mono Lake nitrification activity. Because of the parallel physiological and ecological characteristics of methane oxidizers and nitrifiers (Bedard and Knowels, 1989; Holmes et al.1995; Bodelier and Frenzel, 1999), we expected a similar pattern of nitrification and methane oxidation activity. Integrated methane oxidation rate profiles in Mono Lake displayed steadily increasing rates from winter to summer (Carini et al., 2005; Chapter 2) with the highest overall activity occurring in September (unpublished data). In contrast, nitrification rates integrated over depth indicated peak activity occurred during November (5.3 μmol m^2 d^{-1}) with the lowest rates measured in the summer (the average Aug. integrated nitrification rate was 1.2 μmol m^2 d^{-1}).

A review of the literature suggests that a consistent seasonal pattern of NTR has failed to emerge. Studies of NTR in marine systems (both water column and sediment) such as the Tay Estuary in Scotland and the mid-Narragansett Bay have reported summer rate maxima (Henrickson et al., 1981; Seitzinger et al., 1984). A positive correlation between rates and seasonal temperatures indicated that temperature might be the major force driving NTR in those systems (Herbert, 1999). However, reports of nitrification rates in Danish coastal waters, the upper- Narragansett Bay, and Chesapeake Bay described summer NTR minima. The authors hypothesized that reduced O_2 concentrations and competition for NH_3 inhibited activity during the summer at those sites (Hansen et al., 1981; Ward, 1984). Physio-chemical and biological

factors that may influence nitrification activity have been derived, by and large, from culture studies. Furthermore many of these parameters, such as those mentioned above, are interdependent to varying degrees and it is difficult to extrapolate the role of individual affects between systems (Herbert, 1999). For example, increases in temperature may stimulate enzymes associate with nitrification (Hansen, 1980) but could also decrease O_2 solubility, which could indirectly reduce nitrification rates. Similarly interdependent variables that can affect activity may explain some of the apparent contradictory data reported. We hypothesize that maximal nitrification rates observed in November resulted from increased NH_3 availability due to the breakdown of seasonal stratification.

AOB Phylogenetic Succession

The bacterial ammonia-oxidizing community in Mono Lake documented during this study was dominated by AOB affiliated with nitrosomonads and consisted mainly of *Nitrosomomas* species. Only sequences associated with the β-Proteobacteria were identified (Fig. 3.3). Screening of Mono Lake water samples with the NOC primer pair indicated a lack of detectable AOB affiliated with the γ-Proteobacteria. These results are counterintuitive because it would appear likely that ammonia-oxidizing γ-Proteobacteria and *Nitrosospira* species from the β-Proteobacteria could better adapt to the hypersaline conditions in Mono Lake. The first AOB isolated from a saline (marine) environment, *Nirosococcus oceani*, is affiliated with the γ-Proteobacteria (Watson, 1965) and related species are widely distributed in marine environments (Ward and O'Mullan, 2002). *Nitrosospira* species outnumber *Nitrosomonas* species (by a factor of 2-3) in marine environments (Stephen et al., 1996; Bano and Hollibaugh, 2000; Hollibaugh et al., 2002). However, the genus *Nitrosomonas* does include some marine species identified in the

polar oceans (Bano et al., 2000; Hollibaugh et al., 2002), and other halotolerant species including *N. halophila* and some *N. europaea* strains (Koops et al., 1991),

In fact, *Nitrosomonas* sequences dominated the Mono Lake AOB population in 1995 (Ward et al., 2000). The bacterial ammonia-oxidizing community in 1995 consisted of sequences with very high affinity to *N. europaea* and *N. eutropha* sequences (Ward et al., 2000). Thirty-four unique sequences derived from NitAB amplifications were most closely related to *N. europaea* (> 95% similarity) and 5 were not differentiable (< 2.5% different) from the sequence of *N. eutropha* (Stackebrandt and Goebel, 1994; Ward et al., 2000). Primers specific for γ-Proteobacteria did not produce amplicons from any samples and only sequences associated with the β-proteobacteria were identified (Ward et al., 2000).

The robustness of results from Mono Lake in 1995 was tested by the authors using multiple, independent PCR reactions with several different primer sets at two separate laboratories and combined with DGGE analysis. They concluded that environmental parameters such as salinity and temperature most likely exerted greater selection on functional rather than ribosomal genes that may have accounted for physiological diversity that could have allowed sub-species of *N. europaea* to become acclimated to alkaline, high-salinity environments such as Mono Lake (Ward et al., 2000). These results suggest that *N. europaea*- and *N. eutropha*-like bacteria were present and most likely the dominant members of the AOB population of Mono Lake in 1995.

Although the phylogenetic data from this study and the data presented in Ward et al. (2000) were collected from different sampling sites (see above), general cross-site phylogenetic comparisons between these two stations revealed very similar population distributions (Hollibaugh et al. 2001; Humayoun et al., 2003) with minor differences resulting more from

improved methodology than to true differences between the sites or sampling times. Sequence data from this study relative to 1995 Mono Lake data indicated a decrease in diversity and a shift in community composition at the species level. Only 8 unique clone sequences were retrieved during 2002-2003 and a single *Nitrosomonas* sequence accounted for approximately 48% of the nitrifier clone library. While the population was still dominated by *Nitrosomonas* species (Fig. 3.3), none of the sequences were most closely related to *N. europaea* strains (7-11% difference). Re-examination of the phylogenetic affiliations of the 1995 sequences (using BLAST) confirmed the original results (Ward et al., 2000) ensuring that new additions to the sequence database were not responsible for the observed phylogenetic differences between the two studies. Pair-wise sequence alignments also established that sequences retrieved during the current study were distinct from 1995 sequences. The possibility that the observed difference in nitrifier phylogeny between the two studies was due to the different sampling locations cannot be ruled out. However, it seems highly unlikely that two divergent populations with no detectable common affiliations could co-exist within the proximity of these sites given the hydrological mixing model referenced above.

The combination of geochemical profiles, nitrification rate measurements, and phylogenetic information all suggest a discontinuity in Mono Lake nitrification activity and nitrifier populations between 1995 and 2003-2003. Contrasting hydrological mixing regimes resulting in differential NH_3 availability in the mixolimnion may have contributed to the observed pattern. The 1995 study was conducted at the end of an extended period of monomixis during which the annual fall turnover redistributes nutrients (NH_3) that have been compartmentalized by seasonal stratification. Ammonium concentrations in Mono Lake generally increase in late November as nutrient-rich bottom water is entrained into the upper

water column. Elevated NH_3 concentrations (20-40 μM) decline slowly throughout the winter (due mostly to the efflux of volatile NH_3) until the spring algal bloom in March (Melack and Jellison, 1998). Ammonia concentrations during 1995 decreased to 1.3 μM during the March algal bloom but then increased (likely due to *Artemia* excretion) through June and July to a peak concentration of 5.5 μM. As a result of this annual cycle, mixolimion NH_3 concentrations during monomictic periods (and in particular 1995) were > 3 μM for up to 60 % of the year (Melack and Jellison, 1998).

The transition to a meromictic regime in 1996 changed the nitrogen dynamics in the mixolimnion considerably. Ammonium that accumulated in the monimolimnion remained confined there due to a strong pycnocline. Mixolimion NH_3 concentrations were limited by diffusion across the pycnoline, which drastically reduced NH_3 availability to the upper water column. Ammonium concentrations became chronically low and by 1997 mixolimnetic concentrations were < 1μM throughout the year (Melack and Jellison, 1998). Nitrifier populations became undetectable in the first few years (1997-1998) following 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 mixolimion NH_3 availability. Ammonia-oxidizing bacteria are notoriously slow growing (Prosser, 1989) and recovery from environmental perturbations may proceed slowly. The observed shift in nitrifier phylogeny from 1995 to 2002-2003 may reflect the development of a nitrifier population more adapted to meromictic conditions and the associated decrease in NH_3 availability.

Mono Lake Archaea

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 evidence demonstrating the ubiquity of AOA in the environment (Francis et al., 2005; Leininger et al., 2006; Park et al., 2006). In fact, no archaeal sequences were obtained using the general archaeal 21F / 1492R primer set despite the fact that Mono Lake has an abundant archaeal population, as demonstrated by high rates of methanogenesis and anaerobic methane oxidation, and whole cells in water samples hybridizing to archaeal specific FISH probes (Joye et al., in preparation). Furthermore, archaeal-specific biomarkers have been documented in Mono Lake (Turic et al. submitted). There are 4 likely explanations for the lack of successful amplification. First, the extraction method employed was not rigorous enough to liberate Mono Lake archaeal DNA. Second, the archaeal component of the DNA extract was contaminated or degraded. Third, Mono Lake general archaeal and archaeal *amo*A sequences are too divergent for current primer sets to bracket. Fourth, there were no archaeal ammonia oxidizers present in Mono Lake.

Several different standard extraction methods were evaluated including the phenol/chloroform method (Murray et al., 1998), bead-mill homogenization (method adapted from the MOBIO Ultraclean Soil DNA kit protocol), and combinations of these methods that also included freeze / thaw cycles. None of the different methods or combinations seemed to be more effective, and archaeal sequences are routinely retrieved from other environments using these extraction techniques. The Mono Lake DNA extract from these samples was aliquoted and stored at -80°C. It was used successfully as template for several different PCR applications before and after attempts at archaeal *amoA* amplification and so is considered of good quality. There is also almost no doubt that Mono Lake does support an Archaeal population based on

measurements of processes known to be mediated by archaea and whole cell hybridizations of several different archaeal specific FISH probes. Yet, to date, no evidence of archaeal DNA recovered from Mono Lake water column samples has been reported. It is certainly possible that there are no AOA in Mono Lake; however, FISH analysis described below indicated the presence of Crenarchaeota, the phylum that encompasses known archaeal ammonia oxidizers. Crenarchaeotes were present in all samples examined and their abundance correlated with peak nitrifying activity suggesting some AOA may occur in Mono Lake and therefore archaeal *amo*A gene sequences should be present. It follows from this reasoning that sequence divergence is the most likely candidate for the inability to retrieve archaeal 16S or functional gene (*amo*A) sequences from Mono Lake and that further investigation and methods development is needed to address the distribution of archaea in Mono Lake.

Potential Importance of Archaeal vs. Bacterial Ammonia Oxidizers

The relationship between AOB distribution and nitrification rates demonstrated a negative correlation between AOB abundance and nitrification activity. Absolute AOB cell numbers between the two August samplings (2002 and 2003) were not significantly different from each other but did differ significantly from the November and May cell numbers (ANOVA, $p < 0.01$). However, AOB abundance did not vary significantly (ANOVA, $p > 0.05$) over depth on specific dates (Fig. 4.4). Ammonium, O_2, and DOC concentrations are key factors that may control AOB distribution (Kowalchuk et al., 1998; Bothe et al., 2000; Okabe et al., 2003). AOB in this study were exposed to a range of NH_3 and O_2 concentrations over time and depth (Fig. 3.4) and their distributions did not correlate with either NH_3 or O_2 concentrations (regression $p > 0.05$). DOC concentrations may indirectly affect AOB abundance and distribution. Previous

studies have shown that heterotrophic bacteria have a higher affinity for O_2 and may out-compete AOB under conditions of high DOC and low O_2 concentrations (Macfarlane and Herbert, 1984). DOC concentrations (10-80 mg l^{-1}) in Mono Lake are consistently high (Domagalski et al., 1989; Hollibaugh et al., 2001; Humayoun et al., 2003; Giri et al., 2004), including through the oxycline where O_2 falls below detection limits, yet AOB abundance remains constant over depth for every sample period.

Regression analysis was used to evaluate the correlation between temperature (T) and AOB abundance. However, analysis based on T vs. AOB cell numbers did not suggest any pattern (regression $p > 0.05$ for each sampling and the sum of all data points). However, when cell numbers were plotted against season, a linear regression yielded an R^2 value of 0.992 ($p < 0.05$) indicating that AOB cell numbers followed a seasonal progression with the highest abundance occurring during August (Fig. 3.4). However, this seasonality is inversely correlated with the seasonal pattern of integrated nitrification activity, driving a negative correlation between AOB abundance and nitrification activity.

FISH analysis using probe Cren537 indicated that Crenarchaeotes were present in all samples and their abundance varied over space and time (Fig. 3.4). Absolute Crenarchaeota cell numbers exhibited a strong correlation with NTR rate measurements (regression $p < 0.05$ for Aug, 2002 and May 2003 and $p < 0.01$ for the sum of all data points excluding Nov. 2002; Table 3.1). Similarly, an enrichment culture of Crenarchaeota from North Sea water demonstrated that Crenarchaeota abundance, but not that of AOB, correlated with NTR (Wuchter et al. 2006). A similar time series study also revealed that the abundance of the gene encoding for the archaeal amoA was strongly correlated with a decline in ammonium concentrations and with the abundance of Crenarchaeota (Wuchter et al., 2006). The North Sea data provides robust

presumptive evidence that Crenarchaeota provide a good proxy for AOA. Although an AOA contribution to nitrification in Mono Lake cannot be ruled out, the correlative nature of the data and the lack of archaeal *amo*A amplification make it impossible to assess the relative contribution to nitrification of archaeal and bacterial ammonia oxidation in Mono Lake.

SUMMARY

Geochemical profiles and ^{15}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, evidence from a study conducted during1997 and 1998 suggests a discontinuity in Mono Lake nitrification activity and nitrifier populations occurred between 1995 and 2003-2003. Contrasting hydrological mixing regimes resulting in differential NH_3 availability in the mixolimnion may have contributed to the observed shift in AOB phylogeny. FISH data revealed that AOB cell numbers followed a seasonal progression inversely correlated with the seasonality observed in integrated nitrification rates suggesting a negative correlation between AOB abundance and nitrification activity. Crenarchaeota cell numbers exhibited a strong correlation with NTR rate measurements but in itself did not provide enough evidence to attribute any quantifiable nitrification activity to AOA.

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Table 3.1. Regression R^2 values and statistical significance of NH_3 oxidation rates versus AOB abundance, Crenarchaeota abundance, and NH_4^+, NO_3^-, and N_2O concentrations. Values are presented for individual sampling dates as well as the sum of all data points. AOB cell specific nitrification rates are also listed.

Sample	Depth (m)	Rate (nM NH_3 d^{-1})	AOB (10^4 ml^{-1})	Cren (10^5 ml^{-1})	fmol NH_3 h^{-1} AOB^{-1}	NH_4^+ (μM)	NO_3^- (nM)	N_2O (nM)
Aug. 2002	3	26.6				1.1	88.8	10.9
	5	62.7	5.8	3.6	0.05	1.3	208.8	17.7
	10	86.8	5.8	5.8	0.06	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	5.7	8.2	0.34	5.9	482.7	25.5
	13.5	219.3				7.9	302.2	19.6
	14	140.9	5.6	3.6	0.10	9.5	237.2	17.5
	15	76.0				14.6	175.7	14.9
* P < 0.05	16	30.3	5.8	3.0	0.02	20.6	82.8	9.1
R^2			0.25	0.76 *		0.02	0.49 *	0.28
Nov. 2002	3	228.9	3.2	4.4	0.30	10.9	204.0	25.2
	8	336.6	3.0	4.0	0.46	8.2	300.0	32.8
	13	370.6	3.2	6.2	4.84	4.5	350.0	41.3
	15	357.2	3.2	2.8	0.47	4.8	340.0	32.7
	18	322.8	3.3	2.5	0.41	5.4	210.0	25.5
* P < 0.05	20	55.5				9.8	180.0	19.0
R^2			0.01	0.0		0.55	0.61	0.69 *
May-03	3	20.8				1.1	66.0	15.8
	5	54.8	4.6	5.1	0.05	1.1	174.2	22.8
	10	90.9	4.4	6.5	0.09	1.2	356.5	33.0
	12	204.0				1.6	431.2	34.8
	13	320.7	4.6	8.5	0.29	2.1	439.1	27.0
	14	417.3	4.7	9.4	0.37	4.2	257.0	17.8
	15	205.6				10.5	146.0	9.3
* P < 0.05	16	29.6	4.6	6.5	0.03	16.1	94.2	7.2
R^2			0.48	0.89 *		0.02	0.29	0.03
Aug. 2003	3	4.57				1.0	59.2	20.3
	5	3.95	5.8	5.6	0.00	0.8	57.0	20.7
	10	14.71	5.8	6.1	0.01	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	6.2	8.2	0.05	1.2	58.6	31.7
	13.5	279.57				1.2	164.0	36.4
	14	479.98	6.1	9.4	0.33	2.2	441.4	39.7
** P < 0.01	15	356.92				6.8	406.9	34.8
* P < 0.05	16	62.83	6.2	8.1	0.04	24.0	190.9	19.2
R^2			0.13	0.6		0	0.86 **	0.80 **
Total R^2			0.18	0.06		0.004	0.48 **	0.32 **
		w/o Nov.		0.45 **				

FIGURE LEGENDS

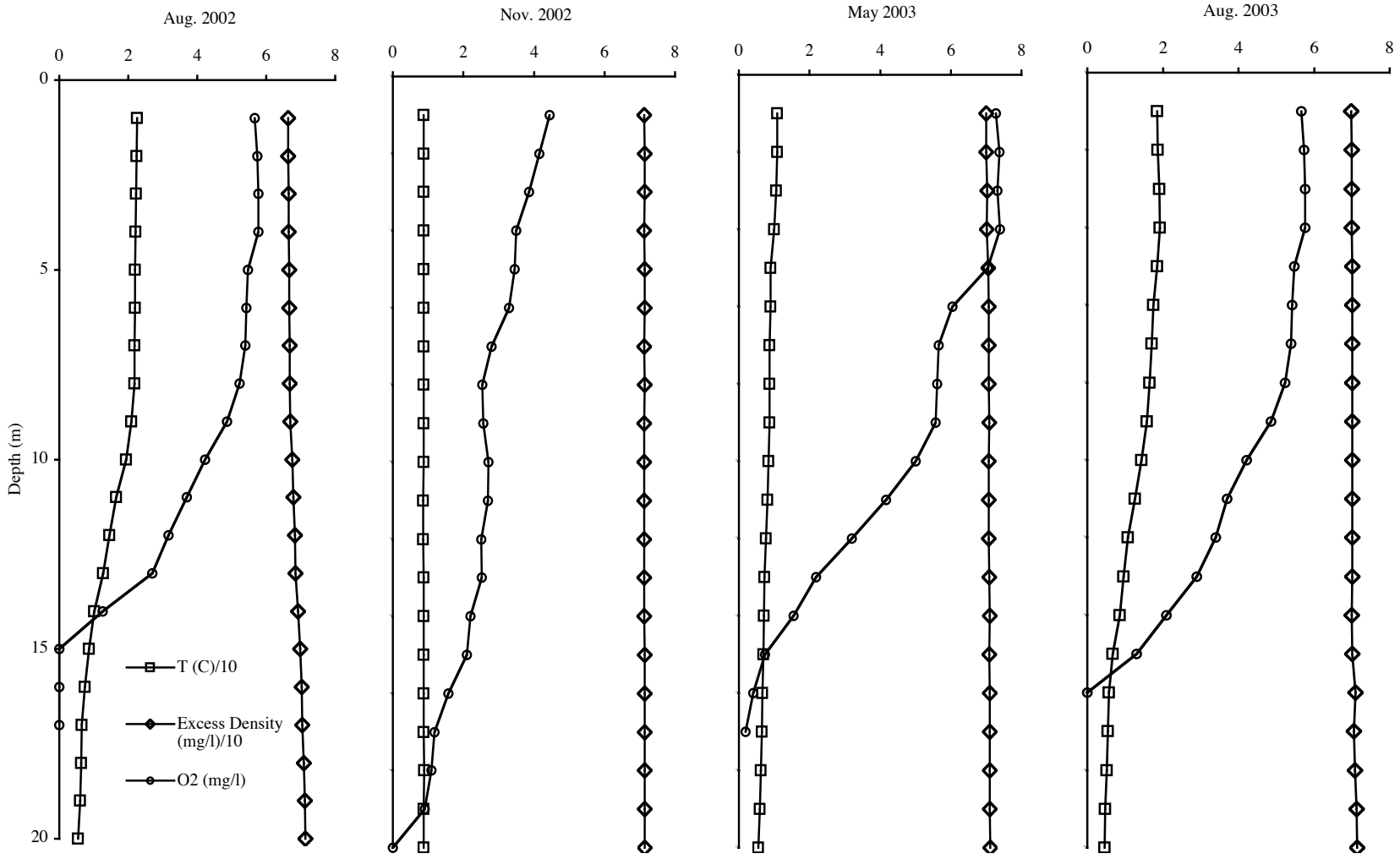
Figure 3.1. Vertical depth profiles of temperature (-□-), oxygen (-O-), and excess density (-◇-) in Mono Lake from August 2002 (a), November 2002 (b), May 2003 (c), and August 2003 (d). Temperature and excess density values are scaled down by a factor of 10.

Figure 3.2. Vertical depth profiles of nitrification rate (NTR) and biogeochemical parameters in Mono Lake from August 2002, November 2002, May 2003, and August 2003: Nitrification Rates (a), Ammonium concentration (b), Nitrate +nitrite, (c), and nitrous oxide (d).

Figure 3.3. 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 5% sequence divergence.

Figure 3.4. Vertical profiles of ammonia-oxidizing bacterial, Crenarchaeota abundance, and corresponding nitrification rates. Error bars are standard deviations around the mean. (a) August 2002, (b) November 2002, (c) May 2003, (d) August 2003.

Figure 3.1.



a. b. c. d.

Figure 3.2.

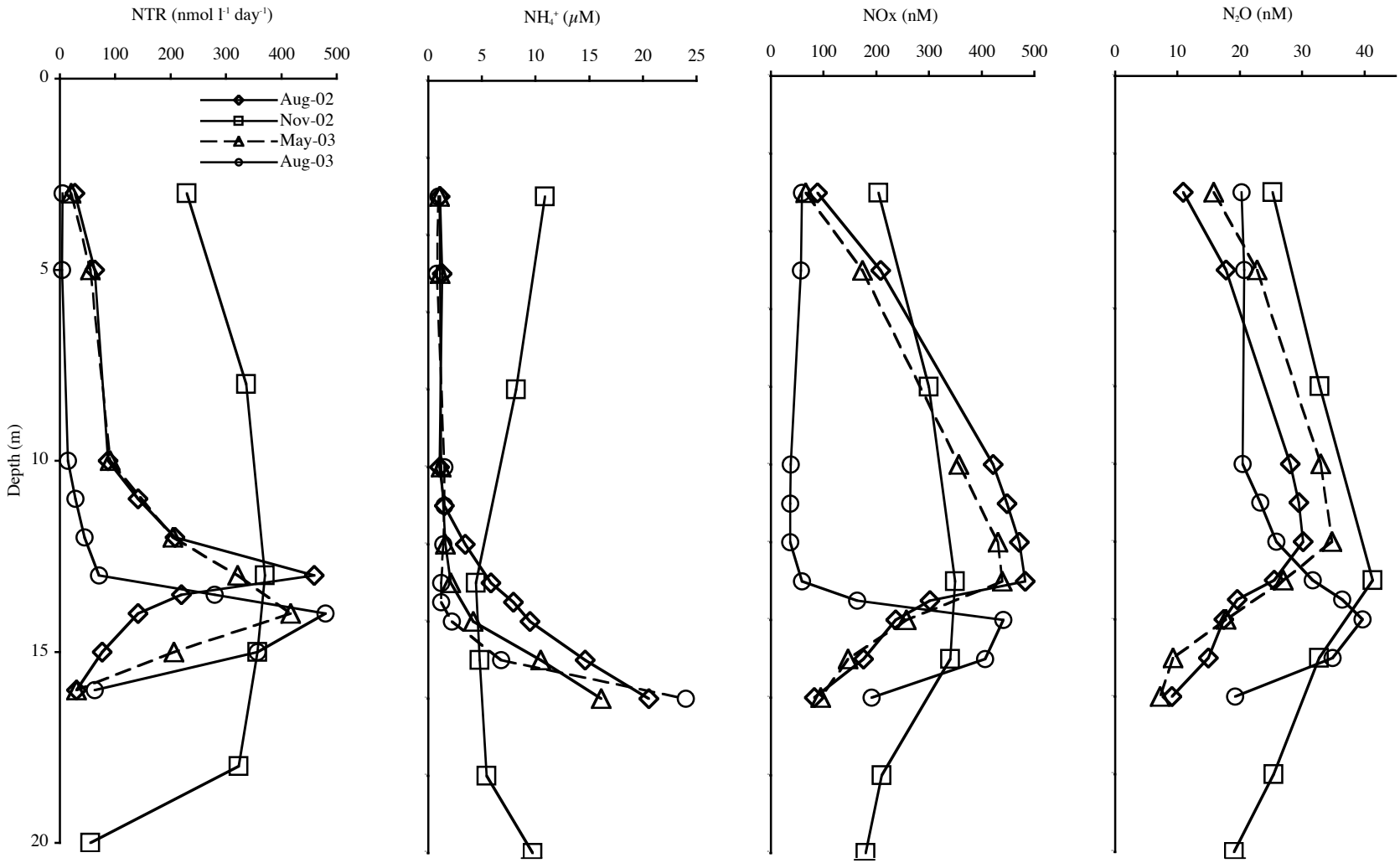

NTR (nmol l-1 day-1)
NH4+ (μM)
NOx (nM)
N2O (nM)
Depth (m)
Aug-02
Nov-02
May-03
Aug-03
a.
b.
c.
d.

Figure 3.3.

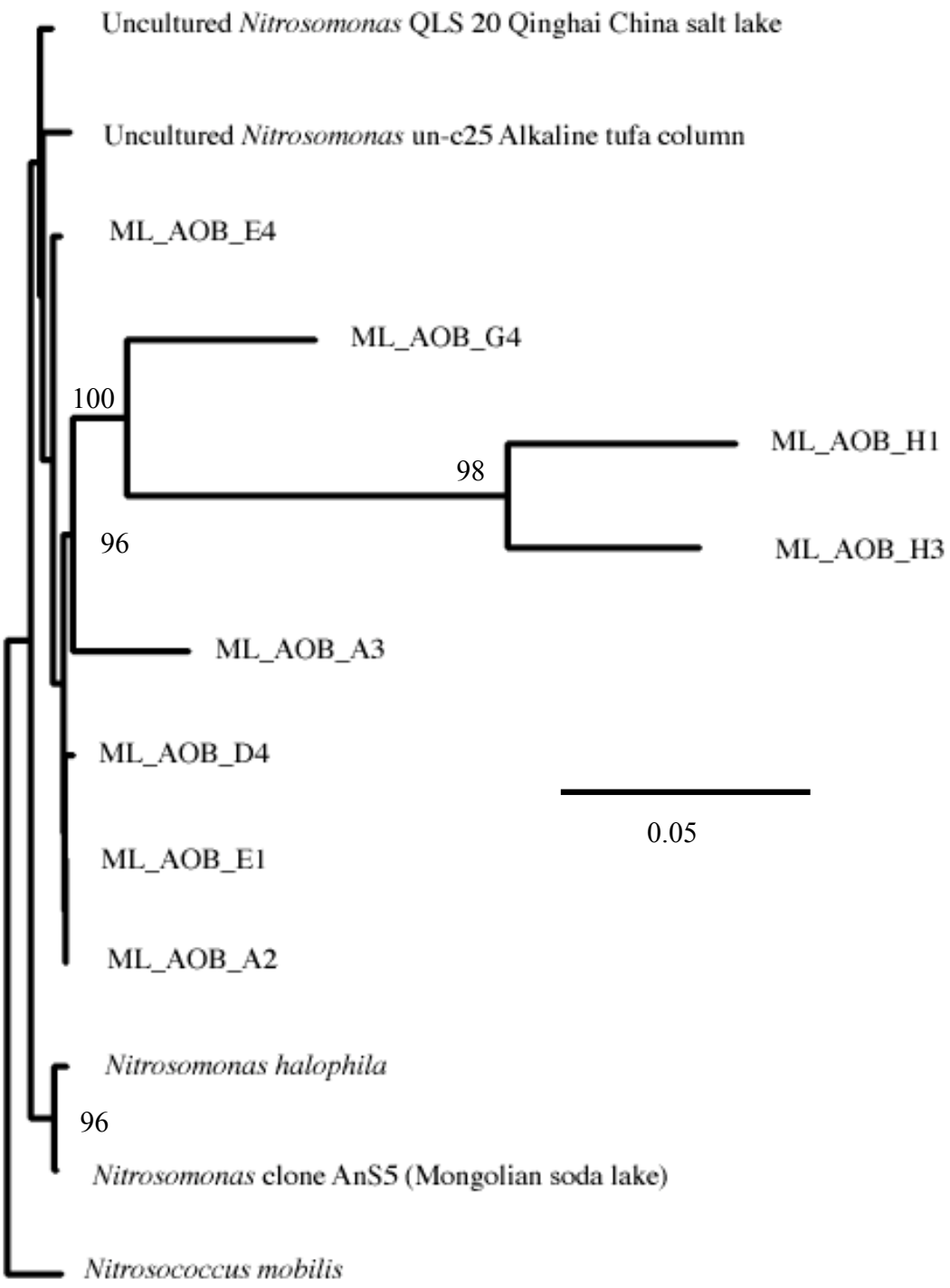
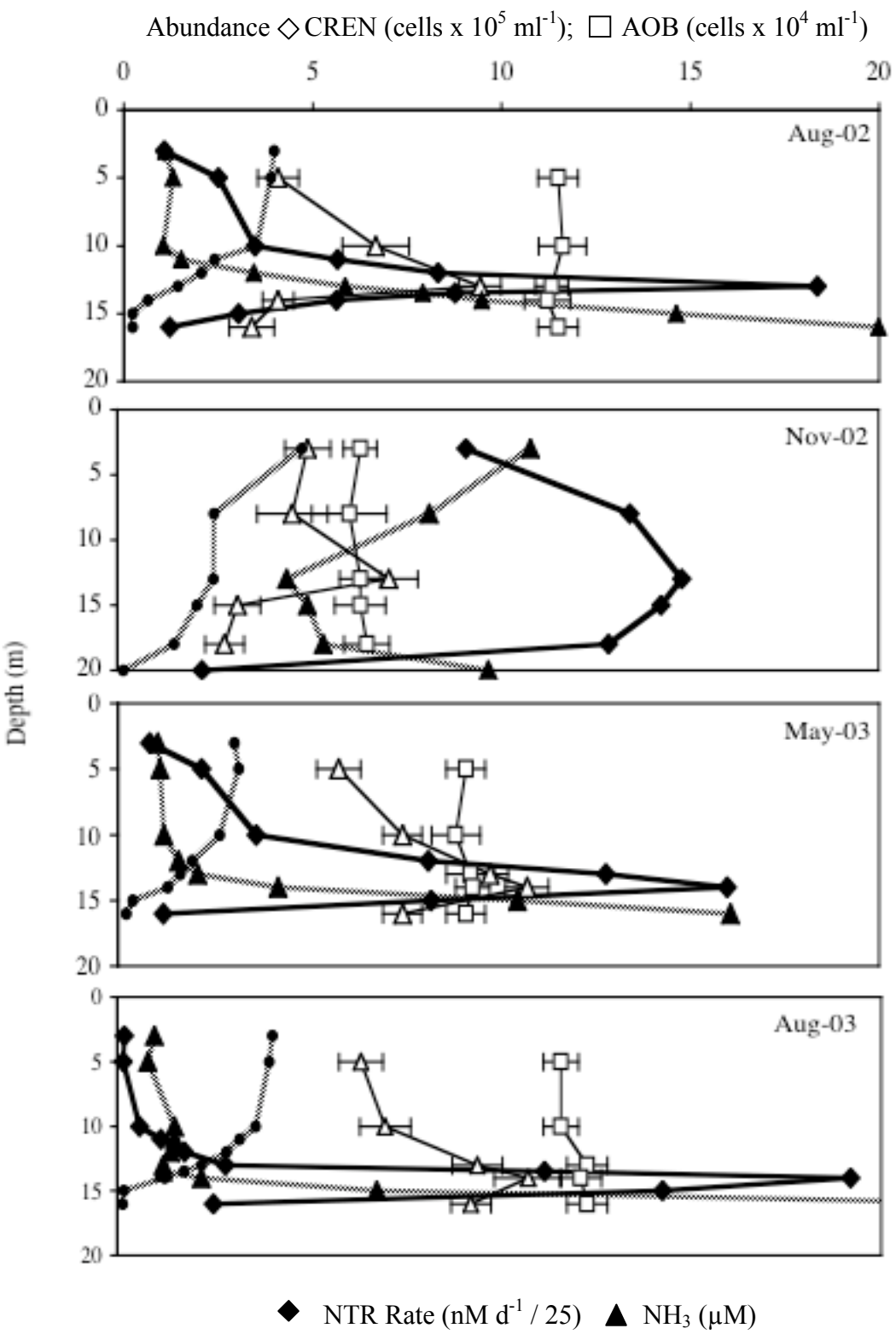

Uncultured *Nitrosomonas* QLS 20 Qinghai China salt lake
Uncultured *Nitrosomonas* un-c25 Alkaline tufa column
ML_AOB_E4
ML_AOB_G4
100
ML_AOB_H1
98
96
ML_AOB_H3
ML_AOB_A3
ML_AOB_D4
0.05
ML_AOB_E1
ML_AOB_A2
Nitrosomonas halophila
96
Nitrosomonas clone AnS5 (Mongolian soda lake)
Nitrosococcus mobilis

Figure 3.4.


Abundance ◇ CREN (cells x 10^5 ml^{-1}); □ AOB (cells x 10^4 ml^{-1})
0
5
10
15
20
Aug-02
Nov-02
May-03
Aug-03
Depth (m)
◆ NTR Rate (nM d^{-1} / 25) ▲ NH_3 (μM)

CHAPTER 4

INTRACTIONS BETWEEN METHANE OXIDATION AND NITRIFICATION ACTIVITY AND COMMUNITY COMPOSITION IN MONO LAKE[1]

[1] Carini, S.A., L. Jorrel, M. Bowles, and S.B. Joye**,** Prepared for submission to *Applied and Environmental Microbiology*

ABSTRACT

We examined the effects of relative substrate availability on methane oxidation, nitrification, and associated microbial community dynamics using aerobic enrichment cultures. Methane and ammonia oxidation activity and associated geochemistry were compared between an un-amended control and cultures that were amended with methane (CH_4), ammonium (NH_4^+), or methane and ammonium (CH_4+NH_4^+) to determine how group-specific diversity and abundance were affected by enrichment conditions and whether the differential responses of group-specific populations may have correlated with measured oxidation rates. Microbial community composition and relative group-specific abundance in each enrichment was monitored using denaturing gradient gel electrophoresis (DGGE), sequence analysis, and fluorescence *in situ* hybridization (FISH). Methane and NH_4^+ oxidation rates increased over time in enrichments amended with CH_4 and NH_4^+, respectively. The highest CH_4 oxidation rates occurred in the NH_4^++CH_4 enrichment, inferring that methanotrophs benefited from nitrogen (N) addition. However, FISH and DGGE analysis demonstrated that the methanotroph population in the CH_4-only enrichment was able to take advantage of elevated CH_4 absent supplemental NH_4^+ through increased overall methanotroph abundance and a shift in dominance of specific type II methanotroph species. Ammonia oxidation occurred in both NH_4^+-only and CH_4 + NH_4^+ enrichments. Crenarcheota abundance correlated with NO_X accumulation and nitrification activity in the NH_4^+-only enrichment while AOB abundance decreased, suggesting that ammonia-oxidizing archaea (AOA) contributed to nitrification under N-replete conditions. In contrast, there was a decrease in crenarchaeal abundance and an increase in AOB abundance in the CH_4+NH_4^+ enrichment that correlated with increased nitrification

activity. The reversal in the correlation between AOB and nitrification relative to that observed in the NH_4^+-only enrichment plus the significant decline in crenarchaeal abundance suggests that AOB were likely more active nitrifiers in the CH_4+NH_4^+ enrichment. We suspect that methanotrophs may have out-competed Crenarcheotes for NH_4^+ in the CH_4+NH_4^+ enrichment.

INTRODUCTION

Methane- and ammonia-oxidation occur extensively in terrestrial and aquatic habitats and play key roles in global carbon (C), nitrogen (N), and oxygen (O) cycles (Cicerone and Oremland, 1988). The biochemistry, ecology, and molecular biology of methane- and ammonia-oxidizing bacteria (hereafter referred to as MOB and AOB, respectively) have been documented previously (Hanson and Hanson, 1996; Kowalchuk and Stephen, 2001). Biological CH_4 oxidation by methanotrophs is the predominant sink mitigating the flux of CH_4, a radiative trace gas, to the atmosphere (Topp and Hanson, 1991). Ammonia oxidation to nitrite (NO_2^-) is the first step in the process of nitrification, which ultimately generates nitrate (NO_3^-) via the coupled action of nitrite-oxidizing bacteria (Prosser, 1989). Nitrification provides critical links to other nitrogen cycling processes (e.g. denitrification and anammox) that regulate concentrations and bioavailability of N in the environment.

Ammonia-oxidizing bacteria were long considered the predominant prokaryotic contributors to aerobic ammonia oxidation (Bock and Wagner, 2001). However, a marine group I Crenarchaeota (SCM1), possessing a unique archaeal ammonia monooxygenase subunit (*amo*A) and the capacity for growth with ammonium chloride and bicarbonate as the sole energy and carbon sources, has demonstrated the existence of ammonia-oxidizing archaea (AOA; Könneke

et al., 2005). More over, correlations between the abundance of *amo*A, Crenarchaeota, and ammonia oxidation in the North Sea suggests that AOA may play a significant role in N cycling in the environment (Wütcher et al., 2006). Although a direct link between *amo*A, Crenarchaeota, and nitrification in the environment remains to be shown (Nicole and Schleper, 2006), the widespread recovery of archaeal *amo*A sequences from diverse aquatic and terrestrial environments (Francis et al., 2005; Lenninger et al., 2006) highlights the need to document their role in the nitrogen cycle. What about the Lam et al paper that I sent you? That is pretty convincing.

Physiological, biochemical, and ecological similarities between aerobic methane and ammonia oxidizers provide opportunities for interactions that may significantly affect process rates and group-specific population dynamics. Both MOB and AOB are obligate aerobes that mediate contributory or predominant processes that link the reduced and oxidative phases of the C and N cycles, respectively (Hanson and Hanson, 1996; Prosser, 1989). Methane-oxidizing bacteria use methane as their sole source of structural carbon as well as energy. Methanotrophs were initially grouped into two families characterized by their internal membrane arrangements and specific C assimilation pathways. Phylogenetic relationships, derived mostly from 16S rRNA analysis, have generally validated the original phenotypic and physiochemical taxonomy and type I and type II methanotrophs are now recognized as distinct assemblages in the γ- and α-Proteobacteria, respectively (Hanson and Hanson, 1996). Ammonia-oxidizing bacteria fix CO_2 using energy derived from the conversion of ammonia to nitrite and are classified as members of the β- or γ-Proteobacteria (Prosser, 1989; Head et al., 1993). Although AOA are taxonomically divergent from MOB and AOB, they demonstrate a predominantly chemoautotrophic

metabolism deriving energy from the oxidation of reduced N compounds to fix inorganic C (Wuchter et al., 2003; Könneke et al., 2005; Ingalls et al., 2006).

Substantial sequence identity exists between the genes encoding the mono-oxygenase systems of methane (*pmmo*) and ammonia (*amo*) oxidizers. Predicted amino acid sequences of these genes revealed substantial conservation within and between sub-classes of the Proteobacteria and suggest that bacterial *pmmo* and *amo* are evolutionarily related (Holmes et al. 1995). Although there is no significant nucleotide homology between AOB and AOA *amo*A sequences, 25% sequence identity and 40% sequence similarity at the protein level suggests that AOB and AOA *amo* enzymes may share an evolutionary history with respect to amino acid residues that influence particular enzymatic metal co-factors (Nicol and Schleper, 2006).

As a result of physiological and biochemical similarities, MOB, AOB, and AOA co-occur where both oxygen and reduced substrates (CH_4 and NH_3) are available, virtually ensuring interaction through competition for substrates. Although many studies have focused on the metabolism of CH_4 or NH_3 oxidation or the effect of NH_3 concentrations on CH_4 oxidation and vice versa, few have focused on the interaction between these processes (Megraw and Knowles, 1987; Roy and Knowles, 1994; Carini et al., 2003) and none have incorporated AOA in the analyses.

Laboratory enrichment experiments provide a means to elucidate interactions between methane and ammonia oxidation and the organisms responsible. We used samples from the Mono Lake water column for the enrichment cultures described here. Mono Lake is an alkaline salt lake located just east of the Sierra Nevada range in northern California (38°N, 119°W). At the time of water collection (see methods), conditions in Mono Lake were hydrologically and biogeochemically conducive for methane and ammonia oxidation activity and microbial

interactions. The lake was meromictic and the monimolimnion (bottom water) had been isolated below the chemocline (i.e. salinity gradient or pycnocline) since 1995. This isolation resulted in persistent anoxia and accumulation of high concentrations of dissolved CH_4 (50-100 μM) and NH_3 (60-113 μM) in the monimolimnion.

Seasonal stratification resulted in a mixolimnion that penetrated to the thermocline at approximately 12 m. The oxycline created a microaerophilic zone that encompassed an area between 13 and 15 m at which point the O_2 concentration dropped below detection limits. Methane and NH_3 concentrations in the upper water column were similar (~ 1 μM) to concentrations measured earlier in the year. However, concentrations in the seasonally anoxic zone (between the pycnocline and the bottom of the oxycline) and microaerophilic zone increased, suggesting greater flux of these reduced species from the monimolimnion. Mono Lake also has a very high dissolved inorganic carbon (DIC) concentration (~ 400 mM) ensuring AOB and AOA would not be C limited during the enrichment. In addition, *in situ* methane and ammonia oxidation activity and microbial population data are available (Joye et al., 1999; Ward et al., 2000; Carini et al., 2005 – Chapter 2; Carini and Joye, in prep. – Chapter 3) to provide context for relating aspects of the enrichment study results to process activity and population interactions in the environment.

Molecular techniques provide phylogentic and population information that facilitates the elucidation of group-specific responses to the varying experimental conditions. Denaturing Gradient Gel Electrophoresis (DGGE) has been used to compare different community "fingerprints" (Muyzer et al. 1997; Murrell et al. 1998) as well as to obtain specific phylogenetic information by identifying specific clones of interest for subsequent sequencing (Carini et al., 2005 – Chapter 2). Overall community analysis via DGGE traditionally employs PCR primers

that target the variable 3 region of the 16S rDNA (Muyzer et al. 1993). Group-specific diversity has been investigated by nesting the variable 3 primer set within template from primer pairs designed to target group-specific regions of 16S rDNA such as nitAB for AOB (Voytek and Ward, 1995; Bano and Hollibaugh, 2000) and MethT1F/R and MethT2F/R for MOB (Wise et al., 1999). Diversity of AOA has been investigated by phylogentic alignment of sequences of the functional archaeal ammonia monooxygenase gene (*amo*A) detected using the primer sets Cren-amo1F/Cren-amo1R (Konneke et al., 2005) and Arch-amoAF/Arch-amoAR (Francis et al, 2005).

Quantitative information complimentary to the DGGE and sequence data has been obtained using fluorescently labeled group-specific probes (FISH) to enumerate individual MOB (Bourne et al., 2000), AOB (Mobarry et al., 1996), and Crenachaeota. FISH probes specific for AOA have not as yet been developed. However, correlations between Crenarchaeota , archaeal *amo*A gene abundance, and ammonia oxidation rates have led others to use FISH probe Cren 537 (Herndl et al., 2005) as a general proxy for AOA abundance (Könneke et al., 2005; Wuchter et al., 2006).

We used ammonium and/or methane enrichments to examine the impact of relative substrate availability on rates of ammonia and methane oxidation and on patterns of associated group-specific microbial community composition. Molecular techniques enabled us to examine whether group-specific diversity and abundance was affected by enrichment conditions and how the differential responses of group-specific populations may have correlated with measured oxidation rates.

METHODS

Study Site and Enrichment Culture Preparation

Water for the enrichment cultures was collected at station 6 in Mono Lake's central basin (37°57.822'N, 119°01.305'W) on August 8, 2003. When the samples were collected Mono Lake was meromictic and the monimolimnion (bottom water) had been isolated below the pycnocline since 1995. The lake was also seasonally stratified with an oxycline and thermocline spanning 14 to 16 m. The surface water was 19.2°C with an O_2 concentration of 5.52 mg l^{-1} and an excess density of 70.1 mg l^{-1}. Ammonia and CH_4 concentrations were <1 μM above the oxycline but increased through the base of the oxycline to 17.2 and 5.5 μM respectively (Carini and Joye, in prep - Chapter 3). Six depths ranging from the surface to the bottom of the oxycline were chosen to contribute to a homogenized water-mix that was used to prepare the different enrichment treatments. The water was collected with a Niskin sampler at approximately 3 m intervals between 2 to 17 meters. Water samples from each depth were transferred via a funnel, equipped with a screen to exclude *Artemia*, into acid cleaned, sample rinsed 1 liter PETG media bottles (n=3 per depth) and stored at 4°C for shipment to the UGA lab where the experiment was run.

The individual samples were combined in a clean (acid soaked, milliQ water rinsed and then air-dried) 20 L carboy and mixed well to create a homogenized blend that integrated chemical and biological elements representative of the upper water column above the base of the oxycline. Sub-samples were collected from the homogenized water to determine initial potential methane and ammonia oxidation rates, nutrient and dissolved gas concentrations and community structure (sampling and analyses described below). One liter of the mixture was then placed into each of twelve PETG media bottles (1.3 L volume) and sealed with gas-tight septa screw caps

(Nalgene®). The samples were amended to create four enrichment regimes (n=3 each). One set was used as a control and received no amendment. A second set was amended with CH_4 (80 μmol l^{-1} dissolved CH_4), a third set was amended with NH_4^+ (80 μmol l^{-1} using NH_4Cl), and a fourth set was amended with both CH_4 and NH_4^+ (80 μmol l^{-1} each). All treatments were incubated in the dark and maintained at 20°C. The enrichments were kept aerated (O_2 ~ 4 mg l^{-1}) by daily venting of the headspace followed by resealing and gentle mixing. Amendments of CH_4 (daily) and NH_4^+ (every second day until day 22, then daily) were made following aeration to approximate a steady substrate flux (changes in headspace and enrichment culture volume were accounted for when re-amending). Weekly time series sub-samples from each treatment were analyzed for potential methane and ammonia oxidation rates and dissolved inorganic nitrogen (DIN) concentrations (e.g. NH_4^+, NO_2^-, and NO_3^-) for a total of 35 days. Concurrent samples were taken from each enrichment for molecular analyses to determine microbial community compositions.

Methane and Ammonia Oxidation Rates

Potential methane oxidation rates were determined by measuring the consumption of CH_4 over time. A three ml sub-sample of enrichment culture from each bottle was transferred to an 8 ml headspace vial. The vial was closed with a butyl rubber stopper and aluminum crimp seal. A 10% CH_4 standard was injected into the headspace through the stopper using a gas tight syringe to achieve 300 μM headspace CH_4. An initial, time = 0 headspace CH_4 concentration was determined after a 12-hour equilibration period (to account for solubility induced concentration changes in the headspace) and a second measurement was made 24 hrs later. Bottles were shaken gently at 75 rpm and maintained at 20°C during the incubation. Methane concentrations were

determined using a Shimadzu 14-A gas chromatograph equipped with a Porpak® T column and a flame ionization detector (Joye et al. 1999).

Potential ammonia oxidation rates were measured by quantifying the production of nitrate (NO_3^-) + nitrite (NO_2^-) (hereafter $NO_X = NO_3^- + NO_2^-$) following a 24 hour incubation (Joye and Hollibaugh, 1995). A three ml sub-sample of water from each enrichment bottle was transferred to individual 10 mL serum vials and amended to 300 μM NH_4^+. The serum vials were kept in the dark, shaken gently at 75 rpm, and maintained at 20°C during the incubation. Ammonia oxidation rates were calculated as the difference between final and initial NO_X concentrations (quantification described below).

Dissolved Inorganic Nitrogen (DIN)

Nutrient samples from each enrichment bottle were filtered (Millipore Acrodisc, 0.2 μm) and stored at 4°C. Ammonium concentrations were quantified within 4 hrs using the phenol-hypochlorite method (Solarzano, 1969). To account for matrix effects, standards were prepared using He purged Mono Lake surface water and internal standards were run. Ammonia concentrations in samples were calculated from linear regressions of known standard concentrations. Nitrite and NO_3^- concentrations were quantified within 48 hrs. The concentration of NO_X was determined using an Antek®745 Nitrate/ Nitrite Reducer (vanadium reduction assembly) inline with an Antek ®7050 chemiluminescent nitric oxide detector (Álvarez-Salgado & Miller 1998). Reduction efficiency checks of the vanadium solution were run after every 10 samples. Nitrite concentrations were determined by spectrophotometry (Bendschneider and Robinson, 1952) modified for Mono Lake water chemistry (samples and standards were adjusted to ~pH 7 with concentrated HCl before reagent addition). Nitrate

concentrations were calculated as the difference between NO_X and NO_2^-. Standards for these analyses were made using artificial Mono Lake Water that reproduced *in situ* ionic strength and pH conditions (Oremland et al., 2002) to account for matrix effects.

Molecular Analyses

Samples for molecular analyses were collected at four time points during the experiment. Following an initial time point (t_0), sub-samples were collected on days 15, 22, and 35. Each of those days corresponded with a potential rate assay and geochemical sampling. Microbial biomass was collected for community profiles (DGGE), sequence analysis and phylogeny, and whole cells were fixed for enumeration using fluorescence *in situ* hybridization (FISH).

DNA Collection and Extraction

Sub-samples (150 ml) from each treatment replicate were pooled (total volume = 450 ml) and the microbial biomass from each treatment was collected with individual Sterivex filter cartridges (0.2 µm). Excess water in the filter cartridge was replaced with an extraction buffer and stored at -70°C until processed (Ferrari and Hollibaugh, 1999).

DNA was extracted from Sterivex filters using a modified technique of Murray et al. (1996) and Ferrari and Hollibaugh (1999). Filter cartridges containing extraction buffer (1.5 M NaCl, 5 mM $MgCl_2$, 100 mM Tris HCl - pH 7, 100 mM EDTA - pH 8, 100 mM Na_2HPO_4 - pH 8) were thawed at room temperature. Forty µl of lysozyme (50 mg/ml) was added and the cartridges were incubated at 37°C for 30 min on an orbital shaker (25 rpm). Lysate was expelled from the cartridge and then the cartridge was rinsed with extraction buffer to ensure maximum yield. DNA was further liberated from the lysate using bead-mill homogenization (method

adapted from the MO BIO® Ultraclean Soil DNA kit protocol; Carini and Joye, in prep – Chapter 3). The DNA obtained was suitable for the PCR and no subsequent purification was necessary.

PCR Amplification of Microbial Community DNA

Previously designed oligonucleotide primers were used to characterize group specific community composition over time. Methane-oxidizing bacteria were detected using MethT1dF/MethT1bR and F27/MethT2b primers that target type I and type II methane oxidizer specific regions of 16S rDNA respectively (Wise et al., 1999). Bacterial ammonia-oxidizers were identified using a nested approach: EUB1/EUB2 (Liesack et al. 1991) was used to amplify total bacterial rDNA and the product was subjected to a second amplification using Nit-A/Nit-B (NitAB; Voytek and Ward 1995). NitAB is internal to the EUB primers, targets a region of 16S rDNA specific for ammonia oxidizing β-Proteobacteria, and this nested approach has been shown to be more sensitive when screening for less abundant microorganisms (Voytek and Ward, 1995). Archaeal ammonia oxidizer sequences were detected using the primer sets Cren-amo1F/Cren-amo1R (Konneke et al., 2005) and Arch-amoAF/Arch-amoAR (Francis et al., 2005) that target a putative archaeal ammonia monooxygenase (*amo*A) gene. The primer pair 341f and 534r (*E.coli* position) was used to amplify the variable 3 region of rDNA from bacterial group-specific amplicons for subsequent DGGE analysis (Muyzer et al., 1993). The DGGE primers were modified with a GC clamp and labeled with fluorescein.

All PCR reactions were prepared in a UV sterilized laminar flow hood. 4-10 ng of genomic template was added to a master-mix (adjusted to 1µl of PCR product for nested amplifications) and a "hot start" procedure was followed: after 5 min at 95° C in the thermocycler (MJ Research DNA Engine®), 2.5 units of Taq DNA polymerase was added while

the reaction tubes were held at 80° C. A "touchdown" thermal cycling program (Don et al. 1991) was used for each of the primer pairs and each PCR was run for 30 cycles. All reactions were run with appropriate positive and negative controls and the PCR product was verified for amplification and fragment size by agrose gel electrophoresis.

DGGE, Cloning, and Sequencing

DGGE was used to monitor the group specific community composition of the methanotroph and ammonia-oxidizing bacterial populations over the course of the incubation. Denaturing gels were 6.5% polyacrylamide with a denaturant gradient ranging from 40 to 70%. The denaturants in a 100% solution were 40% deionized formamide and 7 M urea. PCR products were concentrated by ethanol precipitation and resuspended. Samples (250 ng DNA) were mixed 1:1 with neutral loading dye and loaded onto the gel. Gels were run at 80 V for 15 hrs in a 60° C, 1x TAE buffer and visualized on a Hitachi FMBIO III scanner equipped for fluorescein detection.

A clone library was constructed using the original PCR product generated by the group specific primer pairs. PCR products were isolated by agarose gel electrophoresis, extracted from the agarose (QIAquick® Gel Extraction Kit; Qiagen), inserted into the pCR®4-TOPO® plasmid vector and transformed into chemically competent One Shot®TOP10 Escherichia coli cells as per the manufacturer's instructions (Invitrogen Corp). Transformed cells were cultured and a portion of each individual culture was re-extracted for PCR using DGGE primers. DGGE was then run to identify unique clones for sequencing of the original PCR product.

Bands suggestive of unique clones were cut out, re-amplified with the PCR and re-run using DGGE. Clones that produced bands which did not have the same electrophoretic mobility

as the original bands (based on multiple lanes of standard distributed across the gel) or that produced more than one DGGE band were discarded. Frozen, glycerol preserved whole-cell clone cultures (2x96 well micro-titer plates) were sequenced (primer M13F) by SeqWright (Houston, TX) and yielded robust sequence fragments of 550-800 bp. The obtained 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 (GCG) package (Madison, WI). A phylogenetic tree was constructed using the neighbor-joining method and Jukes-Cantor distances (PHYLIP package v. 3.5).

Fluorescence *in situ* Hybridization

Fluorescence *in situ* hybridization (FISH) was used to determine the abundance of both type I and type II methanotrophs, AOB and Crenarcheota. 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; Schleper et al., 2004; Konneke et al., 2005; Francis et al., 2005; Wüchter et al., 2006; Leininger et al., 2006; Hallum et al., 2006). Although it is almost certain that not all crenarchaeotes oxidize NH_3, their enumeration is used as a proxy for AOA and may provide valuable information about the potential involvement of AOA in nitrification (Wüchter et al., 2006; Carini and Joye, in prep – Chapter 3).

Formalin (4%) preserved samples were thawed and mixed well, then 500 µl 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 µm polycarbonate membrane filter (Osmonics). The tube was then rinsed with PBS (2 x 15 ml) and this wash solution 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 (2x) and allowed to air dry in a Petri dish. Sections of individual filters were separated for DAPI only staining as well as hybridization with specific probes and DAPI counterstaining (50 µl of 2 mg ml^{-1} solution for 3 min). Oligonucleotide probes Mγ84, Mγ705, and Mα450 were used to detect type I and type II methanotrophs, respectively (Eller *et al.,* 2001). Probes were synthesized by MWG Biotech (Ebersberg, Germany) and labeled with fluorochromes Oregon green (Mα450, type II) and Texas Red (Mγ84 and Mγ705, Type I). Simultaneous hybridization with these three probes on a single filter section allowed for differentiation between type I (red) and type II (green) abundance and total methanotroph cell counts (red + green). Ammonia-oxidizing bacteria were enumerated using Texas Red labeled oligonucleotide probes NEU, Nso190, and Nsm156 (MWG Biotech) designed to detect halophilic/halotolerant *Nitosomonas* spp., ammonia-oxidizing β-Proteobacteria, and *Nitosococcus* spp. respectively. Successive hybridization reactions were performed on single filter sections with hybridization solutions (formamide concentrations) optimized for each particular probe (Mobarry et al., 1996). Enumeration of Crenarchaeota was carried out using probe Cren537, which specifically targets marine group I Crenarchaeota, as described by Herndl et al. (2005).

Total microbial (DAPI stained) and hybridized methanotroph, 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 per sample filter section). The number of cells per milliliter (cells ml^{-1}) in each sample was 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 control for loss of cells from the filters that may have occurred during hybridization.

AOB Cell Specific Nitrification rates

Cell specific rates of NH_3 oxidation were calculated by dividing the moles of NH_3 oxidized per liter of sample by the total number of AOB cells per liter of sample as determined by FISH at each time point and in each treatment.

Statistical Analyses

Differences in NH_3 and CH_4 oxidation rates between treatments and over time were compared using paired t-tests assuming unequal variances (n = 3). Differences in MOB, AOB, and Crenarchaeota abundance between treatments and over time were compared using a combination of ANOVA and Tukey's analyses. Correlations between oxidation rates and associated microbial abundances were assessed by linear regression.

RESULTS

Enrichment Culture Methane Oxidation and Nitrification Activity

The potential CH_4 oxidation (pMOx) rate in the initial (t_0) homogenized Mono Lake water used to prepare enrichment cultures was 12.6 (±1.8) μmol CH_4 l^{-1} day^{-1} (hereafter noted as μM CH_4 d^{-1}). Potential CH_4 oxidation rates in the control (no enrichment) generally decreased throughout the experiment with no detectable activity on days 28 and 35. The NH_4^+-only enrichment had slightly increased pMOx activity through day 8, then declined and maintained

relatively low rates between 6.0 and 9.7 μM CH_4 d^{-1}. Treatments amended with CH_4-only and CH_4 + NH_4^+ exhibited significant ($p < 0.05$) increases in pMOX activity by day 8 (Fig. 4.1). The pMOx rates in both the CH_4-only and CH_4 + NH_4^+ enrichment increased steadily through day 35 to a maximum of 142.3 and 196.4 μM CH_4 d^{-1}, respectively. From day 15 through the end of the time course, the CH_4 + NH_4^+ enrichment demonstrated consistently higher ($p < 0.05$) pMOX rates relative to the CH_4-only treatment (Fig. 4.1).

The potential NH_3 oxidation (pNTR) rate in the initial (t_0) homogenized Mono Lake water used to prepare the enrichment cultures was 0.9 (±0.6) μmol NH_3 l^{-1} d^{-1} (μM NH_3 d^{-1}). Rates of pNTR in the control (no enrichment) and the CH_4-only enrichment did not differ significantly ($p > 0.05$) from the initial (t_0) pNTR rate throughout the experiment. The pNTR rates in NH_4^+-only and NH_4^+ + CH_4 enrichment both showed higher NH_3 oxidation activity by day 15 (Fig. 4.1). The pNTR rates in those two treatments increased through the course of the experiment to a maximum of 22.3 and 24.8 μM NH_3 d^{-1}, respectively, and were only significantly different on day 22, when the pNTR rate was significantly higher ($p = 0.004$) in the NH_4^+ + CH_4 enrichment (Fig. 4.1).

Time-Course of Dissolved Inorganic Nitrogen (DIN) Concentrations

The initial NH_4^+ concentration in the homogenized Mono Lake water-mix was 11.2 (± 0.9) μM. Ammonium concentrations in the control and CH_4-only enrichment steadily declined over the course of the incubation to 4.2 and 1.3 μM, respectively. Ammonium concentrations in the NH_4^+-only and NH_4^+ + CH_4 enrichments decreased rapidly from 75 (±3.7) μM after the initial enrichment to 48 (±2.6) μM by day 8 (Fig. 4.2a). Between day 8 and day 35, NH_4^+ concentrations in both NH_4^+ enrichments were about 43 (± 4.1) μM, with the exception day 22

when the NH_4^+ concentration for the NH_4^+-only and $NH_4^+ + CH_4$ enrichments decreased to 2.9 and 0.6 μM, respectively (Fig. 4.2).

There was no detectable NO_2^- in the initial homogenized Mono Lake water-mix. Nitrite concentrations remained below detection limits in all treatments until day 22 (Fig. 4.2). On day 22, NO_2^- concentrations had increased to 1.1 μM in both the NH_4^+-only and $NH_4^+ + CH_4$ enrichments. Nitrite concentrations remained low throughout the time-course (< 0.6 μM) in the control and CH_4-only enrichment but increased in both the NH_4^+-only and $NH_4^+ + CH_4$ enrichments to 1.7 and 1.9 μM, respectively by day 35 (Fig. 4.2).

The initial NO_3^- concentration in the homogenized Mono Lake water-mix was 0.7 (± 0.2) μM. Nitrate concentrations in all treatments remained low (< 2 μM) through day 15 (Fig. 4.2). Nitrate concentrations in the NH_4^+-only and $NH_4^+ + CH_4$ enrichments increased rapidly between days 15 and day 28 to over 100 μM. By day 35, NO_3^- concentrations in the control and CH_4-only enrichments had increased slightly to an average of 22.5 μM but NO_3^- concentrations in the NH_4^+-only and $NH_4^+ + CH_4$ enrichments had increased 3 fold to 348 and 310 μM respectively (Fig. 4.2).

Microbial Community Structure

The initial (t_0) type I methanotroph community was represented by six distinct bands with relatively similar intensity (Fig. 4.3). Three of the bands (ML_MI_1, ML_MI_2, and ML_MI_4,) represented sequences most closely related to *Methylomicrobium* species. Two additional bands that displayed slightly greater electrophoretic mobility (ML_MI_7, and ML_MI_8) had sequences that were most closely related to *Methylobacter* species (Fig. 4.3).

On day 15, the type I community profile in the control was unchanged. However, observable differences had developed in the type I community structure in the enrichments. The type I community in the CH_4-only enrichment was less diverse and was dominated by band (ML_MI_4) representing a sequence most closely related to *Methylomicrobium* sp. ML1 (Accession No. DQ496231). Bands ML_MI_1 and ML_MI_2, representing sequences most closely related to other *Methylmicrobium* spp., and bands ML_MI_7, and ML_MI_8, affiliated with *Methylobacter* spp. became less apparent (Fig. 4.3a). The NH_4^+-only enrichment retained more of the diversity from t_0 relative to the other enrichments but band ML_MI_8, representing a sequence most closely related to *Methylobacter*, displayed increased intensity (Fig. 4.3).

By day 22, the diversity of type I methanotrophs in the control had decreased. Bands ML_MI_1, ML_MI_2 most closely related to *Methylomicrobium* species became less distinct in all treatments. Band ML_MI_4 had become dominant in all enriched treatments. Minor bands representing other *Methylomicrobium*-like sequences were still somewhat apparent but substantially less intense and by day 35, the *Methylomicrobium*-like bands were no longer apparent in enriched treatments. Diversity at the species level was nevertheless apparent on day 35 as a minor *Methylobacter*-like band ML_MI_7 was still observable (Fig. 4.3).

The initial (t_0) type II methanotroph community was represented by five distinct bands (Fig. 4.3b). Three bands appeared to show greater intensity and represented sequences most closely related to *Methylobacterium* (ML_MII_2 and ML_MII_3) and *Methylosinus* species (ML_MII_10). Minor bands represented sequences that were also most closely related to *Methylobacterium* (ML_MII_1) and *Methylosinus* (ML_MII_7, 8, 9, and10) species (Fig. 4.3).

On day 15, the type II community profile of the control appeared unchanged. However, observable type II methanotroph community differences had developed in the enrichments since

t_0 and differences between treatments were noted. Banding patterns in the CH_4-only and NH_4^+-only treatments were similar to each other but band ML_MII_3 was no longer evident in either enrichment and bands ML_MII_7, 8, 9, and 10 had increased in intensity (Fig. 4.5). The CH_4 + NH_4^+enrichment maintained the same diversity as the t_0 profile but bands ML_MII_3, 7, 8, 9, and 10 appeared more intense (Fig. 4.3).

By day 22, the type II community displayed differences from day 15 as well as more differences between treatments. The bands (ML_MII_3, 7, 8, 9, and 10) representing *Methylosinus* species that exhibited increased intensity on day 15 were less intense (Fig. 4.3b). While the CH_4-only enrichment maintained similar diversity to the control, new bands appeared in the NH_4^+-only (ML_MII_5 and 6) enrichment (Fig. 4.3). By day 35, the type II community profiles demonstrated additional shifts. Most of the bands in the control had become considerably less apparent and the three different enrichments each displayed unique profiles. The CH_4-only enrichment was dominated by band ML_MII_3 representing a *Methylobacterium*-like species and band ML_MII_8 was more intense. The three bands ML_MII_3, 6, and 7 were most prominent in the NH_4^+-only enrichment while in the CH_4+NH_4^+ enrichment, *Methylosinus*-like bands ML_MII_10 and 12 showed the most intensity (Fig. 4.3).

The initial (t_0) ammonia-oxidizing bacterial community was represented by five distinct bands. No dominant bands were apparent with each band exhibiting relatively similar intensity. All bands were representative of sequences most closely related to *Nitrosomonas*-like microbes from hyper-saline and/or alkaline environments (Fig. 4.3). The AOB community profile in the control did not vary significantly over the course of the experiment. There was, however, variation between the other treatments over time (Fig. 4.3).

On day 15, diversity had decreased in the CH_4-only and NH_4^+-only enrichment with the loss of bands ML_AOB_2, 7, and 8. AOB community profiles in the CH_4 + NH_4^+ enrichment indicated decreased diversity by day 15 with band ML_AOB_8 becoming less apparent (Fig. 4.3). On day 22, all three enrichments displayed almost identical profiles. The only difference may have been a slight increase in intensity in band ML_AOB_9 in the NH_4^+-only and CH_4 + NH_4^+ enrichments (Fig. 4.3). By day 35 the AOB profile from the CH_4-only enrichment displayed the most diversity with all 9 bands apparent and with band ML_AOB_9 showing the greatest intensity. The NH_4+-only and CH_4 + NH_4^+ enrichments displayed further decreases in diversity and were very similar with only bands ML_AOB_1, 4, and 9 apparent (Fig. 4.3).

Microbial Abundance

The initial (t_0) abundance of type I and type II methanotrophs, AOBs, and Crenarchaeota was 6.04 (± 3.07) x 10^4 cells ml^{-1}, 4.60 (± 2.33) x 10^4 cells ml^{-1}, 9.60 (± 1.63) x 10^4 cells ml^{-1}, and 3.70 (± 0.25) x 10^5 cells ml^{-1}, respectively (Fig. 4.4). Crenarchaeota represented 5.2% of the total (DAPI stained) population. Methane- and ammonia-oxidizing bacteria represented 2.1 and 1.3 % of the total population, respectively. In the control (no enrichment), DAPI stained cells decreased significantly ($p = 0.031$) by day 8 but did not change significantly through the end of the incubation. Crenarchaeota abundance decreased significantly ($p = 0.002$) between day 8 and day 15 but remained the same for the remainder of the incubation. In contrast, the abundance of both types of methanotrophs and the AOB in the control did not change significantly over time (Fig. 4.4).

In the CH_4-only enrichment, the total number of methanotrophs increased through day 22 but did not change significantly thereafter. However, the ratio of type I to type II methanotroph

abundance decreased significantly over time ($p < 0.05$), a trend observed in each type of enrichment. Type II methanotrophs were less abundant at t_0 but increased rapidly, out numbering type I methanotrophs by day 15, and increasing to a maximum of 8.8×10^4 cells ml^{-1} on day 22. Type I methanotroph abundance declined to a minimum at day 22 and then increased slightly to 5.3×10^4 cells ml^{-1} on day 35 (Fig. 4.5). Ammonia-oxidizing bacteria and Crenarchaeota abundance also varied inversely to each other. Crenarchaeal cell numbers decreased drastically (by approx. 85%) between t_0 and day 15 and their abundance remained low through the end of the experiment. In contrast, AOB abundance increased 40% by day 22 and continued to increase slightly by day 35 (Fig. 4.5).

The relative crearchaeal and AOB abundance observed in the NH_4^+-only enrichment was opposite that observed in the CH_4-only enrichment. Crenarchaeota abundance increased significantly ($p < 0.05$) between days 15, 28, and 35 while AOB abundance decreased over the same time period. Also, similar to the pattern observed in the CH_4-only enrichment, there was an inversion of relative type I and type II methanotroph abundance. However, in the NH_4^+-only enrichment, both type I and type II methanotrophs decreased between t_0 and day 15 and type II methanotrophs did not out number type I methanotrophs until day 22 (Fig. 4.5).

In the $CH_4 + NH_4^+$ enrichment, type I methanotroph cell numbers decreased as type II methanotrophs increased, however, reversal of type specific abundance occurred on a time scale intermediate to that observed in the CH_4-only and NH_4^+-only enrichments. Type I methanotrophs decreased while type II methanotrophs increased between t_0 and day 15 but did not outnumber type I methanotrophs until day 22 (Fig. 4.5). Creanarchaeal and AOB abundances also demonstrated a pattern similar in relative abundance to the CH_4-only enrichment. Crenarchaeota cell numbers decreased by ~70% on day 15 (compared to 85% in the CH_4-only enrichment) and

their abundance remained relatively low for the remainder of the experiment. AOB abundance increased slightly but significantly ($p < 0.05$) by day 15 but did not increase significantly after that point (Fig. 4.5).

DISCUSSION

Enrichment Cultures and the Control

The homogenized nature of the Mono Lake water used to prepare the enrichment cultures and incubation and sampling of the different treatments under identical conditions ensured that variations in process rates and population dynamics observed between treatments were a result of the enrichment regime to which the samples had been exposed. Nonetheless, "bottle effects" are an unavoidable consequence of long-term incubations and may complicate data interpretation. Bottle effects stem from isolation of the water sample, which alters material exchange as well as grazing pressure and/or viral induced mortality. Thus, the changes in oxidation activity and population dynamics observed in the different enrichment regimes were evaluated in light of trends noted in the un-amended control.

Potential CH_4 and NH_3 oxidation activity in the control remained comparatively low and variations in pMOx and pNTR were negligible relative to changes in oxidation rates observed in amended treatments (Fig. 4.1). MOB and AOB cell numbers remained constant in the control throughout the entire incubation with type I methanotrophs being more abundant than type II methanotrophs (Fig. 4.3). This same trend of relative abundance and persistence of these two groups of methanotrophs in the control is typical of *in situ* Mono Lake conditions (Carini et al., 2005 – Chapter 2). Also consistent with reported data, AOB abundance comprised a relatively small percentage of the initial bacterial population (Ward et al., 2000; Carini and Joye - Chapter

3) and total methanotroph abundance in the control was generally higher than AOB throughout the experiment. Furthermore, both MOB and AOB diversity in the control, characterized by group- specific DGGE fingerprints, remained relatively unchanged (Fig. 4.3). Consequently, the changes we observed in those group's activity, abundance, and diversity in the various enrichment cultures can be attributed largely to the effects of variable substrate availability.

There were, however, changes in total microbial and Crenarchaeota abundance in the control treatment. Overall microbial abundance (DAPI counts) decreased significantly by day 15 while Crenarchaeota abundance remained unchanged for the first two weeks before decreasing significantly between days 15 and 22. These patterns may have resulted from either bottle influenced viral activity and/or altered grazing pressures. Viral abundance in Mono Lake is among the highest reported in any natural aquatic system (Jiang, et al., 2004). Both strong viral seasonality and related host specificity in Mono Lake (Sabet et al., 2006) suggest that the rapid decrease and subsequent stability in overall microbial cell numbers in the control may represent a new host-phage equilibrium based on a decrease in host abundance and degradation of a large portion of host specific viral progeny (Furman, 1999). The time lag preceding the decrease in Crenarchaeota abundance was more suggestive of an alteration in grazing pressures. Protists are present in Mono Lake and proliferate during periods when *Artemia* abundance is low (R. Jellison, pers. comm.). An increase in protozoan abundance and grazing following the removal of *Artemia* from the water used for the enrichments (by screening) may have differentially affected Crenarchaeota abundance based on size selective grazing coupled with the frequency of suitable prey encounters (Jürgens et al., 1999). Fortunately the shifts in Crenarchaeota abundance observed in the enrichments were both temporally and quantitatively very different than the changes noted in the control. Decreased crenarchaeal abundance in the CH_4-only and CH_4 +

NH_4^+ enrichments occurred more rapidly and were of significantly greater magnitude than in the control. Moreover there was an increase in Crenarchaeota abundance observed in the NH_4^+- only treatment that was contrary to the trend documented in the control. Therefore we maintain that, as with the MOB and AOB, the changes in Crenarchaeota abundances observed in the enrichments may also be attributed to the varying treatment regimes.

Substrate Induced Stimulation of CH_4 Oxidation and Associated Methanotroph Population Dynamics

Methane oxidation rates in enrichments amended with CH_4 increased significantly throughout the incubation relative to the control (Fig. 4.1). Increased CH_4 oxidation rates often correlate with increased CH_4 availability in the environment. Methane oxidation rates increased in landfill soils (Whalen et al., 1990; Jones and Nedwell, 1993), rice planted microcosms (Bodelier and Frenzel, 1999), and estuarine sediment (Carini et al., 2003) when exposed to elevated CH_4 concentrations. Increased CH_4 oxidation rates may result from an accumulation of methanotrophic biomass as a consequence of exposure to elevated CH_4 concentrations during seasonal lake stratification (Rudd et al., 1974; Harrits and Hanson, 1980). Observed increases in methanotroph abundance also correlated with higher CH_4 oxidation rates in soils (Bender and Conrad, 1994) and rice paddies (Henckel et al., 2000). However, other evidence has suggested that stable methanotroph populations may persist under taxing conditions such as extended periods of very low CH_4 concentrations (Holmes et. al., 1999; Knief et. al., 2003) or anoxia (King, 1996). In these cases, standing stocks are able to increase activity during transitory periods of favorable geochemical conditions without a corresponding increase in abundance (Eller and Frenzel, 2001; Carini et al., 2005 – Chapter 2). Community composition data from our

CH_4 enrichments suggest a combination of additional methanotroph abundance and increased activity of the initial *in situ* methanotroph population likely contributed to the higher CH_4 oxidation rates observed in those treatments.

Methanotroph diversity and abundance in Mono Lake samples varied between the control and enriched treatments. In the control, diversity and relative type abundance remained constant over time; the DGGE fingerprints of both types remained very similar relative to their respective t_0 fingerprints throughout the incubation and type I methanotroph abundance was consistently higher than that of type II methanotrophs. In contrast, as CH_4 oxidation activity increased in the CH_4 amended treatments, diversity and abundance of type I methanotrophs decreased while different type II methanotrophs became more dominant and overall type II methanotroph abundance increased (Fig. 4.5). This data is consistent with the hypothesis that type I methanotrophs generally outnumber type II methanotrophs in environments with low CH_4 concentrations, such as the control (< 0.5 μM dissolved CH_4), while type II methanotrophs tend to dominate environments with high CH_4 concentrations, such as our CH_4 enriched cultures (Amaral and Knowles, 1995; Henckel et al., 2000; Macalady et al., 2002).

However, it is unlikely that the increases in type II abundance were entirely responsible for the observed increase in CH_4 oxidation activity in the CH_4 amended treatments. Although type II abundance increased significantly (nearly doubled), CH_4 oxidation rates in the CH_4 amended treatments increased on a much larger scale (12 to 16 fold). Moreover, the greatest increase in type II abundance (in the CH_4-only enrichment) did not coincide with the highest recorded CH_4 oxidation rate (in the CH_4+NH_4^+ enrichment). CH_4 oxidation rates in both CH_4 enriched treatments also increased significantly during periods when methanotroph abundance remained constant (Fig. 5.5). Furthermore, the incorporation of C from isotopically labeled CH_4

into family specific lipid biomarkers (PFLAs) has revealed that methanotroph abundance does not necessarily correlate with activity (Henckel et al., 2000; Macalady et al., 2002) and that the generally accepted paradigm concerning the distribution and activity of type I vs. type II methanotrophs based solely on environmental parameters may be too broad (Knief et al., 2006). Therefore, it is likely that increased activity of a portion of the initial methanotroph population (including type I methanotrophs) in addition to type II biomass accumulation contributed to the increase in overall CH_4 oxidation rates observed in the CH_4 amended enrichments.

The Effects of Supplemental Ammonia on Nitrification Rates and the Relative Contributions of Ammonia-Oxidizing Bacteria and Crenarchaeota

The rate of NH_3 oxidation increased in the NH_4^+-only enrichment relative to the control (Fig. 4.1), demonstrating that nitrification was N limited. The Mono Lake mixolimnion has an extremely low N:P ratio (<0.01), indicating chronic nitrogen limitation (Joye et al., 1999). Increased NH_3 availability in the NH_4^+-only enrichment should have benefited an existent nitrifying population as observed previously in soils (Mendum et al., 1999; Bruns et. al., 1999) and sediments (Carini et al., 2003) that demonstrated increased nitrification coincident with increased NH_3 availability. However, detectable nitrification rates and production of NO_2^- and NO_3^- in our NH_4^+ amended treatment were temporally offset from high initial NH_4^+ consumption (Fig 4.2), indicating fierce competition for available NH_3. Nevertheless, the detection of NH_3 oxidation activity and eventual NO_X production in the NH_4^+-only amended treatment revealed the presence of a viable nitrifying population. Mono Lake supports nitrification *in situ* and populations of both AOB and Crenarchaeota are present. While the pattern of AOB abundance and correlation between crenarchaeal abundance and nitrification profiles suggests that AOA

may contribute to NH_3 oxidation in Mono Lake, the lack of verifiable archaeal ammonia monooxygenase genes and the sufficient abundance of AOB to account for all of the measured nitrification activity leaves open the question of relative contributions between AOA and AOB *in situ* (Carini and Joye, 2007 – Chapter 3).

Significant changes in both crenarchaeal and AOB abundance in the NH_4^+-only enrichment provided stronger correlative data supporting potential AOA activity in Mono Lake relative to *in situ* observations. Crenarchaeota abundance increased by 91% as the nitrification rate increased, while AOB abundance decreased by 46% (Fig. 4.5). The gross increase in crearchaeal abundance was likely even higher if adjusted for the decreased abundance observed in the control. Regression analysis between nitrification rates and AOB and Crenarchaeota abundance yielded R^2 values of 0.99 and 0.98, respectively ($p < 0.01$ for each; Table 4.1). Clearly, members of the Crenarchaeota proliferated over the AOB population under N-replete conditions and were strongly correlated with nitrifying activity. AOB cell specific nitrification rates in the NH_4^+-only enrichment were ~ 8 to 18 fmol $cell^{-1}$ hr^{-1} by the end of the incubation (Table 4.1). Cell specific rates reported for pure culture (*Nitrosospira*) and amended soils were generally less than 10 fmol $cell^{-1}$ hr^{-1} (Belser and Schmidt, 1978; Jiang and Bakken, 1999; Okano et al., 2004) indicating that the AOB population may not have had the physiological capacity to account for all the measured nitrification in the NH_4^+-only enrichment. Taken together, these findings suggest an AOA contribution to nitrification in the NH_4^+-only enrichment.

Patterns of Group Specific Activity and Microbial Interactions Under CH_4+NH_4^+ Enriched Conditions

To this point the discussion has been limited to the effect of primary substrate enrichment on a specific group's oxidation activity and population dynamics. Simultaneous enrichment with both CH_4 and NH_4^+ elicited particular variations in specific oxidative activities and associated populations relative to the responses observed in the individual substrate enrichments. Methane oxidation potential increased in response to NH_4^+ addition but was primarily regulated by CH_4 availability. Potential CH_4 oxidation rates in the NH_4^+-only enrichment were not significantly different from the control demonstrating that nitrifiers did not contribute to CH_4 oxidation and suggesting that the stimulation of CH_4 oxidation in the CH_4+NH_4^+ treatment was due to the effect of supplemental N on the methanotrophic community. A comparison of CH_4 oxidation trends and methanotroph community population dynamics between the different CH_4-augmented treatments suggested two distinct responses to supplemental N and the respective mechanisms involved.

A more rapid onset of increased CH_4 oxidation appeared to be the initial, relatively short-term effect triggered by additional N availability. The CH_4 + NH_4^+ enrichment was the only treatment that experienced significantly higher CH_4 oxidation during the first week of the incubation. Moreover, by day 15, CH_4 oxidation in the CH_4 + NH_4^+ enrichment was 89% higher than in the CH_4-only enrichment. Similar methanotroph diversity and relative family abundance in both treatments during this time period suggests that the initial increase of CH_4 oxidation rates in the CH_4 + NH_4^+ enrichment was due to the alleviation of N limitation of the existing methanotroph population. Recent studies have also demonstrated a similar stimulatory effect of

N availability on CH_4 oxidation (Bodelier et al., 2000; Eller and Frenzel, 2000; Carini et al., 2003).

As the incubation progressed, relative CH_4 oxidation activity and methanotroph community structure between the $CH_4 + NH_4^+$ and CH_4-only enrichments shifted. Although methane oxidation rates remained significantly higher ($p = 0.0001$-0.0003) in the CH_4+NH_4^+ enrichment, the magnitude of increased CH_4 oxidation attributed to the initial N induced surge of activity lessened. The 89% higher CH_4 oxidation activity in the CH_4+NH_4^+ enrichment relative to the CH_4-only enrichment on day 15 decreased to <38% by day 35 (ANOVA $p < 0.05$). During the same time frame, significant differences developed between treatments in the magnitude of relative type abundance, overall methanotroph abundance, and type II community species dominance.

Although type II methanotroph cells grew to outnumber that of type I methanotrophs in both treatments, type I methanotroph abundance declined approximately the same amount as type II methanotrophs increased in the $CH_4 + NH_4^+$ enrichment, resulting in a relatively steady total methanotroph abundance in that treatment over time. In contrast, type I methanotroph abundance decreased only slightly in the CH_4-only enrichment while type II methanotrophs increased substantially, resulting in an overall methanotroph increase in that treatment of 33%. Type II specific methanotroph diversity also responded differently to the particular mode of enrichment applied. By the end of the incubation, type II methanotrophs displayed different DGGE fingerprints between treatments. Bands ML_MII_3 and _8, representing sequences affiliated with *Methylosinus*-like species, were more dominant in the CH_4-only enrichment. In contrast, bands ML_MII_7 and _10 (significantly different but also most similar to *Methylosinus*-like species) were more dominant in the $CH_4 + NH_4^+$ enrichment (Fig. 4.3).

Thus, the Mono Lake microbial populations exposed to increased CH_4 concentrations reacted differently depending on whether additional N was available. Clearly, the methanotroph population of the CH_4 + NH_4^+ enrichment had a higher potential for CH_4 oxidation due to readily available NH_3. However, the methanotroph population in the CH_4-only enrichment was also able to take advantage of higher CH_4 availability even without supplemental N through community composition adaptation. Specifically, higher overall methanotroph abundance developed and a different set of type II methanotrophs, presumably those microbes more able to take advantage of particular N pools available in the un-amended enrichment dominated the CH_4-only enrichment. The concentration of dissolved organic nitrogen (DON) in Mono Lake is approximately 145 μM (Joye et al., 1999) and DON conversion of NH_4^+ could contribute additional N to support increased CH_4 oxidation of specific type II methanotrophs without supplemental NH_4^+ enrichment.

A comparison with other group's specific activity and population dynamics revealed little, if any interaction or influence on CH_4 oxidation and methanotroph populations in any of the enrichments. Methanotrophs appeared to be able to adapt to their benefit regardless of the activity or population dynamics of the other microbial groups examined here. In fact, methanotrophs or methanotrophic activity seemed to have a detrimental effect on the Crenarchaeota (discussed below). AOB activity and/or population dynamics may have had some slight effect on the methanotrophic activity or populations in the CH_4-only enrichment, however no consistent trends of interaction or influence were evident between treatments. For example, although there was a significant increase in AOB abundance in the CH_4-only treatment, there was little pNTR and both methanotroph abundance and CH_4 oxidation rates increased. In contrast, a smaller increase in AOB abundance in the CH_4+NH_4^+ enrichment supported a

significant increase in pNTR yet CH_4 oxidation achieved the highest rates observed in the experiment.

Quite the opposite was true of nitrification. While activity was consistently elevated concurrent with NH_4^+ enrichment or negligible in treatments not amended with NH_4^+, Crenarchaeota and AOB population dynamics varied significantly between the different treatments. Although there was minimal pNTR activity in the CH_4-only enrichment, crenarchaeal abundance experienced an immediate and considerable decrease (-89%) while AOB abundance increased 77%. In contrast to the population data from the NH_4^+-only enrichment, there was a decrease in crenarchaeal abundance and an increase in AOB abundance in the CH_4+NH_4^+ enrichment that correlated with increased nitrification activity ($r = -0.73$ and 0.69 for Crenarchaeota and AOB, respectively). However, the change in abundance of both groups in the CH_4+NH_4^+ enrichment was mitigated in relation to the CH_4-only enrichment (16% less of a decrease in Crenarchaeota and 56% less increase in AOB). While the data suggests that AOA may have been involved in nitrification in the NH_4^+-only enrichment, the relationship between the different nitrifier populations and activity in the CH_4+NH_4^+ enrichment is more ambiguous. The reversal in the correlation between AOB and nitrification relative to that in the NH_4^+-only enrichment and the significant decline in crenarchaeal abundance suggest that AOB were likely more active in nitrification in the CH_4+NH_4^+ enrichment. However, because crenarchaeal abundance did not decrease as drastically in the CH_4+NH_4^+ enrichment and absolute crenarchaeal and AOB cell numbers were similar, an AOA contribution to nitrification in the CH_4+NH_4^+ enrichment cannot be ruled out.

SUMMARY AND CONCLUDING REMARKS

Methane and NH_3 oxidation rates increased over time in enrichments amended with their primary substrate. Community composition data from our CH_4 enrichments suggest a combination of additional methanotroph abundance and increased activity of the initial *in situ* methanotroph population likely contributed to the higher CH_4 oxidation rates observed in those treatments. The rate of NH_3 oxidation increased in the NH_4^+-only enrichment relative to the control, demonstrating that nitrification was N limited. Trends in community composition data together with data from previous studies of AOA suggested AOA may have contributed to nitrification in the NH_4^+-only enrichment.

Methanotrophic activity and community composition reacted differently dependent on the presence or absence of additional NH_4^+. The methanotroph population of the $CH_4 + NH_4^+$ enrichment had a higher potential for CH_4 oxidation due to readily available NH_3. However, the methanotroph population in the CH_4-only enrichment was also able to take advantage of higher CH_4 availability even without supplemental N through community composition adaptation. Nitrification activity was not significantly affected by supplemental CH_4, however, nitrifier community composition reacted differently dependent on the presence or absence of additional CH_4. Contrary to the response in the NH_4^+-only enrichment, Crenarchaeota abundance decreased and AOB increased in the $CH_4 + NH_4^+$ enrichment suggesting that AOB were likely more active in nitrification in the CH_4+NH_4^+ enrichment.

The integration of results from individual enrichments with the responses elicited in the dually enriched $CH_4 + NH_4^+$ treatment are crucial to beginning to understand the interactions between these ecologically intertwined processes and populations, particularly in light of the

widespread potential for archaeal involvement in NH_3 oxidation and concomitant influence on global N, C, and O cycles.

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Table 4.1. Regression R^2 values and statistical significance of CH_4 and NH_3 oxidation rates versus relevant group-specific abundance. AOB cell specific nitrification rates are also listed.

Sample	Day	pMOx Rate (nM CH_4 d^{-1})	Type I (10^4 ml^{-1})	Type II (10^4 ml^{-1})	MOB (10^4 ml^{-1})	pNTR Rate (μM NH_3 d^{-1})	AOB (10^4 ml^{-1})	Cren (10^5 ml^{-1})	fmol NH_3 h^{-1} AOB^{-1}
Control	0	12.6	6.04	4.60	10.64	0.9	9.58	3.69	0.39
	8	17.8				0.5			
	15	6.3	6.58	4.78	11.36	1.0	9.09	3.75	0.46
	22	6.7	5.98	4.88	10.86	1.5	8.86	1.76	0.71
	28	0.0				1.0			
	35	0.0	6.28	5.18	11.46	0.8	8.49	2.00	0.39
R^2			0.14	0.65	0.74		0.01	0.20	
CH4-only	0	12.6	6.04	4.60	10.64	0.9	9.58	3.69	0.39
	8	23.9				0.1			
	15	74.3	6.00	7.13	13.14	0.9	14.15	0.44	0.28
	22	98.9	5.15	8.84	13.99	1.5	16.48	0.49	0.38
	28	133.7				0.0			
* P < 0.05	35	142.3	5.33	8.80	14.13	0.2	16.99	0.48	0.05
R^2			0.60	0.88 *	0.83				
NH4+-only	0	12.6	6.04	4.60	10.64	0.9	9.58	3.69	0.39
	8	14.6				0.7			
	15	10.5	5.88	4.18	10.06	3.9	9.35	3.71	1.73
	22	5.8	3.88	6.18	10.06	14.6	6.92	5.45	8.79
	28	6.9				20.0			
** P < 0.01	35	9.7	3.88	6.18	10.06	22.3	5.18	7.00	17.95
R^2							0.99 **	0.97 **	
CH4+NH4+	0	12.6	6.04	4.60	10.64	0.9	9.5	3.69	0.39
	8	21.9				0.4			
	15	139.4	5.66	4.91	10.56	4.4	11.465	1.18	1.61
	22	170.8	3.88	6.18	10.06	21.9	11.465	1.03	7.94
	28	192.5				23.6			
	35	196.4	3.28	7.00	10.28	24.8	11.465	1.00	9.01
R^2			0.70	0.69	0.55		0.45	0.51	

FIGURE LEGENDS

Figure 4.1. Time course of potential methane (A) and ammonia (B) oxidation rates from sub-samples of Mono Lake enrichment cultures. Error bars are standard deviation around the mean (n=3).

Figure 4.2. Time-course measurements of Mono Lake enrichment culture DIN concentrations: ammonia (A), nitrite (B), and nitrate (C).

Figure 4.3. Bar graph representation of total and group specific absolute cell counts in the un-amended control over the course of the incubation. Total (DAPI stained) and Crenarchaeota cells (A) and comparative functional group-specific abundance (B).

Figure 4.4. Time-course DGGE analysis of the variable 3 region of PCR products from Mono Lake enrichment culture DNA amplicons produced using: MethT1 (A), MethT2 (B) and NitAB (C) primer sets.

Figure 4.5. Time course measurements of potential rate measurements and corresponding functional group abundance from Mono Lake enrichment cultures: CH_4-only (A), NH_4^+-only (B), and both NH_4^++CH_4 (C). Group specific cell numbers are scaled with retained proportion. See Fig. 4.3 for absolute initial abundance.

Figure 4.1.

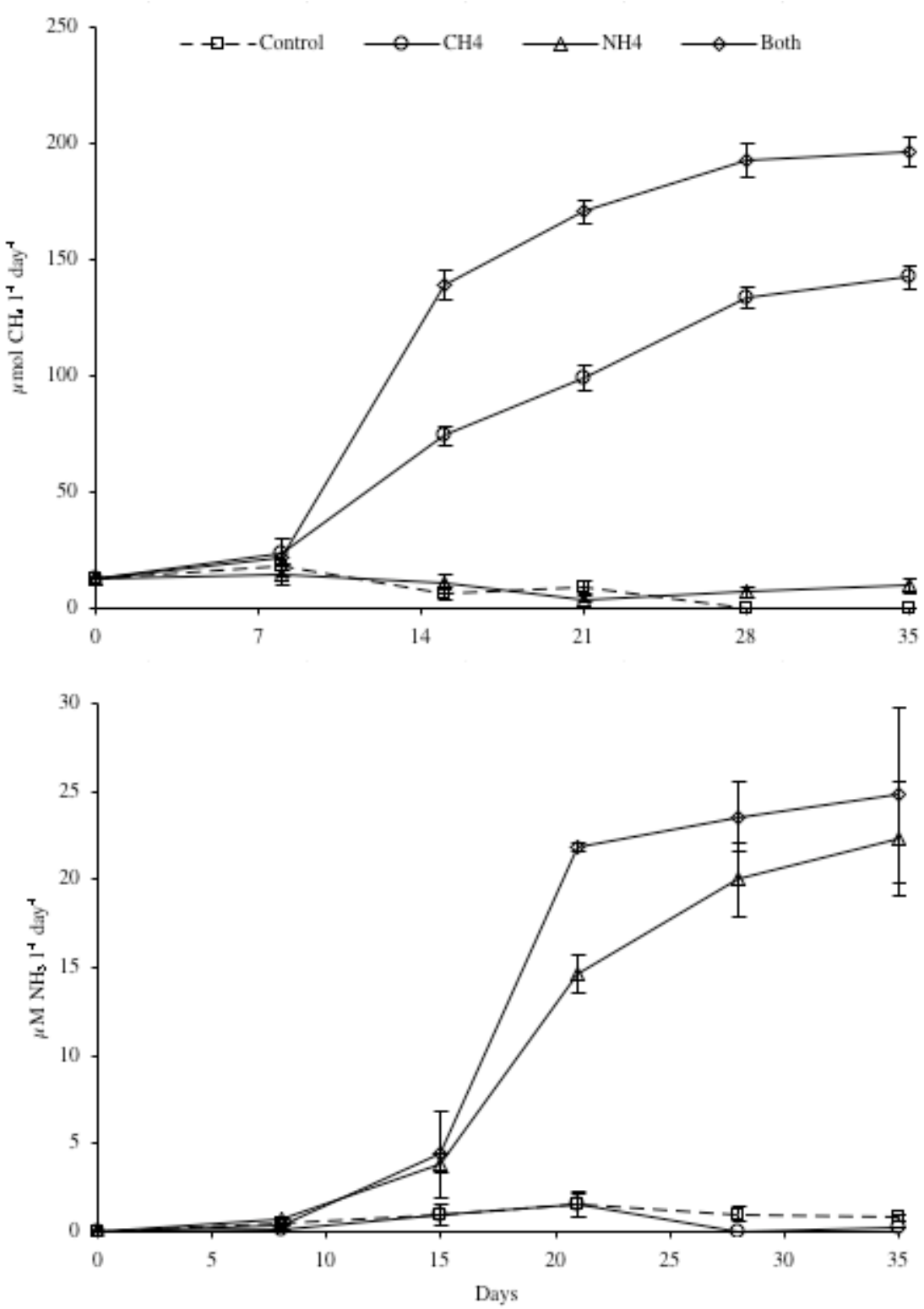

Control
CH4
NH4
Both
250
200
150
100
50
0
μmol CH4 l-1 day-1
0
7
14
21
28
35
30
25
20
15
10
5
0
μM NH3 l-1 day-1
0
5
10
15
20
25
30
35
Days

Figure 4.2.

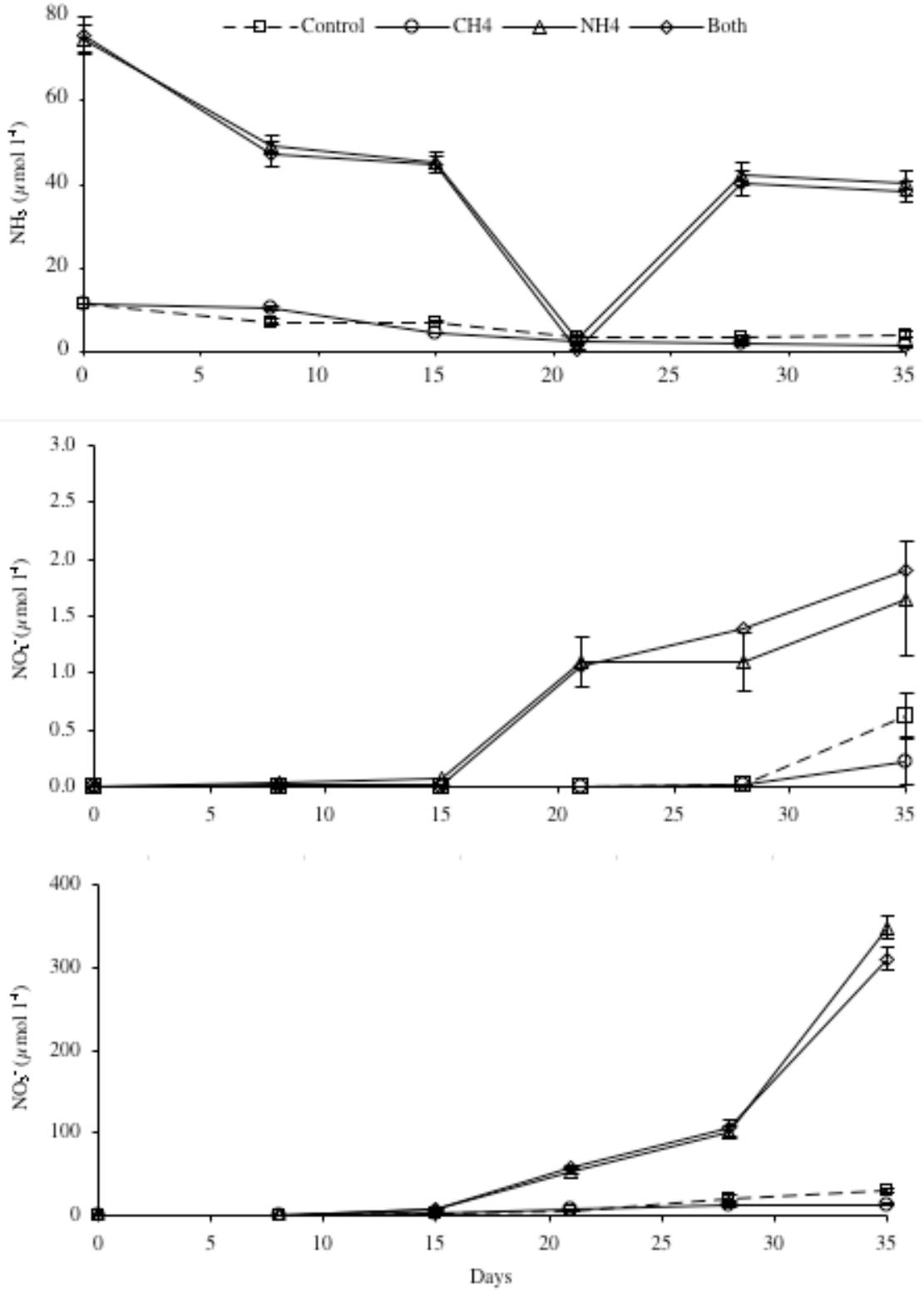


Figure 4.3.

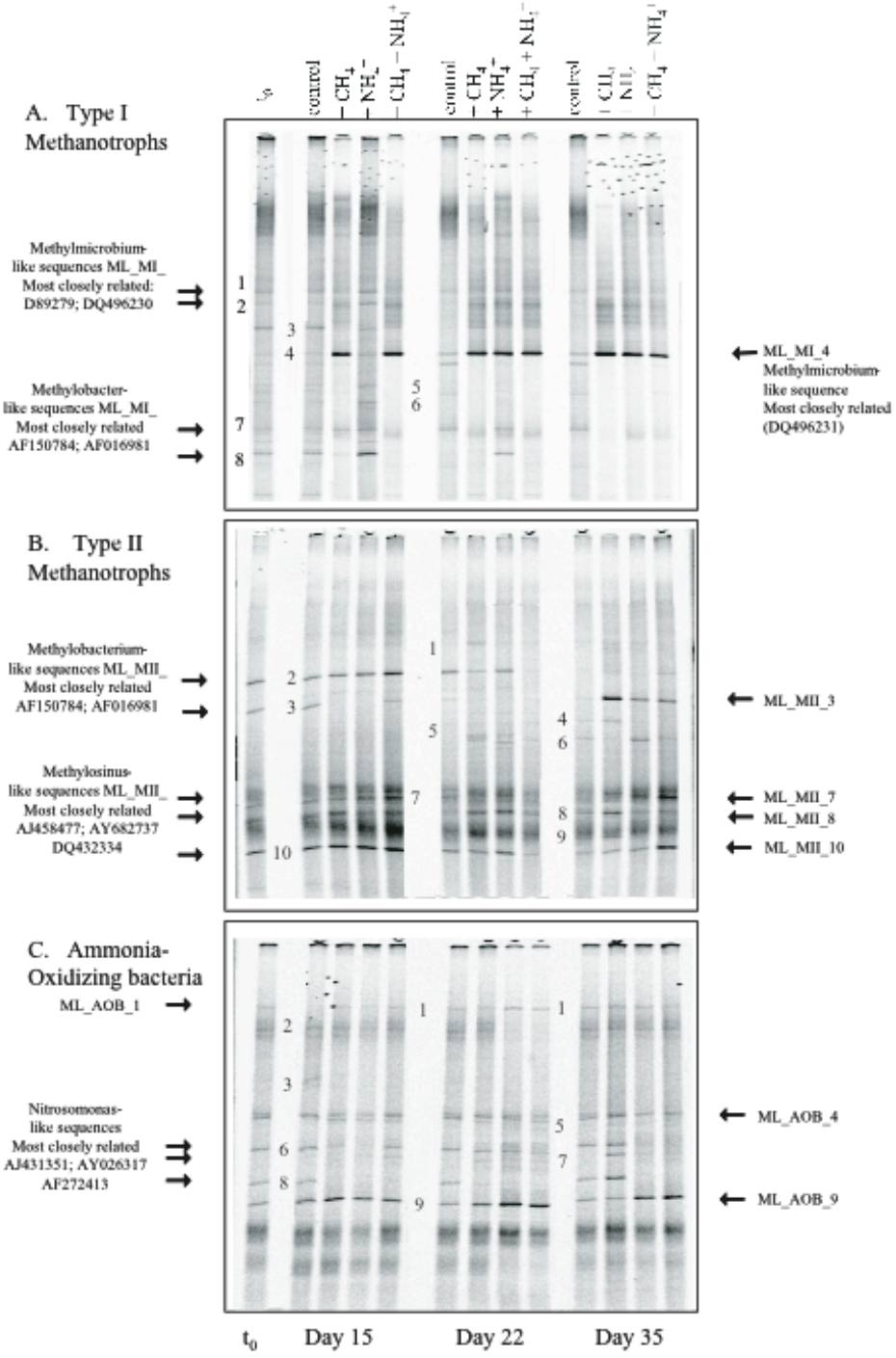

A. Type I Methanotrophs
Methylmicrobium-like sequences ML_MI_ Most closely related: D89279; DQ496230
Methylobacter-like sequences ML_MI_ Most closely related AF150784; AF016981
ML_MI_4 Methylmicrobium-like sequence Most closely related (DQ496231)
B. Type II Methanotrophs
Methylobacterium-like sequences ML_MII_ Most closely related AF150784; AF016981
Methylosinus-like sequences ML_MII_ Most closely related AJ458477; AY682737 DQ432334
ML_MII_3
ML_MII_7
ML_MII_8
ML_MII_10
C. Ammonia-Oxidizing bacteria
ML_AOB_1
Nitrosomonas-like sequences Most closely related AJ431351; AY026317 AF272413
ML_AOB_4
ML_AOB_9
control
CH_4 – NH_4^+ –
CH_4 – NH_4^+
CH_4 + NH_4^+
t_0
Day 15
Day 22
Day 35

Figure 4.4.

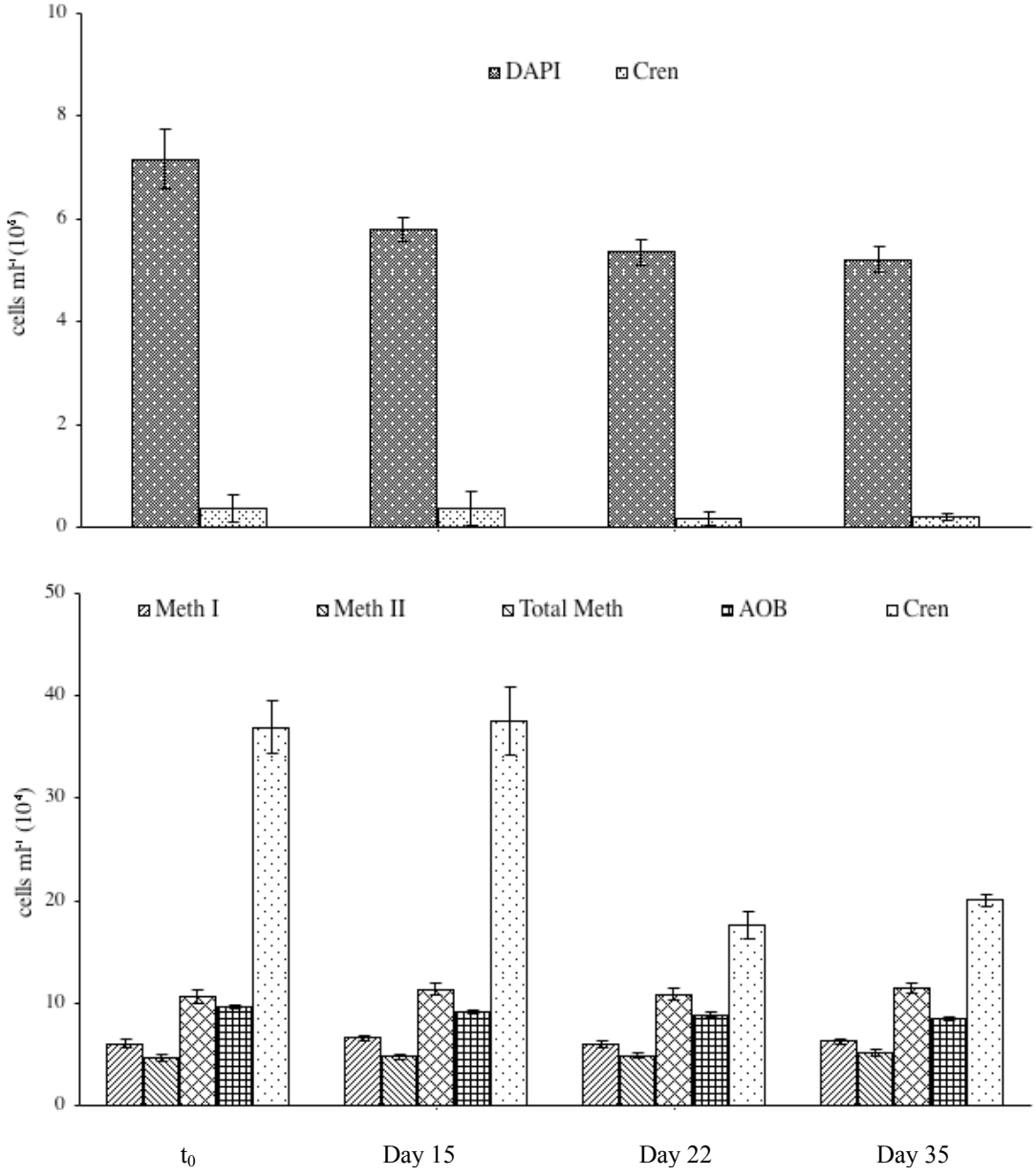
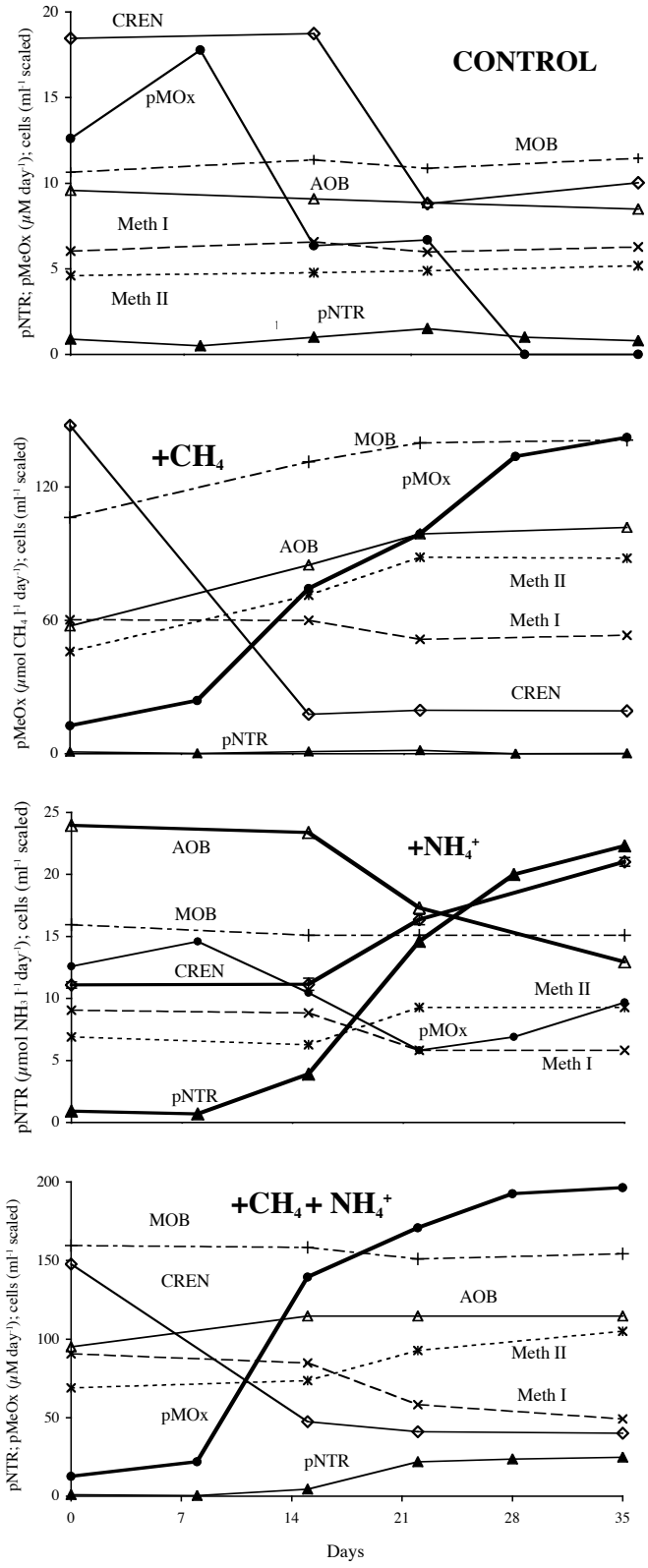

DAPI
Cren
cells ml⁻¹(10⁶)
Meth I
Meth II
Total Meth
AOB
Cren
cells ml⁻¹ (10⁴)
t0
Day 15
Day 22
Day 35

Figure 4.5.


CONTROL
CREN
pMOx
MOB
AOB
Meth I
Meth II
pNTR
pNTR; pMeOx (μM day⁻¹); cells (ml⁻¹ scaled)
+CH4
MOB
pMOx
AOB
Meth II
Meth I
CREN
pNTR
pMeOx (μmol CH4 l⁻¹ day⁻¹); cells (ml⁻¹ scaled)
+NH4+
AOB
MOB
CREN
Meth II
pMOx
Meth I
pNTR
pNTR (μmol NH3 l⁻¹ day⁻¹); cells (ml⁻¹ scaled)
+CH4 + NH4+
MOB
CREN
AOB
Meth II
Meth I
pMOx
pNTR
pNTR; pMeOx (μM day⁻¹); cells (ml⁻¹ scaled)
Days

CHAPTER 5

CONCLUSIONS

The data presented here has furthered our understanding of the ecology of methanotrophs and ammonia oxidizer populations. We addressed controls on both activity and community composition *in situ* (**Chapters 2 & 3**) and under conditions of manipulated substrate availability designed to illicit interactions through competition for mutually required metabolites (**Chapter 4**). While some of the data provided support for generally accepted physiological and populational paradigms, a surprising wealth of novel information was uncovered (**Chapters 2, 3, & 4**).

In situ Mono Lake CH_4 oxidation measurements confirmed a widely held tenet of methanotroph preference for low O_2 tension. Peak CH_4 oxidation activity was maintained in the microaerophilic zone proximal to the bottom of the oxycline even as it migrated upward through the water column during seasonal stratification. On the other hand, community abundance data supported a small but growing body of evidence suggesting a widespread strategy of stable methanotroph populations persisting through adversity and subsequently taking advantage of ephemeral advantageous conditions vs. population growth-induced activity only during periods optimal growth conditions (**Chapter 2**). We also expanded the range of environments (hypersaline lakes) where type II methanotrophs may be expected to be found.

Nitrification in hypersaline Mono Lake by nitrosomonads was reconfirmed following a discontinuity in activity that occurred between 1995 and 2003-2003. Contrasting hydrological mixing regimes resulting in differential NH_3 availability in the mixolimnion may have

contributed to the observed pattern. However, the consequences of the transition to a monomictic mixing regime likely shifted nitrosomonad species composition from N. *europea*-like organisms to nitrosomonads most closely related to alkaline/hypersaline-adapted species (**Chapter 3**). AOB abundance was inversely correlated with the seasonality observed in integrated nitrification rates and indicated a negative correlation between AOB abundance and nitrification activity. In contrast, Crenarchaeota cell numbers exhibited a strong correlation with NTR rate measurements. While the pattern of AOB abundance and correlation between crenarchaeal abundance and nitrification profiles suggests that AOA may contribute to NH_3 oxidation in Mono Lake, the lack of verifiable archaeal ammonia monooxygenase genes and the sufficient abundance of AOB to account for all of the measured nitrification activity leaves open the question of relative contributions between AOA and AOB *in situ* (**Chapter 3**).

Manipulation of substrate availability in individual enrichment cultures facilitated the elucidation of controls and interactions between methanotroph and nitrifier populations and activity (**Chapter 4**). Consistent with previous results, methane and NH_3 oxidation rates increased over time in enrichments amended with their primary substrate. While additional methanotroph abundance and increased activity of the initial *in situ* methanotroph population likely contributed to the higher CH_4 oxidation rates observed in those treatments, NH_3 oxidation rates likely increased due to the alleviation of N limitation. Trends in community composition data in the NH_4^+-only enrichment provided more compelling support for potential AOA contribution to nitrification relative to the *in situ* data.

The presence or absence of additional NH_4^+ influenced the response in methanotrophic activity and community composition. The methanotroph population of the CH_4 + NH_4^+ enrichment had a higher potential for CH_4 oxidation due to readily available NH_3. However, the

methanotroph population in the CH_4-only enrichment was also able to take advantage of higher CH_4 availability through community composition adaptation. Nitrification activity was not significantly affected by supplemental CH_4, however, Crenarchaeota abundance decreased and AOB increased in the CH_4 + NH_4^+ enrichment suggesting that AOB were likely more active in nitrification in the CH_4+NH_4^+ enrichment.

The integration of results from individual enrichments with the responses elicited in the dually enriched CH_4 + NH_4^+ treatment provided preliminary insight into the interactions between these ecologically mingled processes and populations which may prove significant to the current reassessment of relative influences on global N, C, and O cycles in light of the widespread potential for archaeal involvement in NH_3 oxidation.