THE ABUNDANCE OF AMMONIUM-OXIDIZING BACTERIA IN LAKE BONNEY, ANTARCTICA DETERMINED BY IMMUNOFLUORESCENCE, PCR AND IN SITU HYBRIDIZATION

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Previous studies of biogeochemical cycling in Lake Bonney, Antarctica, suggest that nitrification plays a central role in controlling the depth distributions of oxidized and reduced inorganic nitrogen in both east and west lobes. For example, there is a mid-depth N_2O-N maximum of 41.6 μg at l^{-1} (> 500,000% saturation) in the east lobe, the highest level reported for a natural system. The source of this N_2O peak is thought to be nitrification under conditions of low oxygen tension. Although nitrifying bacteria have been detected, attempts to isolate and culture them have been unsuccessful. This study examines three techniques for the determination of abundance of nitrifying bacteria in this lake. Applying a polymerase chain reaction (PCR) assay developed for the detection of ammonium-oxidizing bacteria belonging to the beta and gamma subclasses of the Proteobacteria, immunofluorescent antibody assay (IFA) and fluorescent probe *in situ* hybridization (FISH) techniques, the distribution and relative abundance of ammonium-oxidizers was examined. In general, nitrifiers were detected at depths above the pycnocline and usually associated with decreasing concentrations of NH_4^+ and increasing concentrations of NO_3^- or NO_2^-. These data are consistent with the chemical distributions and the role of nitrifying bacteria in determining the distribution of nitrogen compounds in this lake.

INTRODUCTION

Lake Bonney, a perennially ice-covered, glacial-fed lake within the Taylor Valley, provides an unusual system in which to study the microbially mediated processes of nitrification and the bacteria responsible. Physical processes, such as turbulent mixing, are severely limited due to the ice cover and in situ biogeochemical reactions and diffusion are the dominate processes controlling chemical distributions. The lake is divided into two lobes by a narrow, shallow strait which results in the hydrographic isolation of the deeper layers of each basin. As a result of this isolation and important differences in the recent history (1000–2000 years) of the two lobes, their deep water chemistry differs markedly [*Spigel and Priscu*, 1996; *Lyons et al.*, 1997]. Both lobes are chemically stratified; the chemocline in each basin is located below the sill depth and separates the upper trophogenic zone which is fresh, well-oxygenated, and low in nitrogenous compounds from the hypersaline, anoxic lower zone where photoautotrophic activity is absent and inorganic nitrogen is regenerated [*Priscu et al.*, 1995]. In the west lobe, the distribution of inorganic nitrogen species

below the chemocline is consistent with a typical stratified system in which the surface layer is nitrogen depleted, and the deep anoxic layer has high NH_4^+ concentrations but the oxidized forms of nitrogen are depleted presumably due to denitrification. Denitrification has been measured in the deep layer of this lobe [*Priscu et al.*, 1995]. In contrast the deep anoxic layer of the east lobe has high concentrations of NO_2^-, NO_3^-, and NH_4^+. Denitrification was not detectable below the chemocline in the east lobe [*Priscu et al.*, 1995]. Additionally N_2O appears at very high levels (exceeding 580,000% over air saturation) at the oxic/anoxic interface of the east lobe, while trace levels exist in the west lobe [*Priscu et al.*, 1996; *Priscu*, 1997]. These observations suggest that denitrification dominates chemical distributions in the west lobe and that nitrification, rather than denitrification, dominates the chemical distributions in the east lobe.

It is widely accepted that microorganisms are the primary mediators in most biogeochemical processes of the nitrogen cycle in aquatic systems. Nitrification is the two-step process of oxidation of NH_4^+ through NO_2^- to NO_3^- and is carried out by two separate groups of chemolithotrophic bacteria (NH_4^+ oxidizers and NO_2^- oxidizers). Under low oxygen tensions, nitrification can also result in significant production of trace gases such as N_2O and NO in aquatic environments and their subsequent release into the atmosphere [*Poth and Focht*, 1985; *Downes*, 1988; *Goreau et al.*, 1980]. Nitrification has been studied for its potential importance as an oxygen sink, a mechanism for loss of nitrogen (via coupling to denitrification), and as a source of NO_2^- and NO_3^- supplying new nitrogen to primary producers in surface waters. The presence of nitrifying bacteria and the process of nitrification can be inferred by chemical profiles of the substrates and products of the reactions. However in environments containing oxygen gradients, nitrification is often tightly coupled to the heterotrophic microbial process, denitrification (dissimilatory NO_3^- reduction), sharing many of the same substrates and intermediates. This spatial and chemical overlap confounds direct interpretation. To determine the importance of each process in controlling the biogeochemistry in these complex systems, information on the abundance and distribution of the organism responsible is needed in conjunction with rate measurements and nutrient profiles.

Although nitrifiers have been isolated from diverse environments and are generally ubiquitous in soils, freshwater and marine environments [*Koops and Möller*, 1992], they account for a very small proportion of the total bacterial population in natural environments. Moreover little is known about the diversity or genetic composition of lacustrine nitrifiers. The number of species that have been isolated and described from lakes is small, even compared with the accepted low diversity of nitrifiers in terrestrial and marine environments [*Hall*, 1986]. Sensitive and specific methods of detection and identification are necessary in order to study their ecology and their role in natural systems.

Early efforts to enumerate viable nitrifiers in lakes relied on the MPN (most probable number) technique. This technique is problematic in that it is inherently imprecise and grossly underestimates the population [*Hall*, 1986]. Moreover comparison between studies is difficult due to the sensitivity of bacteria to enrichment conditions. Another method, fluorescent polyclonal antibodies, has been used to study the serological diversity and distribution of nitrifying bacteria in soils [*Besler and Schmidt*, 1978; *Schmidt*, 1974], sewage [*Yoshioka et al.*, 1982], lake sediments [*Smorczewski and Schmidt*, 1991], and marine environments [*Ward and Perry*, 1980; *Ward and Carlucci*, 1985]. This method detects nitrifiers based on cross-reactivity to antibodies directed against cell wall components of known isolates; thus it requires the isolation and culture of bacterial strains. It is widely accepted that, although many bacterial cells present in a natural population appear viable, they cannot be isolated due to the limitation of classical cultivation techniques such as selective media. Moreover in the case of chemoautotrophs, which grow very slowly, and therefore require months or even years for isolation and purification, development of detection methods which do not require culturing would be advantageous.

More recently, studies based on the analysis of 16S rRNA and the use of the polymerase chain reaction have investigated the spatial distribution in the natural environment of microbial taxa which have not been grown in culture [*Giovanni and Cary*, 1993]. This technique has been applied to the detection and analysis of NH_4^+ oxidizers of the beta subclass in natural samples [*McCaig et al.*, 1994; *Nejdat and Abeliovich*, 1994; *Teske et al.*, 1994; *Voytek and Ward*, 1995; *Hiorns et al.*, 1995]. The technique provides mainly qualitative or semiqualitative information and cannot differentiate between dead, dormant, and active cells.

Fluorescent-probe in situ hybridization (FISH) extends the use of comparative 16S rRNA sequencing to rapid determinative and autocological studies of

specific microorganisms in the natural environment [*Giovanni et al.*, 1988; *De Long et al.*, 1989; *De Long*, 1993; *Amann et al.*, 1991, 1995]. The technique relies on the permeability of most bacterial cells, when fixed, to short fluorescently labeled oligonucleotide probes. These probes can be designed to be specific on different taxonomic levels ranging from domains to subspecies [*Giovanni et al.*, 1998; *Amann et al., 1995*]. The detection of individual bacteria is dependent on the presence of sufficient ribosomes per cell (on the order of 10^3 per cell). Therefore not only can bacteria be differentiated on many phylogenetic levels, but the technique also gives information on physiological state of the bacteria on the basis of the number of ribosomes per cell.

A first step in discerning the underlying mechanisms controlling nutrient distributions is to determine the distribution of the bacteria responsible for the processes of nitrification and denitrification. Information on the distribution of denitrifying bacteria and measurements and estimate of rates of transformation in Lake Bonney are presented elsewhere [*Priscu et al.*, 1996; *Priscu*, in press; *Ward and Priscu*, in press]. In this study, we used three techniques (IFA, PCR, and FISH) to determine the abundance of nitrifying bacteria in the east and west lobes of Lake Bonney.

METHODS AND MATERIALS

Study Site

Lake Bonney (77°43′S, 162°20′E) is a large permanently ice-covered (4–5m thick) lake within the Taylor Valley. The lake is separated into two lobes by a narrow sill (approximately 50 m wide and 13 m deep). The area of the east lobe of Lake Bonney is 3.5 km^2 and the maximum depth is 40 m. The temperature increases from 0°C just beneath the ice to 6.1°C at 15 m and decreases to –2°C near the bottom [*Priscu et al.*, 1993; *Priscu et al.*, 1996]. Above 20 m, dissolved O_2 is supersaturated, 45.8 mg l^{-1}, and the water is fresh above 13 m. Below 20 m, the water becomes anoxic and hypersaline, reaching salinities more than 5 times seawater. A strong chemocline occurs at about 17 m (Figure 1A). The concentrations of oxidized forms of nitrogen increase in the deeper layers and remain high to the bottom. A broad N_2O-N peak occurs between 20–24 m, reaching values exceeding 41 μM. The west lobe of Lake Bonney is smaller than the east lobe with a total surface area of 1.3 km^2 and a maximum depth of 40 m. In the fresh layer above 15m, the dissolved O_2 is supersaturated [*Priscu et al.*, 1993; *Priscu et al.*, 1996]. The deep water is anaerobic and salinities reach 6 times seawater. The temperature range is from –2 to 3°C. The chemocline and oxycline are shallower in the west lobe, beginning at 13 m and 15 m respectively (Figure 1B). Nitrogenous compounds are low in the surface waters. NH_4^+ is regenerated beneath the trophogenic zone and steadily increases to 300 μM at the bottom (40m). NO_2^-, NO_3^-, and N_2O-N peak between 13–17 m, with concentrations reaching 0.6 μM, 25 μM, and 1.1 μM respectively, and then decrease rapidly.

Sample Collection

Sampling was done through a 25 cm diameter hole in the surface ice (4 m thick) at a central site in each lobe during the 1993-1994 and 1994-1995 austral spring and summer (October–December). Bacterioplankton samples were collected in Niskin bottles from ten depths. Depths are reported relative to the free water surface, i.e., the level to which water rose in the sampling hole. Samples (200 ml) for IFA and total bacterial counts were preserved in buffered formalin, 2% final concentration, and stored at 4°C. Subsamples (1–2 ml) were fixed in 4% freshly prepared formaldehyde for in situ hybridization probing (see below). Samples for DNA analysis (approximately 4 liters) were concentrated 100 fold by ultrafiltration using a Filtron (Northborough, MA) open channel Ultrasette with a 300 kd nominal molecular weight cutoff membrane or a Pellicon tangential flow filtration system using GVLP, 0.22 μm pore size ultrafilters (Millipore). The concentrate was filtered onto a 47 mm, 0.2 μm pore size Gelman Supor filter. Filters were stored frozen in EDTA (0.5 M, 0.5 ml) until total DNA was extracted.

Staining and Enumeration of Total Bacteria and Nitrifiers Serotypes

Replicate 5 and 10 ml aliquots of the formalin preserved samples were filter concentrated to 1 ml onto prestained Poretics 0.2 μm polycarbonate filters and stained with DAPI (final concentration 0.05 μg ml^{-1}; Sigma Chemical Corp.) following the protocol of *Porter and Feig* [1980]. Total bacterial counts were obtained by enumeration with a Zeiss epifluorescent scope at x1000, using a 100W HBO mercury lamp and Zeiss filter set: BP365/10 excitation, LP395 barrier, and FT510 dichromatic beam splitting filters.

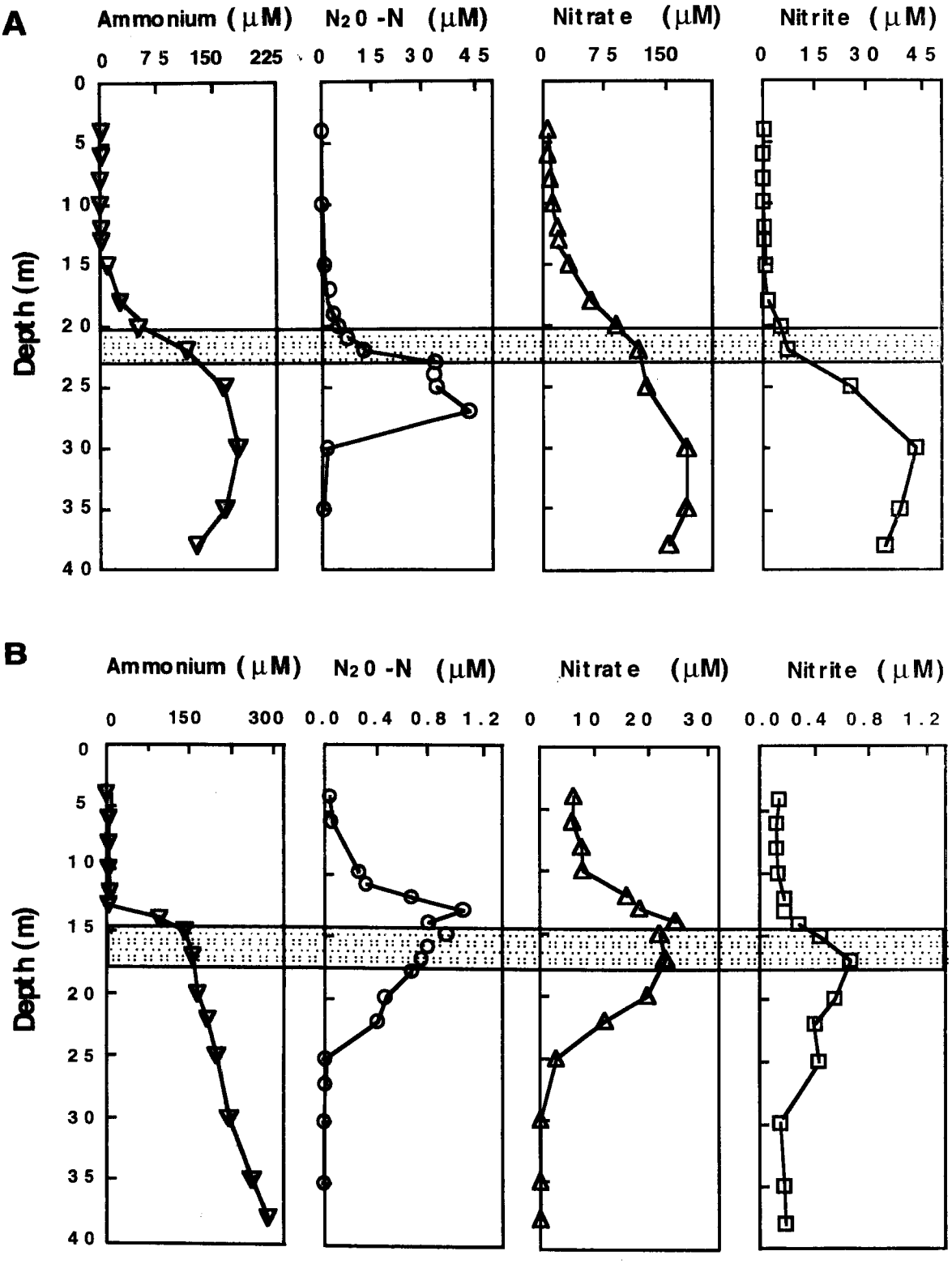

Fig. 1. Dissolved inorganic nitrogen (NH_4^+, N_2O, NO_3^-, NO_2^-) in the east lobe (A) and west lobe (B) of Lake Bonney, November 1993. Stipled bars demarcate the oxic/anoxic transition zone.

Immunofluorescent enumeration of NH_4^+ oxidizing bacteria was done by the method described by *Ward and Carlucci* [1985] using antisera raised against *Nitrosomonas marina* and *Nitrosococcus oceanus*. The antisera used are fully described by *Ward and Carlucci* [1985]. Two sets of duplicate 20 ml aliquots were filtered onto prestained Poretics 0.2 μm polycarbonate filters. Separate filters were stained with each antiserum and duplicates of each serotype were counted. Stained samples were enumerated with a Zeiss epifluorescence scope at x1000, using a 100W HBO mercury lamp and Zeiss filter set: 450DF55 excitation, 505DF35 barrier, and 505drlext02 dichromatic filters. A minimum of 200 cells or 300 fields were counted to calculate abundances.

DNA Extraction and PCR Amplification

High molecular weight DNA was extracted and purified from the frozen filters of concentrated bacteria following a standard protocol with slight modification [*Ausubel et al.*, 1989; *Kerkhof and Ward*, 1993, *Voytek and Ward*, 1995]. The quality of the extracted DNA template was confirmed using the universal 16S rRNA primers EUB 1 and EUB 2 [27 forward and 1525 reverse; *Liesack et al.*, 1991]. PCR prime corresponding to conserved sequences within the 5' and 3' regions of the 16S rDNAs of beta subclass [NITA and NITB; *Voytek and Ward*, 1995] and gamma-subclass [NOC1 and NOC2; *Voytek and Ward*, 1997] NH_4^+ oxidizers were used to detect these groups. Amplification with these primers yields a 1080 bp NIT product and a 1130 bp NOC product. Two stage PCR amplifications were performed following the protocol described in *Voytek and Ward* [1995; 1997] with slight modification. To reduce amplification of non-specific products the total number of cycles was reduced to 30.

Semiquantification of Ammonium Oxidizers

Direct quantification of the number of organisms in a natural sample based on specific PCR products amplified from DNA extracted from these samples is difficult. In all steps of the collection, extraction, and amplification procedures, error and uncertainty are introduced. Some cells are more efficiently collected by ultrafiltration and not all cells readily lyse in standard DNA extraction protocols. Additionally the template copy number per cell may vary almost ten-fold and some templates may amplify more efficiently than others [*Suzuki and Giovanonni*, 1996]. Furthermore *Farrelly et al.* [1995] have shown that without foreknowledge of the genome size and gene copy number in all members, accurate estimations of the abundance of a particular species is impossible. The two-step amplification protocol used here reinforces these errors. For these reasons, an absolute determination of the abundance of NH_4^+ oxidizers with PCR was not possible. In order to determine the relative abundance of NH_4^+ oxidizers in each natural sample, the EUB amplified product was diluted in TE before amplification with the NOC or NIT primers. The dilution series include 5 dilutions (undiluted, 1:2, 1:5, 1:10, and 1:50). The greater the dilution factor, the higher the number of the original template molecules, and thus the higher the number of NH_4^+ oxidizers present in the original sample. A number was assigned to a sample based on the highest dilution in the series that yielded the correct product (e.g., amplification of undiluted samples was assigned 1: 1–50 was assigned 5).

In Situ Hybridization

Cells collected by centrifugation from subsamples (1–2 ml) were washed in PBS and resuspended in 4% formaldehyde, followed by storage at 4°C for 4–16 h. Preserved cells were spotted on gelatin subbed slides and treated with serial ethanol rinses (50, 75, and 100%) as described by *DeLong* [1993]. The following labeled probes were used: (i) EUB 338 complementary to a region of the 16S rRNA conserved in the domain Bacteria [*Amann et al.*, 1995]; (ii) NIT A, B, and C, oligonucleotides complementary to and specific for selected regions of the 16S rRNA molecules of the beta subclass NH_4^+ oxidizers of the Proteobacteria [*Voytek and Ward*, 1995]. The hybridization properties of the FITC labeled oligonucleotides were determined empirically using fixed whole cells from homologous (*Nitrosomonas europaea* cultures) and heterologous species (*Escherichia coli* and *Nitrosococcus oceanus*, a gamma subclass nitrifier, cultures). The NIT probe was also tested with the pure culture of *Nitrosomonas europaea* diluted approximately 10 to 1 with *E. coli*. The optimal conditions for hybridization are reported in *Voytek* [1996]. DAPI staining was performed following the protocol of *Hicks et al.* [1992] for dual staining with DAPI and fluorescent rRNA probes. Slides were stored desiccated at –20°C in the dark. Epifluorescence microscopy was used to visualize cells using the filter sets described above. A minimum of 200 cells or 300 fields were counted to calculate abundances of hybridized cells.

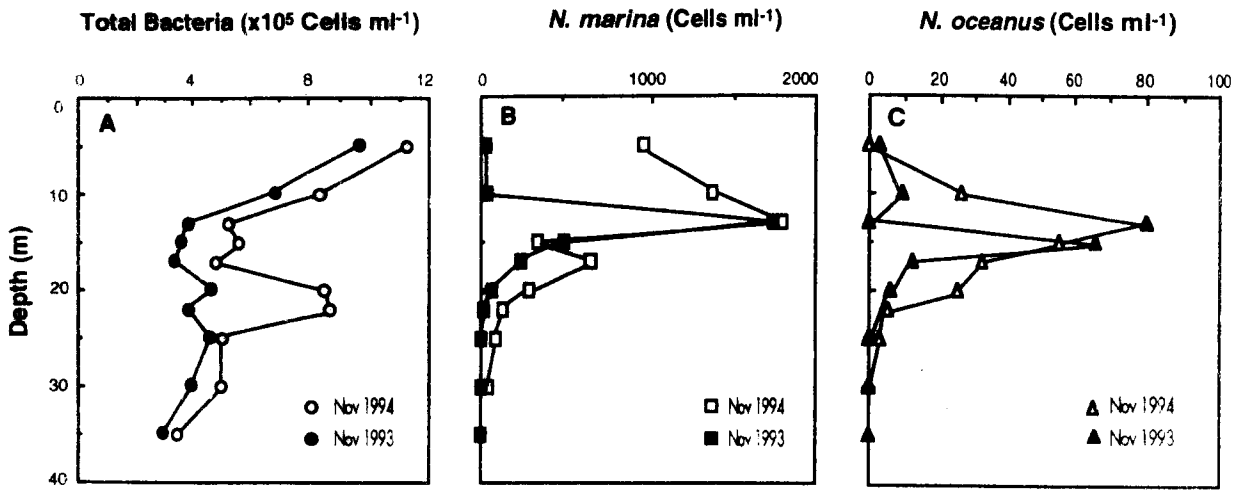

Fig. 2. The depth distribution of total bacteria (A), *Nitrosomonas* serotyped ammonium oxidizers (B) and *Nitrosococcus oceanus* serotyped ammonium oxidizers (C) in the east lobe of Lake Bonney, November 1993 and November 1994. Abundances determined by epifluorescence microscopy using DAPI or FITC. See text for details.

RESULTS

Total Bacterial Community and DAPI Counts

The bacterioplankton population in all samples counted contained a variety of morphotypes but was dominated by rod-shaped cells less than 1 μm in length. The second most abundant cell type was long and filamentous (10 μm). In both lobes and in both years the total abundance of bacteria was between 10^5 and 10^6 cells ml^{-1} (Figures 2A and 3A). Although the absolute numbers of cells are slightly higher in 1994, the depth profiles from year to year did not change substantially. In both lobes, the highest concentrations of cells were observed in the surface and cell number decreased with depth. In the east lobe, there was a small subsurface peak of bacteria at 20 m.

Abundances of N. europaea and N. oceanus Serotypes by IFA

The cell concentrations of *N. marina* serotype were

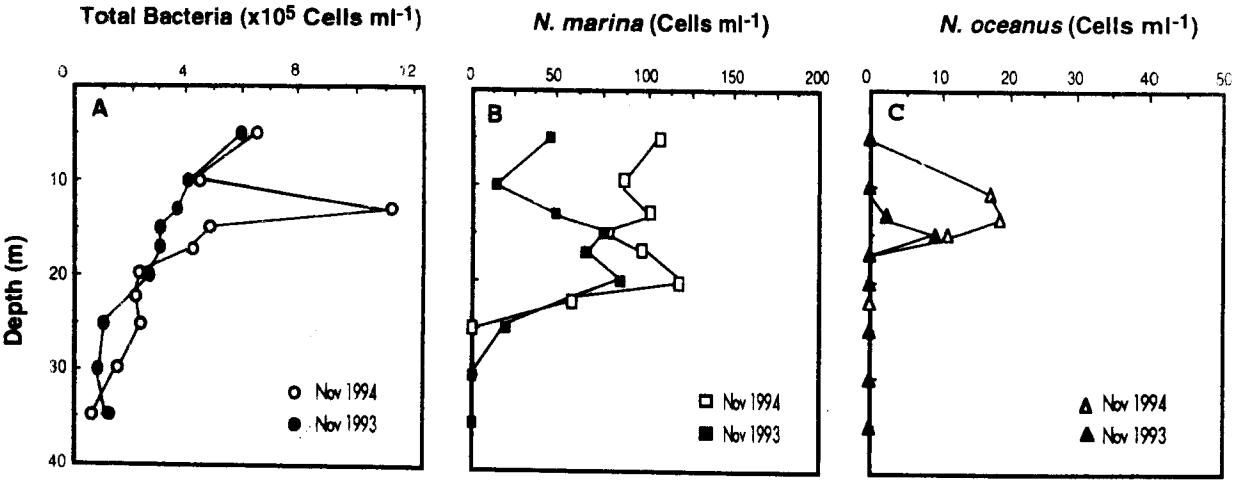

Fig. 3. The depth distribution of total bacteria (A), *Nitrosomonas* serotyped ammonium oxidizers (B) and *Nitrosococcus oceanus* serotyped ammonium oxidizers (C) in the west lobe of Lake Bonney, November 1993 and November 1994. Abundances determined by epifluorescence microscopy using DAPI or FITC. See text for details.

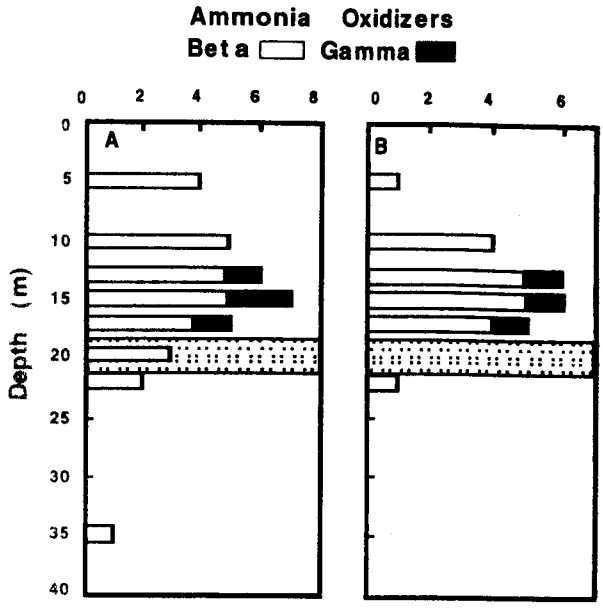

Fig. 4. Relative abundance of beta and gamma subclass ammonium oxidizers in the east lobe of Lake Bonney, November 1993 (A) and November 1994 (B) determined by PCR. Stipled bars demarcate the oxic/anoxic transition zone.

on the order of 10^2 to 10^3 ml^{-1} and were one to two orders of magnitude higher than the *N. oceanus* serotype in the west and east lobes, respectively (Figures 2B, C and 3B, C). In general, cell concentrations of both serotypes were much higher in the east lobe. *N. marina* had a broad surface maximum in the west lobe (Figure 3B) and a large peak at 13m in the east lobe (approximately 2000 cells ml^{-1}; Figure 2B). The distribution of *N. oceanus* was much more limited; cell densities were low in the surface and peaked at 13 m in both lobes. At depths below the peak abundances, concentrations fell off steeply. Virtually no NH_4^+ oxidizers of either serotype were detected deeper than 22 m.

Semiquantification of Ammonia Oxidizers by PCR

In both 1993 and 1994, nitrifying bacteria were detected by the PCR assay in samples taken from seven depths above 25 m in the east lobe of Lake Bonney (Figure 4). In 1993, the relative abundances of both groups were somewhat higher and beta nitrifiers were also detected at 35 m (Figure 5B). In both years, the beta NH_4^+ oxidizers were far more abundant than the gamma and had a broader distribution pattern. The highest abundances of beta nitrifiers were observed in the aerobic waters above the chemocline while gamma nitrifiers had a small peak around 15 m, just above the oxygen transition zone (Figure 4). The overall abundance of nitrifiers in the west lobe was considerably lower (Figure 5). Beta nitrifiers were most abundant above the oxycline and the distribution of the less abundant gamma nitrifiers was limited to the oxygen transition zone. During the 1993 sampling season, NH_4^+ oxidizing bacteria were detected throughout the water column (Figure 5A). The distribution of nitrifiers was more limited in samples taken at the same time the following year (Figure 5B).

In Situ Hybridization

In probe hybridizations of pure cultures, close to 100% of the cells were detectable using the EUB 338 for cells in both *E. coli* and *N. europaea*. The stained *N. europaea* cells were much dimmer than the *E. coli* cells. The NIT A, B, and C probes hybridized to >98% of the *N. europaea* cells in culture. In the mixed sample of 10% of the *N. europaea* culture diluted in *E. coli*, the NIT probes hybridized to >96% of the *N. europaea* added (approximately 10% of the total bacterial cells present). None of the nitrifier specific probes hybridized to the heterologous strains. Samples collected from the east lobe, November 1993, were probed with fluorescently labeled oligonucleotides. Cell concentrations were estimated using DAPI counts for

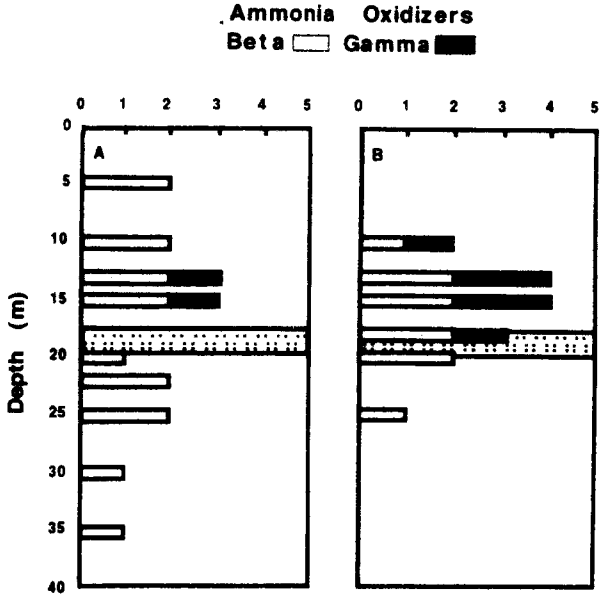

Fig. 5. Relative abundance of beta and gamma subclass ammonium oxidizers in the west lobe of Lake Bonney, November 1993 (A) and November 1994 (B) determined by PCR. Stipled bars demarcate the oxic/anoxic transition zone.

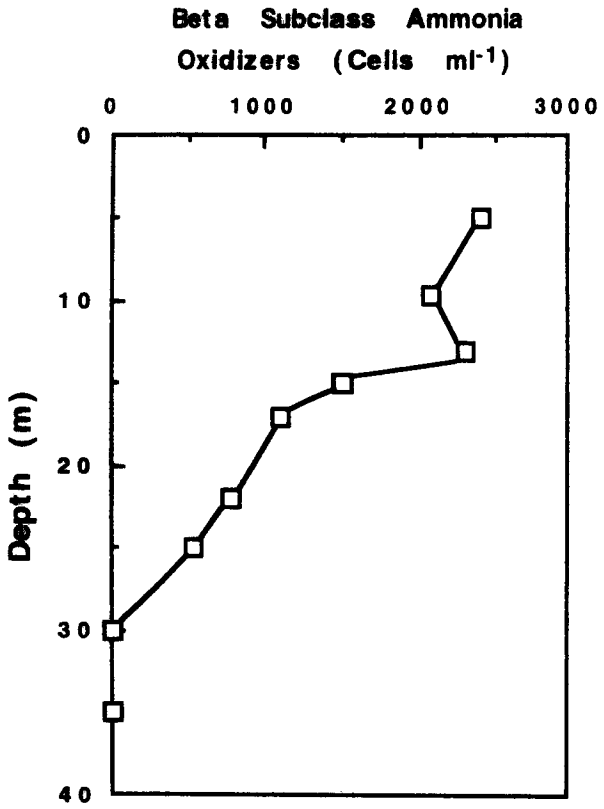

Fig. 6. The distribution of beta subclass ammonium oxidizers in the east lobe of Lake Bonney, November 1993. Abundances determined by fluorescent in situ hybridization (FISH).

total bacteria to correct for any loss of cell material during processing. Beta-subclass NH_4^+ oxidizers were observed in 7 of the 10 samples taken at depths in the east lobe (Figure 6). The highest cell density observed was approximately 2300 cells ml^{-1} at 5 m. This peak was a part of a broad maximum of FISH labeled cells between 5–13 m. Cell densities decreased sharply below 13 m. The overall brightness (i.e., signal strength) of the individual cells appeared to decrease with depth and increasing salinity. Background fluorescence also increased in the deeper samples.

DISCUSSION

The overall patterns of nitrifier abundances found in the two lobes of Lake Bonney were very similar with both the IFA and PCR detection techniques. In general, there were more beta subclass than gamma subclass NH_4^+ oxidizers present in this lake, and the beta subclass was more widely distributed. One reason for this difference is simply that there are many genera of NH_4^+ oxidizers in the beta subclass and they have been found in soils, marine, and freshwater environments. The gamma subclass has one genetically confirmed member, thus far, and it is the marine species *Nitrosococcus oceanus*. It is not surprising that one or two species from a limited environmental niche may be less abundant and have a restricted distribution compared to a group with environmentally diverse members.

The IFA and PCR techniques are expected to detect similar subsets of the total NH_4^+ oxidizer population. Overall the pattern of nitrifiers was similar, but there were several samples in which PCR detected nitrifiers where IFA did not. Most of the observed discrepancy was in the distribution of beta-nitrifiers. The PCR assay was based on primers derived from the sequences of many genera of beta nitrifiers while the antiserum used in the IFA assay was raised to a single species of beta subclass NH_4^+ oxidizer. Therefore we might expect the IFA assay to pick up only a narrow subset of the total beta nitrifier population present while the PCR technique could detect a broader species assemblage. Additionally the efficacy of the IFA assay is dependent on the specificity of the antisera used. As mentioned above, IFA has been used to study nitrifying bacteria in many environments [e.g. *Schmidt*, 1974; *Yoshioka et al.*, 1982; *Smorczewski and Schmidt*, 1991; *Ward and Carlucci*, 1985]. Generally in these studies, antiserum is raised to a strain isolated originally from a similar environment to the one being investigated. No NH4+ oxidizers have been isolated from Lake Bonney despite three years of cultivation efforts. The antisera we used in this study were raised against two marine isolates from temperate waters: *N. marina* and *N. oceanus*. The *N. marina* antiserum cross reacts with the soil nitrifier, *N. europaea* and a subset of the nitrifier strains isolated from a variety of marine environments [*Ward and Carlucci*, 1985; *Voytek and Ward*, 1995]. Neither of the antisera cross react with most of the known soil strains or the estuarine nitrifier isolate (*Nitrosococcus mobilis)* available in culture [*Voytek and Ward*, 1995]. In contrast, all of these NH_4^+ oxidizers can be amplified with the NITA-B primers [*Voytek and Ward*, 1995]. The biological habitats of Lake Bonney are quite different from temperate freshwater or marine environments and the nitrifier community probably reflects those environmental differences. Therefore we suspect that our results from the IFA may have been more closely correlated to the PCR results if we had been able to use antisera from nitrifiers isolated from this system.

Less frequently, IFA detected nitrifiers and the PCR technique did not (e.g., gamma subclass nitrifiers at 20 and 22 m in the east lobe; Figures 2C and 4). This may be due to interference of the PCR amplification from competing non-target DNA. PCR primers amplify target DNA less efficiently when the target is rare relative to the total DNA present. Interference would be most severe in samples containing a potentially large quantity of competing template. A peak in total bacterial cells was observed at these depths (Figure 2A), therefore interference might be greatest there. Alternatively, immunofluorescence may be overestimating the number of "live" cells in the surface. Both detection techniques (PCR and immunofluorescence) may overestimate to some extent since they rely on features of the cell that persist after the cell has lost viability. Survival studies have suggested that a metabolic shutdown occurs when bacteria are severely nutrient limited [*Moyer and Morita*, 1989a and 1989b]. In these studies bacterial cells remained physically intact and maintained their cellular protein levels but cellular DNA content decreased to less than 5% and viability dropped to <0.3% [*Moyer and Morita*, 1989b]. Additionally *Zweifel and Hagström* [1995] found that only a small fraction (2–32%) of intact bacteria enumerated by traditional fluorescent staining techniques actually contained nucleoids. It is possible that the immunofluorescence technique picks up intact cells that contain no or substantially degraded genomic DNA, which would not be picked up by PCR. If this is the case, the estimate of abundance obtained by quantitative PCR assays would be expected to correlate more closely with the activity of nitrifiers than the estimates obtained by immunofluorescence.

The depth profile of fluorescently labeled (FISH) beta subclass nitrifiers generally followed the shape of the profile generated by the less quantitative PCR method. The highest numbers of fluorescently stained cells were found in the surface waters above 15m (Figure 6). In the east lobe, cell densities were on the order of 2000 cells ml^{-1} in the surface and decreased sharply with depth. No cells were detected in samples from 30 m or deeper. The high density of fluorescently labeled cells at 13 m corresponds to the peak observed of *N. marina* serotyped cells (Figures 2b and 6); however much lower densities of *N. marina* serotype cells were observed at shallower depths. This suggests that the peak of beta nitrifiers at 13 m is dominated by a nitrifier species of the *N. marina* serotype. Above that depth however, the nitrifiers present were detected by PCR and FISH and are, therefore, genotypically related but are not phenotypically similar to the *N. marina* serotype. Like the PCR technique, FISH is more inclusive and can detect a wider variety of beta-subclass NH_4^+ oxidizers.

The fluorescence signal strength was higher in hybridized cells examined at depths above 20 m. This may be an indication of higher metabolic activity of the cells at these depths. Indeed the profiles of nitrogenous compounds (Figure 1a and 1b) [*Voytek*, 1996] and the rates of nitrification measured in this lake [*Priscu et al.*, 1996] support this hypothesis. Below 20 m, fluorescently labeled cells were present at concentrations of 100–1000 cells ml^{-1}, but the signal strength was significantly attenuated. In recent papers by *Priscu* [1996] and *Priscu et al.* [1996], the lower N_2O peak observed in the east lobe of Lake Bonney was attributed to relict nitrifying activity. No current activity has been detected at this depth; therefore it was postulated that nitrifier cells active years ago were responsible. Presumably cells that are not metabolically active would rapidly lose rRNA and not be detected. Low-temperature, highly-saline waters may serve as an excellent medium for preservation of non-active, intact cells and would explain persistent detection, albeit at a reduced signal, where no activity is found. An alternative explanation of the diminished signal in samples taken from the deeper depths is the effect of the hypersaline waters from which they were harvested. The prehybridization washing steps in the in situ protocol may not have been adequate to remove the excess salts and may have changed the hybridization conditions in these samples. Higher salt concentrations reduces the stringency of the hybridization solution and may cause more non-specific binding of the probes. The higher background fluorescence observed in these samples may be an indication of this.

Small subunit rRNA-based oligonucleotide probes have become increasingly useful as a tool for characterizing microbial cells in environmental samples [*Amann et al.*, 1995]. The in situ identification and enumeration of individual bacteria by FISH is dependent on the presence of sufficient ribosomes per cell. The detection limit is around 10^3 to 10^4 ribosomes per cell [*DeLong et al.*, 1989; *Amann et al.*, 1995], and therefore sensitivity depends in part on the physiological state of the target cells. Faster growing or more physiologically active cells tend to have more ribosomes and hence bind proportionately more probe molecules. One limitation to FISH is the potential inability to detect slow growing cells (e.g., some bacterial symbionts), which may have too few

ribosomes per cell to provide a signal. To gain greater sensitivity, researchers have attempted to label oligonucleotides with multiple fluorescent molecules, but this resulted in poor or non-specific binding [*DeLong*, 1993]. Greater sensitivity may be achieved by employing indirect labeling methods (e.g., biotin/avidin systems [*Singer et al.*, 1986]), coupled with more sensitive detection systems (e.g., chemiluminescent detection). Cell detection and enumeration by flow cytometry [*Amann et al.*, 1991] is another potential technique for improving the efficiency of counting cells that are in low abundance or rare in natural samples.

Overall we found little variation between samples taken in 1993 and 1994 for both total bacterial counts and for the specific subsets of nitrifying bacteria. This is consistent with the observation of little seasonal or year to year variation in total bacterial counts in samples taken over the past ten years of sampling in Lake Bonney (Priscu, unpublished data). The total bacteria counts measured here were on the order of 10^5–10^6 cells ml^{-1}, which is typical for polar lakes [*Vincent*, 1988; *Simmons et al.*, 1993]. The beta subclass nitrifiers represented up to 1% of the total bacterial population in the east lobe of Lake Bonney determined by FISH and IFA. The gamma subclass was a much less significant fraction of the total population in both lobes. These abundances are quite high for aquatic environments. Using IFA, studies in marine systems have estimated the abundance of nitrifying bacteria to be on the order of 1–10 cells ml^{-1}, and, unlike in our studies, the cell densities were relatively constant throughout the water column [*Ward and Carlucci*, 1985; *Ward*, 1986]. Based on MPN or calculations from measuring nitrification activity, the estimated range of nitrifier cell densities in lakes is between 1 and 10^4 cells ml^{-1} [*Hall*, 1986].

The depth distributions of NH_4^+ oxidizers were very similar no matter which of the three methods were used. NH_4^+ oxidizing bacteria were detected throughout the water column in both lobes but were less abundant in the west than in the east lobe. In general the highest cell numbers of beta nitrifiers were found in the surface waters above the oxycline or in association with the nutricline. Despite the abundance of NH_4^+ oxidizers in the surface waters, NO_2^- and NO_3^- do not accumulate, presumably because these nutrients are readily used by phytoplankton and bacteria in the euphotic zone [*Priscu et al.*, 1995]. The distribution of the less abundant gamma nitrifiers was generally limited to the oxygen transition zone. The distribution pattern of nitrifiers suggests that nitrification was probably responsible for the shallow portion of the peaks of N_2O, NO_2^-, and NO_3^- observed below 20 and 13 m in the east and west lobes respectively. Both beta and gamma nitrifiers were low in number or absent in the deep waters. Based on the low FISH signal strength of cells from deeper depths and undetectable nitrification rates for these depths, nitrifiers detected in the anoxic bottom waters were probably inactive.

Single cell analyses have proven extremely useful for determining the abundance, activities, and variability of microbial species in their natural environments. Unfortunately very few microbes are amenable to cultivation and so they remain difficult to differentiate, identify, and characterize. PCR, with its capacity to amplify specific sequences of DNA from a few copies of the target molecule, is a tool which resolves some of the limitations of traditional culturing techniques and immunofluorescence. PCR eliminates the need to isolate individual nitrifiers or to subject them to often inconclusive taxonomic tests. The advantages of PCR have been demonstrated in the detection and analysis of NH_4^+ oxidizers of the beta subclass [*McCaig et al.*, 1994; *Nejdat and Abeliovich*, 1994; *Voytek and Ward*, 1995; *Hiorns et al.*, 1995] and gamma subclass nitrifiers [*Voytek and Ward*, 1997; *Voytek*, 1996] in natural samples. FISH extends our ability to understand the structure and dynamics of natural microbial communities by not only providing quantitative information on community structure but also qualitative information of the physiological state of the bacteria present.

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REFERENCES

Amann, R., N. Springer, W. Ludwig, H.-D. Görtz, and K.-H. Schleifer, Identification *in situ* and phylogeny of uncultured bacterial endosymbionts, *Nature*, 351, 161–164, 1991.

Amann, R.I. W. Ludwig, and K-H Schleifer, Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation, *Microbiol. Rev.* 59, 143–169, 1995.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds.), *Current protocols in molecular biology*. Green Publishing Associates and Wiley-Interscience, New York., 1989

Besler, L.W. and E.L. Schmidt, Serological diversity within a terrestrial ammonia-oxidizing population, *Appl. Environ. Microbiol*, 36, 584–593, 1978.

DeLong, E.F., Single cell identification using fluorescently labeled, ribosomal RNA-specific probes. in *Handbook of Methods in Aquatic Microbial Ecology*, edited by P.F. Kemp, B.F. Sherr, E.B. Sherr, and J.J. Cole, pp. 285–294, Lewis Publishers, Boca Raton, FL, 1993.

DeLong, E.F., G.S. Wickam, and N.R. Pace, Phyolgenetic stains: ribosomal RNA based probes for the identification of single cells, *Science*, 243, 1360–1363, 1989.

Downes, M.T., Aquatic nitrogen transformations at low oxygen concentrations,*Appl. Environ. Microbiol.*, 54, 172–175, 1988.

Farrelly, V., F.A. Rainey, and E. Stackebrandt, Effect of genome size and *rrn* gene copy number of PCR amplification of 16S rRNA genes from a mixture of bacterial species, *Appl. Environ. Microbiol.*, 61, 2798–2801, 1995.

Giovannoni, S.J., E.F. DeLong, T.M. Schmidt, and N.R. Pace, Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells, *J. Bacteriol.*, 170, 720–726, 1988.

Goreau, T.J., W.A. Kaplan, S.C. Wofsy, M.B. McElroy, F W. Valois, and S.W. Watson, Production of NO_2 and N_2O by nitrifying bacteria at reduced concentrations of oxygen,*Appl. Environ. Microbiol.*, 40, 526–532, 1980.

Hall, G.H., Nitrification in lakes, in *Nitrification*, edited by J.I. Prosser, pp 127–156, IRL Press, Washington, D.C., 1986.

Head, I.M, W.D. Hiorns, T. Martin, A.J. McCarthy, and J.R. Saunders, The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences, *J. Gen. Microbiol.*, 139, 1147–1153, 1993.

Hicks, R.E., R.I. Amann, and D.A. Stahl, Dual staining of natural bacterioplankton with 4', 6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences, *Appl. Environ. Microbiol.*, 58,2158–2163, 1992.

Hiorns, W.D., R.C. Hastings, I.M. Head, A.J. McCarthy, J R. Saunders, R.W. Pickup, and G.H. Hall, Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of *Nitrosospiras* in the environment, *Microbiol.*, 141, 2793–2800, 1995.

Kaplan, W.A. and S.C. Wofsy, The biogeochemistry of nitrous oxide: a review. in *Advances in Aquatic Microbiology*, vol. 3, pp. 181–206, Academic Press Inc., London, 1985.

Kerkhof, L. and Ward, B.B., Comparison of nucleic Acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium, *Appl. Environ. Microbiol*, 59, 1303–1309, 1993.

Koops, H.-P. and U.C. Möller, The lithotrophic ammonia-oxidizing bacteria, in *The Prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*, 2nd ed., edited by A. Balows, H.G. Truper, M. Dworkin, W. Harder and K.-H. Schleifer, pp. 2625–2637, Springer-Verlag, New York, 1992.

Liesack, W., H. Weyland, and E. Stackenbrandt, Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria, *Microb. Ecol.*, 21, 191–198, 1991.

Lyons, W.B., S.W. Tyler, R.A. Wharton, Jr., and D.M. McKnight, The late Holocene/Paleoclimate history of the McMurdo Dry Valleys Antarctic as derived from lacustrine iotope data, *Paleogeography, paleoclimatology and paleoecology*, in press.

McCaig, A.E., T.M. Embley, and J.I. Prosser, Molecular analysis of enrichment cultures of marine ammonia oxidisers, *FEMS Microb. Let.*, 120, 363–367, 1994.

Moyer, C.L. and R.Y Morita, Effect of growth rate and starvation-survival on cellular DNA, RNA and protein of a psychrophilic marine bacterium, *Appli. Environ. Microbiol.*, 55, 2710–2716, 1989a.

Moyer, C.L. and R.Y. Morita, Effect of growth rate and starvation-survival on the viability and stability of a psychrophilic marine bacterium, *Appl. Environ. Microbiol.*, 55, 1122–1127, 1989b.

Nejdat, A. and A. Abeliovich, Detection of *Nitrosomonas* spp. by the polymerase chain reaction, *FEMS Microb. Let.*, 120, 191–194, 1994.

Porter, K.G. and Y.S. Feig, The use of DAPI for identifying and counting aquatic microflora, *Limnol. Oceanogr.*, 25, 943–948, 1980.

Poth, M. and D.D. Focht, ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier dentrification, *Appl. Environ. Microbiol.*, 49, 1134–1141, 1985.

Poulsen, L.K, G. Ballard, and D.A. Stahl, Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms, *Appl. Environ. Microbiol.*, 59, 13554–1360, 1993.

Priscu, J.C., The biogeochemistry of nitrous oxide in permanently ice-covered lakes of the McMurdo Dry Valleys, Antarctica, in *Microbially Mediated Atmospheric Change*, Special Issue of Global Change Biology, in press.

Priscu, J. C., Phytoplankton nutrient deficientcy in lakes of the McMurdo Dry Valleys, Anarctica, *Freshwater Biol.*, 34, 215–227, 1995.

Priscu, J.C., M.T. Downes, and C.P. McKay, Extreme supersaturation of nitrous oxide in a non-ventilated antarctic lake, *Limnol. Oceanogr.*, 41,1544–1551, 1996.

Priscu, J.C. and M.T. Downes, Relationship among nitrogen uptake, ammonium oxidation and nitrous oxide concentration in the coastal waters of western Cook Strait, New Zealand, *Estuar. Coast. Shelf Sci.*, 20, 529–542, 1985.

Priscu, J.C., B. B. Ward, and M. T. Downes, Water column transformations of nitrogen in Lake Bonney, a perennially ice-covered Antarctic lake, *Ant. J. U.S.* 28 (5), 237–239, 1993.

Priscu, J.C., W.F. Vincent, and C. Howard-Williams, Inorganic nitrogen uptake and regeneration in perennially ice-covered Lakes Fryxell and Vanda, Antarctica, *J. Plankton Res.*, 11, 335–351, 1989.

Schmidt, E.L., Quantitative autecological study of micro-organisms in soil by immunofluorescence, *Soil Sci.* 118, 141–149, 1974.

Simmons, G.M., J.R. Vestal, and R.A. Wharton,

Environmental regulators of microbial activity in continental Antarctic lakes, in *Antarctic Microbiology*, edited by W. Vincent, pp. 491–541, Wiley-Liss, Inc., 1993.

Singer. R.H., J.B. Lawrence, and C. Villnave, Optimization of *in situ* hybridization using isotopic and non-isotopic detection methods, *Biotechniques*, 4, 230, 1986.

Smorczewski, W.T. and Schmidt, E.L., Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, eutrophic lake sediment, *Can. J. Microbiol.*, 37. 828–833, 1991.

Spigel, R.H. and J.C. Priscu, Evolution of temperature and salt structure of Lake Bonney, a chemically stratified Antarctic lake, *Hydrobiologia*, 1996.

Suzuki, M.T. and S.J Giovannoni, Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes in PCR, *Appl. Environ. Microbiol.*, 62: 625–630, 1996.

Teske, A., E. Alm, J.M. Regan, S. Toze, B.E. Rittmann, and D. A Stahl, Evolutionary elationships among ammonia- and nitrite oxidizing bacteria. *J. Bacteriol*, 176 (21), 6623–6630, 1994..

Vincent, W. 1988. *Microbial Ecosystems of Antarctica*. Cambride University Press, Cambridge, England. 304pp.

Voytek, M.A., Relative abundance and species diversity of autotrophic ammonium-oxidizing bacteria in aquatic systems, Ph.D. Dissertation, University of California, Santa Cruz, 1996.

Voytek, M.A., J.C. Priscu, and Ward, B.B, Detection of the beta and gamma subclass ammonia-oxidizing bacterium in six lakes in Antarctica using the polymerase chain reaction, in press, 1997.

Voytek, M.A. and Ward, B.B., Detection of ammonium-oxidizing bacteria of the beta-subclass proteobacteria in aquatic samples with the PCR. *Appl. Environ. Microbiol.*, 56, 2430–2435, 1995.

Voytek, M.A. and Ward, B.B., Detection of the ammonia-oxidizing bacterium, *Nitrosococcus oceanus*, in seawater using the polymerase chain reaction. *FEMS Microbiol. Lett.*, submitted, 1997.

Ward, B.B., Nitrification in marine environments. in *Nitrification*, edited by J. I. Prosser, pp 157–184, IRL Press, Washington, D.C., 1986.

Ward, B.B., and A.F., Carlucci Marine ammonia- and nitrite-oxidizing bacteria: Serological diversity determined by immunofluorescence in culture and in the environment. *Appl. Environ. Microbiol.* 50: 194–201, 1985..

Ward, B.B., and M.J. Perry,. Immunofluorescent assay for the marine ammonium-oxidizing bacterium, *Nitrosococcus oceanus. Appl. Environ. Microbiol.*, 39, 913–918, 1980.

Ward, B.B., and J.C. Priscu, Detection and characterization of denitrifying bacteria from a permanently ice-covered Antarctic lake, *Hydrobiologia*, in press, 1997.

Ward, D.M., R. Weller, and M.M. Bateson, 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community, Nature, 345:63–65, 1990.

Watson, S.W., Characteristics of a marine nitrifying bacterium, *Nitrosocystis oceanus* sp. n. *Limnol. Oceanogr.*, 10, R274–289, 1965.

Watson, S.W., E. Bock, E. Harms, H. -P. Koops, and A.B. Hooper, Nitrifying bacteria in *Bergey's Manual of Systematic Bacteriology* vol. 3, edited by J.T. Staley, M.P. Bryant, N. Pfennig and J.G. Holt, pp. 1808–1834, The Williams & Wilkins Co., Baltimore, 1989.

Yoshioka, T., H. Hisayoshi, and Y. Saijo, Growth kinetic studies of nitrifying bacteria by the immunofluorescent counting method, *J. Gen. Appl. Microbiol.*, 28, 169–180, 1982.

Zweifel, U.L. and A. Hagström, Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts), *Appl. Environ. Microbiol.*, 61, 2180–2185, 1995.

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