

Potential role of copper availability in nitrous oxide accumulation in a temperate lake

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Abstract

Denitrifying bacteria require copper for the synthesis of nitrous oxide reductase. In the absence of sufficient bioavailable Cu, nitrous oxide (N₂O) may accumulate in natural waters during denitrification. Cultures of *Paracoccus denitrificans* and natural bacterial assemblages collected from a mesotrophic lake (Linsley Pond) were grown at varying Cu concentrations to determine the Cu speciation that results in elevated N₂O accumulation. *P. denitrificans* experienced Cu limitation beginning at inorganic Cu concentrations of 0.6 fmol L⁻¹ (-log of inorganic Cu, pCu', = 15.2). The natural community did not show an effect until pCu' was reduced to 23.7 with 8-hydroxyquinoline, resulting in an approximate 10-fold increase in N₂O concentrations during denitrification. Additions of ethylenediaminetetraacetic acid alone to natural communities did not affect N₂O concentrations. Natural copper-binding ligands detected with competitive ligand exchange-cathodic stripping voltammetry occurred at concentrations of 6 to 25 nmol L⁻¹ with conditional stability constants ($K_{Cu^{2+}L}$) between 10^{14.4} and 10^{15.1}. Although more than 99% of total Cu in Linsley Pond was bound to these ligands, inorganic Cu concentrations remained 10 orders of magnitude above those found to increase N₂O accumulation during denitrification incubations. Measurements of nitrogen species, dissolved oxygen, and sulfide in the water column of Linsley Pond over the spring growing season revealed that N₂O was produced by assimilatory nitrate reduction and nitrification in addition to denitrification, with nitrification generating most of the N₂O found in the surface waters of the lake. The results suggest that inorganic Cu concentrations in Linsley Pond are sufficient to support denitrification. Moreover, some denitrifying bacteria may be able to access organically bound Cu, reducing the potential for this metal to affect N₂O production in other aquatic environments.

Nitrous oxide (N₂O) is a greenhouse gas that can also contribute to the depletion of ozone (Nevison and Holland 1997). As the global nitrogen cycle is modified by human activities, atmospheric concentrations of N₂O are increasing by 0.25% yr⁻¹ (Machida et al. 1995). There are several nitrogen conversion processes that could contribute to this increase. Nitrous oxide is produced as a free intermediate during denitrification and as a side product during nitrification (Goreau et al. 1980; Zumft 1997). It may also be produced by autotrophs during assimilatory nitrate reduction (Weathers 1984) or through a combination of ammonium oxidation and NO₂⁻ reduction (Codispoti and Christensen 1985). Local environmental conditions, including the O₂ concentration (Goreau et al. 1980) and

presence of sulfide (Sorensen et al. 1980), appear to control the yield of N₂O during these biologically mediated processes.

There remain uncertainties about the magnitude of various sources of N₂O to the atmosphere. The ocean is a net source of between 7 and 11 Tg yr⁻¹ N as N₂O (Bange et al. 1996), due to surface waters that are typically supersaturated with respect to the overlying atmosphere. Subsurface N₂O concentrations are generally inversely correlated with apparent oxygen utilization (AOU), implicating nitrification as the source of the N₂O (Cohen and Gordon 1979). Stable isotope data also implicate nitrification as the primary source of N₂O in the ocean (Kim and Craig 1990). However, highly productive upwelling zones in the Arabian Sea and off Peru are characterized by double subsurface N₂O maxima, and denitrification may be the more important source of nitrous oxide in these areas (Codispoti et al. 2001).

Nitrous oxide dynamics have received less attention in freshwater systems than in oceans, but most studies indicate that rivers and lakes serve as sources (Mengis et al. 1997; Cole and Caraco 2001). Nitrous oxide flux from rivers and lakes may be high during certain times of the year, yet freshwater ecosystems likely contribute less than

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20% of total global N₂O emissions annually (Seitzinger et al. 2000). Although it can be consumed in anoxic hypolimnia via denitrification, N₂O is often supersaturated in epilimnia and may be produced at the oxic/anoxic boundaries of lakes (Mengis et al. 1997). In particular, N₂O appears to be produced during denitrification in transition zones where O₂ is present at concentrations between 5 and 30 μmol L⁻¹ (Yoh et al. 1983). Mesotrophic and eutrophic lakes often become anoxic in summer months, resulting in conditions for both the production and consumption of N₂O during denitrification.

Although dissolved oxygen has received the most attention, other environmental factors may also control the yield of nitrous oxide during denitrification in natural aquatic systems. The enzyme that catalyzes the reduction of N₂O to N₂, nitrous oxide reductase, contains four Cu atoms per molecule (Zumft 1997), imparting on denitrifying bacteria an obligate requirement for Cu. Laboratory studies with several strains of denitrifying bacteria have shown that N₂O accumulates in Cu-limited cultures of denitrifying bacteria (Iwasaki and Terai 1982) and that these denitrifiers cannot utilize N₂O in the absence of Cu (Matsubara et al. 1982). More recently, Granger and Ward (2003) estimated the concentration of inorganic copper species that would limit full utilization of N₂O by marine denitrifiers. Inorganic Cu species (Cu'), predominantly carbonate complexes in seawater and lake water above pH 7, are presumed to be the bioavailable form of copper. Granger and Ward (2003) suggested that complexation of Cu by organic ligands and reduced sulfur species in certain environments may reduce concentrations of bioavailable Cu below these necessary levels, resulting in N₂O accumulation. Work by our group has documented the importance of both organic ligands and reduced sulfur compounds to Cu speciation in freshwater environments (Mylon and Benoit 2001; Mylon et al. 2003). Further, Mengis et al. (1997) observed extremely high N₂O concentrations in lakes at oxic/anoxic boundaries where sulfidic compounds would be expected to be abundant. The work described here was initiated to test for the N₂O production during denitrification as a result of low Cu bioavailability in a local mesotrophic lake where suboxic and anoxic conditions are common.

In natural aquatic environments, dissolved Cu may be bound by a variety of ligands, including many produced by protistan organisms. Both eukaryotic and prokaryotic protists may produce ligands to reduce the toxicity of Cu in contaminated environments (Moffett and Brand 1996; Dryden et al. 2004). Copper may also be strongly bound by allochthonous natural organic matter, such as humic materials and reduced sulfur compounds (Rozan et al. 2000), limiting its bioavailability to phytoplankton (Mylon et al. 2003). Although sulfidic compounds are rapidly oxidized in the presence of even trace levels of O₂, recent analytical advances have enabled the measurement of nanomolar concentrations of sulfide in oxic surface waters (Mylon and Benoit 2001). Given the high affinity of sulfide for Cu ($K_{CuS} = 10^{36.1}$) and the stability of these complexes in oxic/suboxic environments (Luther and Rickard 2005), even nanomolar concentrations of sulfide may significantly influence Cu speciation.

Three approaches were used here to determine the role of Cu in N₂O cycling. First, denitrifying bacteria cultures were grown in defined freshwater media to establish the Cu concentrations required for uninhibited N₂O reduction. We then incubated natural bacterial assemblages under denitrifying conditions while manipulating Cu speciation with the synthetic organic ligands ethylenediaminetetraacetic acid (EDTA) and 8-hydroxyquinoline (oxine) to determine the relation between Cu speciation and N₂O accumulation in a natural community. These experiments were then coupled with field sampling of a local Connecticut lake to provide high-frequency measurements of N cycling and Cu speciation over the course of the spring bloom and onset of summer stratification. Taken together, these experiments were designed to test the potential role of Cu speciation in controlling N₂O accumulation in a natural aquatic system.

Methods

Laboratory culture experiments—Batch cultures of the denitrifying bacterium *Paracoccus denitrificans* (ATCC 19367) were grown under controlled conditions to investigate the relation between Cu speciation and N₂O production during denitrification. Labware used in the preparation of experimental media was composed of either Teflon, Tedlar, or polyethylene that was rigorously acid-cleaned. Experimental incubations were conducted in 400-mL gas sampling bags fitted with Teflon-lined silicone septa. Stock cultures were maintained in the defined freshwater media Fraquil (Morel et al. 1975) amended with bacto-peptone and casein hydrolysate as described by Granger and Ward (2003). All media components were passed through cleaned Chelex 100 resin to remove trace-metal impurities. Copper contamination in the media (with no Cu added) was measured to be 0.5 nmol L⁻¹ by graphite furnace atomic absorption spectrophotometry (GF-AAS). Media were sterilized either with filtration (0.2 μm) or microwave, and all manipulations and sampling were performed in a Class-100 laminar flow hood contained within a Class-100 clean room.

Three culture experiments were performed with varying amounts of added Cu buffered by excess EDTA (10 μmol L⁻¹) to determine the pCu' required for full utilization of N₂O by actively growing *P. denitrificans*. In the first experiment, media containing 0, 3, 10, or 30 nmol L⁻¹ Cu (added bound to EDTA) was added to replicate bags, allowed to equilibrate overnight, and then inoculated with *P. denitrificans* grown in media with no added Cu. The experimental bags were incubated in a water bath at 30°C and sampled every 24 h for optical density (absorbance at 660 nm), NO₂⁻, and N₂O. In the second experiment, the protocol was repeated with Cu additions of 0, 1, 2, or 3 nmol L⁻¹ and samples were taken every 12 h. A third experiment was performed with only 1, 0.25, 0.5, and 1 nmol L⁻¹ added Cu. Copper speciation in each experimental treatment was calculated with MINEQL+ (v 4.5 Environmental Research Software) using thermodynamic constants contained in the program database.

Natural assemblage experiments—Growth experiments were also performed with natural bacterial assemblages collected from Linsley Pond (described below). Three incubation experiments were conducted with varying concentrations of added EDTA and oxine to again determine the pCu' required for uninhibited reduction of N_2O during denitrification. In the first experiment, started on 04 May 2004, seven acid-washed, sterilized Tedlar bags were filled with unfiltered water collected from 10 m. The bags were amended with Chelexed $NaNO_3$ ($250 \mu mol L^{-1}$), bactopectone ($0.4 g L^{-1}$), and casein hydrolysate ($0.4 g L^{-1}$) to enhance denitrification. Iron was also added (FeEDTA in 1:1 mix added to a final concentration of $4.5 \mu mol L^{-1}$) to ensure that Fe would not become limiting. Three bags received no additional ligands and served as the control, while $10 \mu mol L^{-1}$ EDTA was added to two bags and $100 \mu mol L^{-1}$ EDTA to the final two bags. All bags were immediately sampled, then incubated in a mesh cage at 10-m ($5.5^\circ C$) depth for 94 h. At the end of the incubation period, the bags were retrieved, returned to the lab on ice, and sampled for bacterial growth, nitrate, and N_2O in a Class-100 hood.

Additional experiments were performed with Linsley Pond water incubated in the lab to facilitate more frequent sampling. A second incubation experiment was initiated on 19 May 2004. Bags were filled with unfiltered water from 10 m ($5.6^\circ C$), immediately transported back to the lab on ice, amended with nutrients and ligands, and then incubated in the lab at $8^\circ C$ in the dark. Control bags received only NO_3^- , bactopectone, casein hydrolysate, and FeEDTA, while replicate treatment bags also received $100 \mu mol L^{-1}$ oxine. The bags were sampled daily for nearly 2 weeks until all nitrate and nitrite had been removed. A third incubation experiment was conducted 3 weeks later with Linsley Pond water collected from 10 m. In this experiment, FeEDTA and added ligands ($10 \mu mol L^{-1}$ or $100 \mu mol L^{-1}$ oxine in the two treatments) were added to the bags and allowed to equilibrate at $8^\circ C$ overnight before the addition of NO_3^- , bactopectone, and casein hydrolysate. Copper speciation in all three experiments was calculated with MINEQL+ using measurements of the major cations, anions, dissolved Cu, and Cu-binding ligands (measured with competitive ligand exchange–cathodic stripping voltammetry [CLE-CSV] as described below).

Two incubation experiments were performed later in summer 2004 with water collected from Linsley Pond to determine the effect of adding Cu to natural bacteria assemblages. Samples were drawn from shallower depths (6 m on 09 August 2004 and 5 m on 10 September 2004; see Table 2) since the depth of denitrification had risen considerably by the end of the summer. On both dates, water was taken from the deepest depth containing both measurable N_2O and low-enough dissolved oxygen levels for denitrification. Nitrate, bactopectone, and casein hydrolysate were added to replicate or triplicate bags, and Cu:EDTA ($3 nmol L^{-1}$) was also added to the +Cu treatment bags. Bags were incubated in the lab at $8^\circ C$ in the dark.

Field sampling—Nitrous oxide was monitored in Linsley Pond during the spring and early summer of 2005 to

determine the biogeochemical factors contributing to its seasonal accumulation. Linsley Pond is a mesotrophic lake located ca. 17 km east of New Haven, Connecticut, in the town of North Branford ($42^\circ 3' N$, $72^\circ 8' W$). The lake has a surface area of $0.09 km^2$, a maximum depth of 14 m, and a mean depth of 6 m. The watershed of Linsley Pond is comprised of industrial quarry (42%), undeveloped forest and wetland (33%), and developed residential (25%) areas (DEP 1991). Linsley Pond undergoes thermal stratification in the summer, which typically begins in April and lasts until late November. Important biological and chemical parameters of the lake have been reported in previous publications (Hutchinson 1975, Hu et al. 2006). The theoretical water retention time of Linsley Pond is approximately 139 d (Canavan and Siver 1995). Measurements of N_2O , O_2 , and sulfides performed during the 2004 growing season as part of a separate study confirm the uniform behavior of the lake during 2004 and 2005 (Twining unpublished data; Hu et al. 2006). The lake typically stratifies beginning in April, with sulfide accumulating in the hypoxic hypolimnion throughout the spring and summer. Sampling was conducted weekly or biweekly over 3 months to intensively sample during the spring phytoplankton bloom.

All samples were collected from the same station near the center of the pond (the point of maximum depth, 13 m) with 1 m depth resolution using a peristaltic pump and acid-cleaned Teflon tubing. Unfiltered water was collected for chlorophyll *a* (Chl *a*), sulfide, and N_2O . Water was filtered in-line through acid-leached $0.45\text{-}\mu m$ Durapore membranes (Millipore) before collection for analysis of cations (Na, K, Ca, Mg, Fe, and Mn), alkalinity, major anions (Cl , SO_4^{2-}), and nutrients (NH_4^+ , NO_2^- , NO_3^-). On certain dates, water was also collected for voltammetric measurements of Cu-binding ligands (described below). Temperature, pH, conductivity, dissolved oxygen, and oxidation–reduction potential were measured with a YSI 556 field sampling meter, and in vivo Chl *a* was measured with a field fluorometer (Aquafluor, Turner Designs). The YSI dissolved oxygen measurements were periodically checked against Winkler titrations to ensure accuracy.

Samples for N_2O analysis were collected in 43-mL glass headspace vials with Teflon-lined silicon septa. More than two volumes of water were allowed to overflow before adding $70 \mu L$ of $10 mol L^{-1}$ NaOH and immediately capping without any trapped air; vials were stored at $4^\circ C$ until analysis within 48 h. Previous studies have shown N_2O to be stable over several weeks when preserved in this manner (Mengis et al. 1997).

Sample analysis—All samples were stored on ice and most were processed in the laboratory within 8 h of collection. Samples for cation analysis were acidified with 5% HNO_3 (Seastar Baseline) and heated prior to analysis by inductively coupled plasma atomic emission spectroscopy. Chloride and sulfate were measured by ion chromatography with an IonPac AS14 anion-exchange column (Dionex) and a bicarbonate exchange medium. Alkalinity was measured with Gran titrations performed on a Metrohm titrator. Sulfides, as methylene blue reactive sulfides

Table 1. Copper speciation and N₂O accumulation during *Paracoccus denitrificans* laboratory incubation experiments (nos. 1–3) and Linsley Pond assemblage incubation experiments (nos. 5–8). Inorganic (Cu') and free aquo Cu (Cu²⁺) are presented as the negative log of the concentration in mol L⁻¹.

	Date	Depth (m)	[Cu] _{tot} (nmol L ⁻¹)	pCu'	pCu ²⁺	N ₂ O (nmol L ⁻¹)
Experiment 1	13 Apr 04					
30 nmol L ⁻¹ Cu		—	30.5	13.7	13.9	15
10 nmol L ⁻¹ Cu		—	10.5	14.2	14.4	15
3 nmol L ⁻¹ Cu		—	3.5	14.6	14.8	15
0 nmol L ⁻¹ Cu		—	0.5	15.5	15.7	8,300
Experiment 2	21 Apr 04					
3 nmol L ⁻¹ Cu		—	3.5	14.6	14.8	13
2 nmol L ⁻¹ Cu		—	2.5	14.8	15.0	14
1 nmol L ⁻¹ Cu		—	1.5	15.0	15.2	15
0 nmol L ⁻¹ Cu		—	0.5	15.5	15.7	42,000
Experiment 3	28 Apr 04					
1 nmol L ⁻¹ Cu		—	1.5	15.0	15.2	16
0.5 nmol L ⁻¹ Cu		—	1.0	15.2	15.4	57
0.25 nmol L ⁻¹ Cu		—	0.7	15.3	15.5	18,000
0 nmol L ⁻¹ Cu		—	0.5	15.5	15.7	29,000
Experiment 4	04 May 04					
Control		10	5.7	11.4	11.6	16
10 μmol L ⁻¹ EDTA		10	5.7	14.5	14.7	15
100 μmol L ⁻¹ EDTA		10	5.7	15.6	15.8	16
Experiment 5	19 May 04					
Control		10	4.2	11.6	11.9	380
100 μmol L ⁻¹ oxine		10	4.2	23.7	23.9	6,600
Experiment 6	09 Jun 04					
Control		10	4.2	11.6	11.8	570
10 μmol L ⁻¹ oxine		10	4.2	21.7	21.9	350
100 μmol L ⁻¹ oxine		10	4.2	23.7	23.9	4,300
Experiment 7	09 Aug 04					
Control		6	2.4	11.1	12.4	15,000
3 nmol L ⁻¹ Cu		6	5.4	10.8	12.1	600
Experiment 8	10 Sep 04					
Control		5	3.3	11.3	12.3	1,500
3 nmol L ⁻¹ Cu		5	6.3	11.0	12.0	110

(MBRS), were quantified by the methods of Cline (1969). Nitrate, nitrite, and ammonium were analyzed on an Astoria2 continuous flow analyzer (Astoria-Pacific International).

Dissolved Cu was measured in filtered water with a Perkin Elmer 3000 GFAAS after acidification and preconcentration with subboiling evaporation (Benoit et

al. 1997). All sample manipulations were performed in a Class-100 clean room. The standard reference material (SLRS-3, National Research Council, Canada) was analyzed during each run to check recovery and accuracy.

Nitrous oxide was analyzed by gas chromatography (Agilent 6890 Plus) in He headspaces equilibrated with preserved samples at 30°C. Headspace samples were

Table 2. Competitive ligand exchange–cathodic stripping voltammetry (CLE-CSV) field results. The conditional stability constants ($K'_{Cu^{2+}L}$) and ligand concentrations are present as the mean \pm range/2 of replicate measurements. Total Cu is presented as means \pm standard deviation (triplicate analyses of the same sample).

Date	Depth (m)	$\log K'_{Cu^{2+}L}$	L (nmol L ⁻¹)	$\log \alpha_{CuL}$	Cu _{TOT} (nmol L ⁻¹)	pCu'	pCu ²⁺
04 Apr 05	3	15.1 \pm 0.5	25.4 \pm 9.3	7.5	6.5 \pm 0.3	14.1	15.5
11 Apr 05	3	14.8 \pm 0.1	16.1 \pm 0.8	7.0	6.6 \pm 0.1	13.5	14.8
25 Apr 05	5	15.1 \pm 0.1	15.1 \pm 1.3	7.3	4.6 \pm 0.2	14.3	15.3
17 May 05	6	14.5 \pm 0.2	23.4 \pm 10.3	6.9	4.5 \pm 0.1	14.8	15.0
31 May 05	6	14.9 \pm 0.1	5.6 \pm 0.8	6.6	5.1 \pm 0.1	13.6	13.8
13 Jun 05	5	14.4 \pm 0.3	9.1 \pm 1.9	6.4	5.5 \pm 0.2	12.9	14.1

injected directly onto a Supelco Carboxen 1010 PLOT capillary column, and N_2O was detected using an electron capture detector (Agilent $\mu\text{-ECD}$) heated to 350°C using P5 as the make-up gas (10 mL min^{-1}). Laboratory air (316 parts per billion) as well as certified standards of 1 part per million (ppm) and 20 ppm (Airgas) were used for calibration and quality assurance/quality control.

Voltammetric analyses—CLE-CSV was used to determine the concentration and binding strength of dissolved organic compounds in Linsley Pond. Voltammetric analyses were performed on filtered water samples using a Metrohm 746 Trace Analyzer, 747 VA Stand, and a 695 VA autosampler all contained in a Class-100 clean room. CLE-CSV analyses were conducted following the procedures of Campos and Van den Berg (1994) with salicylaldoxime (SA) as the competing ligand. Filtered water samples were divided into acid-cleaned high-density polyethylene tubes and 10 mmol L^{-1} chelexed borate buffer was added. Between 0 and 200 nmol L^{-1} Cu (prepared in 0.2% Seastar HNO_3 from AAS standards) was then added to replicate tubes and allowed to equilibrate with natural ligands for several hours before the addition of $10\text{ }\mu\text{mol L}^{-1}$ SA. The solutions then equilibrated overnight at 4°C before analysis. After equilibration, each solution was brought to 10 mmol L^{-1} NaClO_4 to increase current response, and samples were bubbled with N_2 for 5 min to remove oxygen. Cu-SA complexes were measured with a hanging mercury drop electrode (HMDE) using a double-junction $\text{Ag}:\text{AgCl}$ (saturated KCl) reference electrode and a platinum auxiliary electrode. The Cu-SA complexes were deposited for 60 s at -50 mV while rotating the HMDE (0.60 mm^2 surface area) at 800 revolutions per minute. Deposition was followed by a quiescent period of 5 s at -1000 mV and 5 s at -50 mV before scanning in square-wave mode to -700 mV (pulse amplitude 25 mV , scan rate 20 mV s^{-1}). The concentration and conditional stability constant, $K'_{\text{Cu'L}}$, were calculated using the Ruzic linearization (Ruzic 1982). Conditional stability constants were corrected for inorganic complexes with the side-reaction coefficient, $\alpha_{\text{Cu}'}$, resulting in $K'_{\text{Cu}^{2+}\text{L}}$.

Results

Laboratory culture experiments—The facultative denitrifier *P. denitrificans* grew well on the artificial growth medium, reaching stationary phase in 1–2 days (Fig. 1a). As oxygen was depleted in the media, the cells switched from aerobic growth to denitrification, demonstrated by the removal of NO_3^- (data not shown) and subsequent accumulation of NO_2^- in the media during log-phase growth (Fig. 1b). Following or concurrent with NO_2^- accumulation, N_2O accumulated in all treatments (Fig. 1c; note log scale). However, N_2O was reduced to N_2 in treatments containing sufficient Cu, while N_2O remained significantly elevated in cultures with $\text{pCu}' > 15.1$ (Fig. 2). This indicates that bacteria were not able to obtain sufficient Cu for synthesis of nitrous oxide reductase at low Cu' concentrations. The bacteria were able to eventually utilize all accumulated N_2O at pCu' between

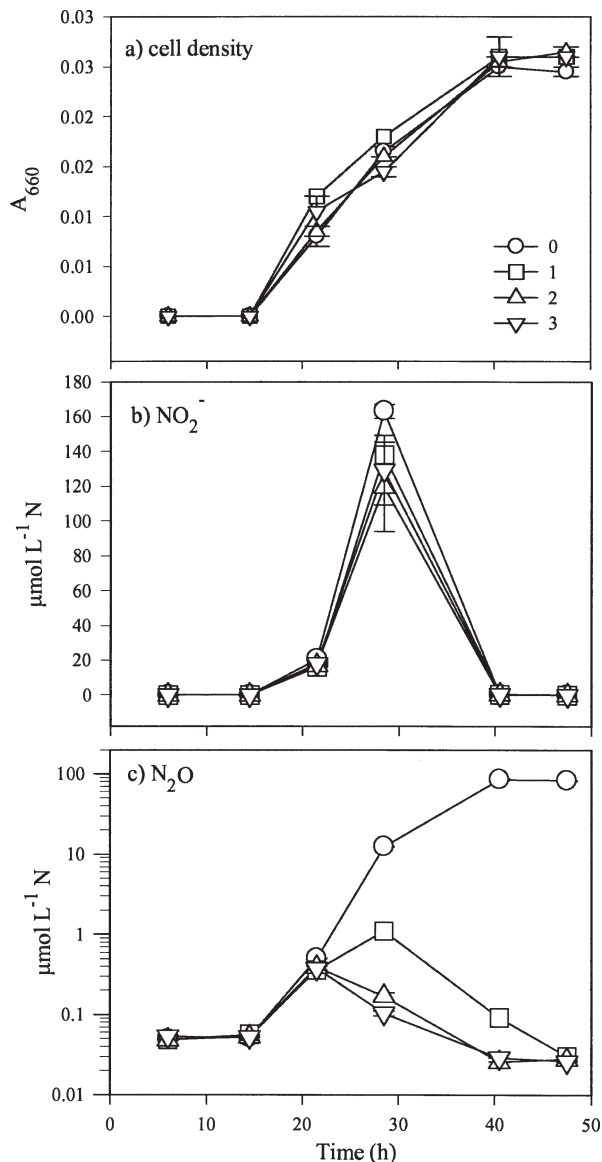


Fig. 1. Time course of (a) cell density, (b) NO_2^- , and (c) N_2O accumulation in denitrifying cultures of *P. denitrificans* grown at varying concentrations of added Cu. Numbers beside symbols are nmol L^{-1} added Cu. All treatments contained $4.5\text{ }\mu\text{mol L}^{-1}$ EDTA. Note the log scale in panel c. Each point is the mean (\pm range/2) of replicate cultures.

15.1 and 15.3, but there was a time delay, indicating limitation of the rate of Cu uptake and subsequent enzyme synthesis.

Natural assemblage experiments—Incubations of natural bacterial assemblages amended with nitrate and labile organic matter resulted in denitrification within approximately 24 h (Fig. 3a). Nitrate was typically depleted within 36 to 48 h, and NO_2^- and N_2O both showed temporary accumulation during denitrification (Fig. 3b,c). Unlike the incubations with *P. denitrificans*, N_2O was eventually utilized in all treatments. The addition of $10\text{ }\mu\text{mol L}^{-1}$ or $100\text{ }\mu\text{mol L}^{-1}$ EDTA did not increase N_2O accumulation,

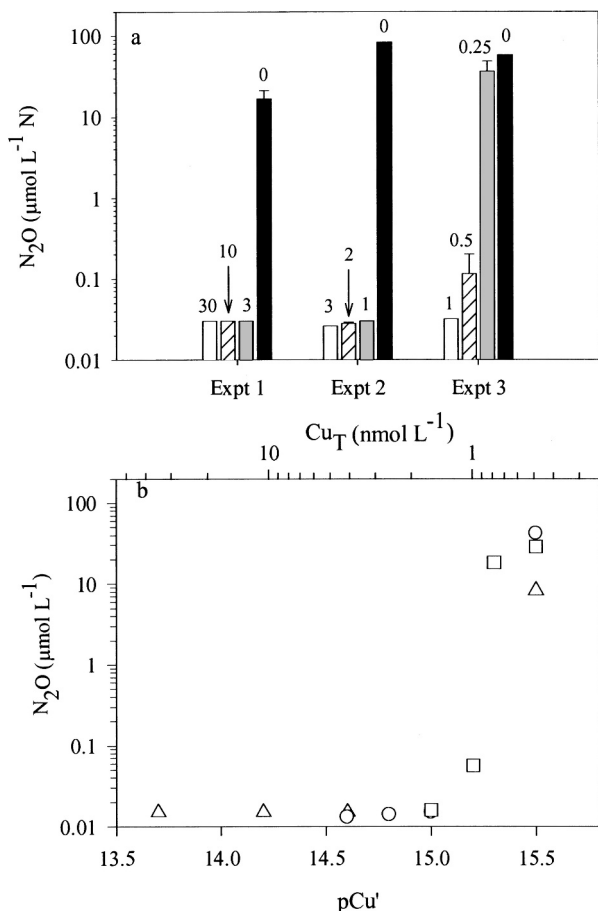


Fig. 2. (a) Nitrous oxide concentrations at the final time point in three separate experiments with *P. denitrificans*. The total Cu concentrations added to the treatments in each of the three experiments are noted above the bars. (b) Accumulated N₂O versus both the total Cu (added Cu plus 0.5 nmol L⁻¹ background contamination; upper scales) and inorganic Cu (pCu') concentrations.

despite lowering pCu' to 15.6 (Table 1, Fig. 4). Addition of 100 µmol L⁻¹ of the strong organic copper ligand oxine (resulting pCu' 23.7) did increase temporary N₂O accumulation during denitrification, while a 10 µmol L⁻¹ addition of oxine (pCu' 21.7) actually reduced N₂O accumulation slightly, relative to control cultures. Complete N₂O removal was eventually achieved in all treatments and experiments, indicating that denitrifiers were able to access sufficient Cu to synthesize nitrous oxide reductase even at extremely low inorganic Cu concentrations (pCu' >20). The added ligands appeared only to slow the rate of N₂O reduction.

Addition of Cu to Linsley Pond water (approximately 3 nmol L⁻¹ background dissolved Cu) reduced the level of N₂O accumulation during denitrification relative to control with no added Cu or ligands (Table 1, Fig. 4). Peak N₂O concentrations in the unamended controls were comparable with those observed in the oxine treatments earlier in the summer (2–15 µmol L⁻¹), but the addition of labile Cu lowered N₂O by more than an order of magnitude to 100–600 nmol L⁻¹. This indicates that organic ligands naturally

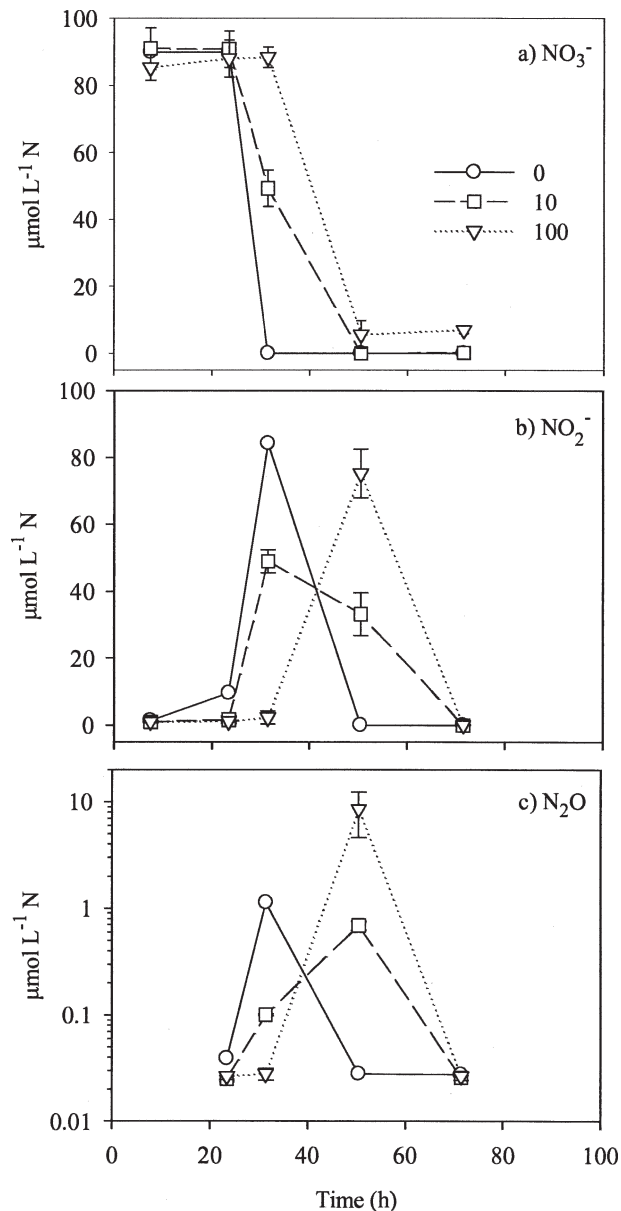


Fig. 3. Time course of (a) NO₃⁻ utilization, (b) NO₂⁻, and (c) N₂O accumulation during incubation of a bacteria assemblage collected from 10 m in Linsley Pond. The synthetic ligand 8-hydroxyquinoline (oxine: $\beta_{\text{Cu(oxine)}_2} = 10^{26.1}$) was added to reduce the availability of the naturally occurring Cu. Nitrate, bactopeptone, casein hydrolysate, and Fe were added to all treatments to promote denitrification. Each point is the mean \pm range/2 of replicate treatments.

present in the water column may cause N₂O to temporarily accumulate during denitrification, presumably by slowing rates of Cu uptake and nitrous oxide reductase synthesis.

Copper speciation in Linsley Pond—Electrochemical measurements revealed the presence of strong Cu-binding ligands in Linsley Pond (Table 2). A class of ligands characterized by conditional stability constants, $K_{\text{Cu}^{2+}\text{L}}$, between 10^{14.4} and 10^{15.1} was measured over the spring. This ligand or class of ligands varied in concentration over

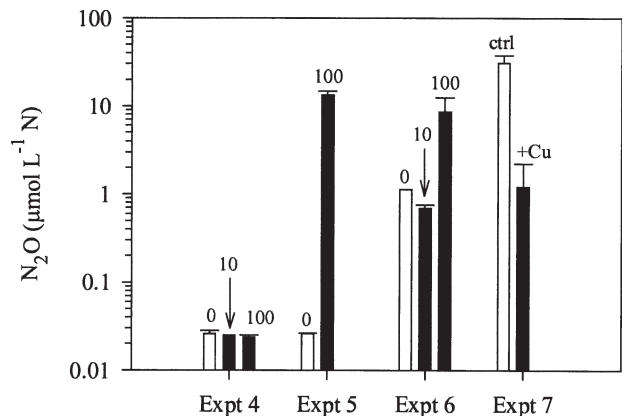


Fig. 4. Maximum N₂O accumulation during four separate experiments with natural bacterial communities collected from 8–10 m in Linsley Pond. In experiments 4–6, various concentrations of either EDTA (expt. 4: 0, 10, or 100 µmol L⁻¹) or 8-hydroxyquinoline (expts. 5 and 6: 0, 10, or 100 µmol L⁻¹) were added to reduce the availability of naturally occurring Cu. Nitrate, bactopectone, casein hydrolysate, and Fe were also added to each treatment to stimulate denitrification. In the final experiment, Cu:EDTA was added to the treatment bags to increase bioavailable Cu.

the 3-month period of observation, declining from a concentration of 25 nmol L⁻¹ on 04 April to 9 nmol L⁻¹ on 13 June. This trend mirrored the drop in water column Chl *a* concentrations following the spring bloom (Fig. 5), suggesting that the ligands were produced by phytoplankton. The measured ligands dominated Cu speciation in Linsley Pond. Much more than 99% of all Cu was bound to these ligands at each sampling point, resulting in pCu' between 12.9 and 14.8 (Table 2).

N biogeochemistry in Linsley Pond—In conjunction with the Cu speciation measurements described above, a suite of chemical and biological (Chl *a*) parameters were measured in Linsley Pond to determine the other biogeochemical processes contributing to the accumulation and depletion of N₂O. The water column was stratified during the entire sampling period, with the epilimnion comprising the upper 4 m, the metalimnion stretching from 4–6 m, and the hypolimnion remaining below 6 m. The spring phytoplankton bloom occurred in late April, resulting in highly elevated Chl *a* and O₂ at the base of the epilimnion. The base of the water column became suboxic (O₂ < 10 µmol L⁻¹) by 25 April, and sulfide species (primarily HS⁻) first appeared above the bottom sediments on 17 May (Fig. 6). Ammonia concentrations increased from approximately 20 µmol L⁻¹ at the surface to 100 µmol L⁻¹ at the base of the water column, indicating a source in the sediments. Nitrate occurred at 10–20 µmol L⁻¹ during the early spring but was removed by phytoplankton growth in the surface and presumably by denitrification at depth. By June, NO₃⁻ was only present in the metalimnion. Nitrite was not detectable on all days, but it was always elevated at the suboxic depths, suggesting production via denitrification.

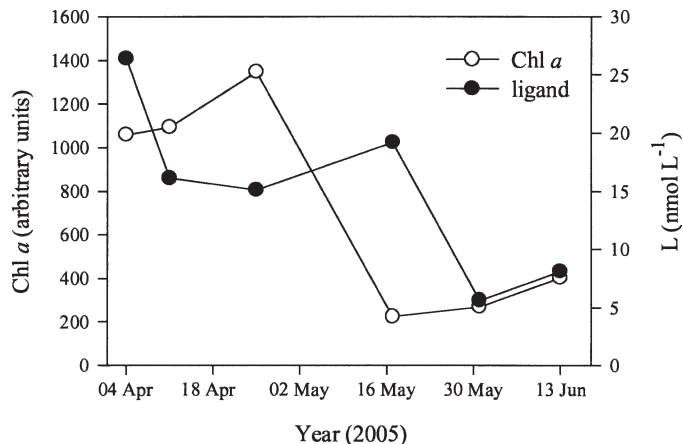


Fig. 5. Concentrations of chlorophyll *a* and Cu-binding ligands in Linsley Pond between 04 April 2005 and 13 June 2005.

Nitrous oxide profiles in Linsley Pond varied substantially over the 3-month sampling period (Fig. 6). On 11 April, N₂O was present at equilibrium with the atmosphere throughout the water column; lower depths had slightly higher concentrations as a result of increased solubility at lower temperatures. Surface concentrations (~12 nmol L⁻¹) remained equilibrated with atmospheric N₂O over the rest of the sampling period, but N₂O was both produced and consumed below the surface as nitrification and denitrification occurred. On 18 April, N₂O concentrations were 44 nmol L⁻¹ at 13 m, indicating production at the sediment–water interface. Dissolved oxygen at this depth was 15 µmol L⁻¹, which is likely too high for occurrence of denitrification. This suggests that the N₂O was either produced by nitrification at the base of the water column or by denitrification within the underlying sediments. One week later (25 April), O₂ at 12 and 13 m dropped below 10 µmol L⁻¹, initiating suboxic conditions favorable to denitrification. Nitrate was depleted in the suboxic zone while nitrite was detected only at these same depths, both indicating the occurrence of denitrification. Nitrous oxide was depleted within the suboxic zone but elevated (37 nmol L⁻¹) immediately above. One week later, on 02 May, both the suboxic zone and the depth of maximum N₂O had risen upward 1 m in the water column. The suboxic zone and the overlying N₂O peak continued to move up the water column for the final 4 weeks of the observation period. By the final sampling day (29 June), the depth of N₂O production had risen to 5 m, where N₂O was four times higher (32 nmol L⁻¹) than at the surface. On three of the sampling dates (17 May through 13 June), a secondary N₂O peak was present within the suboxic zone. In each case, the depth of this peak coincided with that of the NO₂⁻, both of which were likely generated by denitrification.

While N₂O was produced both above and within the suboxic zone, it was clearly removed under anoxic conditions (Fig. 6). Measurable MBRS (ca. 50 nmol L⁻¹) first appeared on 17 May, coinciding with the complete depletion of N₂O at the bottom of the water column. As the anoxic zone moved up the water column, N₂O was

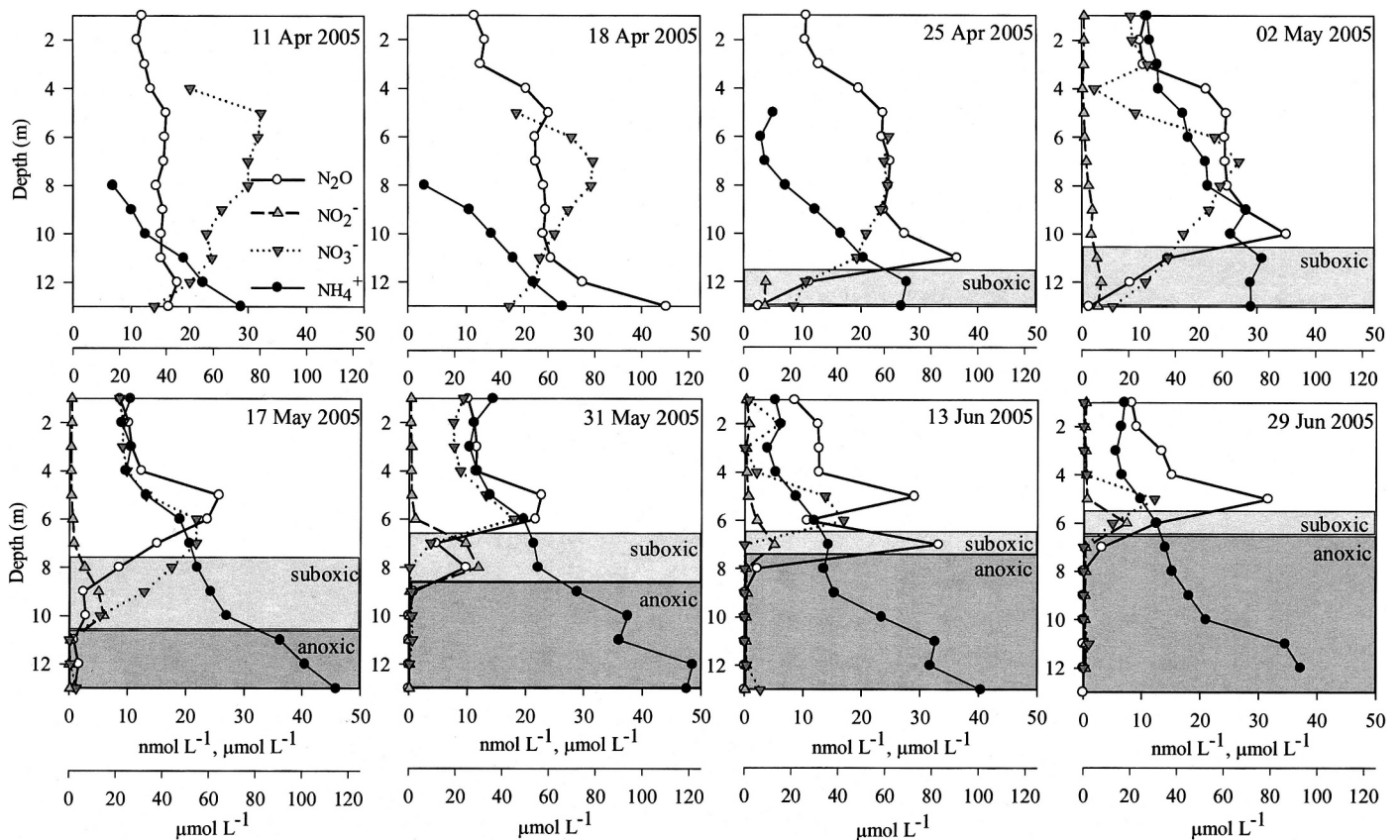


Fig. 6. Depth profiles of dissolved inorganic nitrogen species in Linsley Pond over 3 months in 2005. The upper abscissa corresponds to N₂O (nmol L⁻¹) and NO₂⁻ and NO₃⁻ (μmol L⁻¹), and the lower abscissa indicates concentrations of NH₄⁺ (μmol L⁻¹). Suboxic zones, denoted by light gray shading, are characterized by dissolved oxygen concentrations <10 μmol L⁻¹ but no detectable sulfides. Anoxic zones, denoted by dark gray shading, are characterized by the presence of measurable (>50 nmol L⁻¹) sulfides. Dual N₂O peaks were observed on several dates.

simultaneously removed. Only two samples containing MBRS also showed measurable N₂O, and both of these were at the suboxic/anoxic interface in mid- to late June, indicating that N₂O was mixing down from above and being converted to N₂.

Discussion

Cu speciation and N₂O accumulation—Lab culture studies clearly show that N₂O accumulates during denitrification at low Cu concentrations. Such an effect has been demonstrated previously (Iwasaki and Terai 1982; Matsubara et al. 1982; Granger and Ward 2003), but these earlier studies only compared Cu-replete and Cu-depleted conditions, providing little quantitative information about the Cu speciation conditions where Cu limitation is first observed. Using one of the same species as Granger and Ward (2003), we found N₂O accumulation to increase at total dissolved Cu concentrations of 1 nmol L⁻¹, corresponding to a total inorganic Cu concentration of 0.6 fmol L⁻¹. This concentration is four orders of magnitude lower than that reported by Granger and Ward (2003), who cultured *P. denitrificans* in the artificial marine medium Aquil buffered with 100 μmol L⁻¹ EDTA. Efforts to replicate the speciation calculations of Granger and

Ward with MINEQL were unsuccessful, complicating further direct comparison with their results. However, we calculated pCu' to be 13.5 in their low-Cu media, nearly 200 times lower than reported in the paper and only 50 times higher than pCu' that caused limitation in our cultures (Table 3).

The higher Cu' requirements for *P. denitrificans* grown in Aquil may be due to increased competition for Cu uptake or transport binding sites by Ca²⁺ in marine versus freshwater media. Competition by Ca for Cu binding sites on invertebrates has been shown to reduce Cu bioavailability in studies utilizing the biotic ligand model (Santore et al. 2001; Borgmann et al. 2005). In a study with the freshwater amphipod *Hyaella azteca*, Borgmann et al. (2005) observed Cu bioavailability (toxicity) to decrease by approximately twofold when Ca²⁺ was increased 25-fold. By comparison, Granger and Ward observed sixfold lower bioavailability at 32-fold higher Ca²⁺ concentration (Table 3). Thus variations in Ca may explain only part of the discrepancy.

The Cu speciation in Linsley Pond is controlled by the presence of organic ligands, resulting in inorganic and free hydrated Cu concentrations similar to those used in the culture experiments. Previous studies have observed similarly strong ligands in other lake and estuarine environ-

Table 3. Metal speciation calculated for Aquil media used by Granger and Ward (2003) and Fraquil media used in this study. Complexation was calculated with Mineql+ (v. 4.5) using the stability constants in the default thermodynamic database and total Cu concentrations at which N₂O was observed to accumulate in cultures of denitrifying bacteria. Total concentrations are presented as mol L⁻¹. Also shown are the -log concentrations of the free aquo cations (pMⁿ⁺) predicted by the model.

Component	Aquil		Fraquil	
	Total concentration	pM	Total concentration	pM
Br ⁻	8.4×10 ⁻⁴			
Ca ²⁺	1.1×10 ⁻²	2.1	2.5×10 ⁻⁴	3.6
Cl ⁻	5.6×10 ⁻¹		5.1×10 ⁻⁴	
Co ²⁺	8.0×10 ⁻⁸	10.9	1.3×10 ⁻⁷	10.9
Cu ²⁺	3.0×10 ⁻⁹	14.6	1.0×10 ⁻⁹	15.4
Fe ³⁺	1.0×10 ⁻⁶	19.0	4.5×10 ⁻⁶	18.8
F ⁻	7.1×10 ⁻⁵			
K ⁺	1.0×10 ⁻²		2.0×10 ⁻⁵	
Mg ²⁺	5.5×10 ⁻²		1.5×10 ⁻⁴	
Mn ²⁺	9.0×10 ⁻⁸	8.3	3.8×10 ⁻⁷	7.9
MoO ₄ ²⁻	1.0×10 ⁻⁷		1.0×10 ⁻⁷	
Na ⁺	4.8×10 ⁻¹		4.2×10 ⁻⁴	
NO ₃ ⁻	2.5×10 ⁻⁴		2.5×10 ⁻⁴	
PO ₄ ³⁻	1.0×10 ⁻⁵		1.0×10 ⁻⁵	
SeO ₄ ²⁻	1.0×10 ⁻⁸			
SO ₄ ²⁻	2.9×10 ⁻²		1.5×10 ⁻⁴	
Sr ²⁺	6.4×10 ⁻⁵			
Zn ²⁺	5.0×10 ⁻⁸	10.9	1.0×10 ⁻⁷	10.9
EDTA ⁴⁻	1.0×10 ⁻⁴		1.0×10 ⁻⁵	
DIC	2.4×10 ⁻³		1.5×10 ⁻⁴	
pH	8.10		7.00	
Cu'	3.4×10 ⁻¹⁴	13.5	6.5×10 ⁻¹⁶	15.2

ments. Using CLE-CSV with catechol as the competing ligand, Xue and Sigg (1993) measured conditional stability constants of 10^{13.9} to 10^{14.9} for ligands in a eutrophic lake in Switzerland. The ligands were assumed to have been produced by algae in the lake, given their seasonal covariance with parameters of algal productivity. A similar conclusion was reached regarding Cu ligands of similar strength in an English lake (Achterberg et al. 1997). Very little work has been done to isolate or chemically characterize these strong Cu-binding ligands. Some fraction of the Cu-binding ligands detected in Linsley Pond may be fulvic or humic acids. Shank et al. (2004) found that estuarine humic substances could account for all of the strong ($K'_{CuL} = 10^{13.5}$) Cu-binding ligands in the Cape Fear Estuary. Relatively strong ($K'_{CuL} = 10^{12.0}$) Cu binding was also measured for humic substances by Kogut and Voelker (2001) using a different suite of competing ligands. Very likely the Cu-binding ligands measured in Linsley Pond represent a mixture of organic compounds with autochthonous and allochthonous sources.

Some portion of the Cu ligands in Linsley Pond may be thiols produced by algae in the surface of the lake. It is known that phytoplankton generate thiols in response to Cu stress (Leal et al. 1999), and thiol concentrations in coastal seawater have been found to be correlated with Chl *a* biomass (Al-Farawati and Van Den Berg 2001). At low salinity (<1), copper-thiol complexes have conditional stability constants ($K'_{CuThiol} = 10^{13.5-14.1}$) only slightly lower than those measured in Linsley Pond (Laglera and Van Den Berg 2003), and this difference may be due to

differences in sample pH (Averyt et al. 2004). In fact, a recent study conducted at the same lake detected several thiol compounds and also noted that glutathione—like the Cu ligands in this study—covaried with Chl *a* in surface waters (Hu et al. 2006). However, this same study found Cu(II) to rapidly oxidize certain thiols (3-mercaptopropionic acid), decreasing the likelihood of significant Cu binding by one of the most commonly detected thiols in oxic waters. Heterotrophic and autotrophic bacteria may also serve as sources of detoxifying Cu ligands in natural waters (Moffett and Brand 1996).

As a result of complexation, inorganic Cu concentrations in the oxic meta- and epilimnion of Linsley Pond were reduced below picomolar levels (pCu' = 12.9–14.8). These concentrations are lower than those measured by our group for several rivers in Connecticut (pCu' 8.5–11.6; Rozan and Benoit 1999; Mylon et al. 2003), a difference that may be explained by the lower detection window of the electrochemical technique (differential pulse anodic stripping voltammetry) used in the previous studies. Similar Cu speciation has been reported for other lakes (pCu' 12.7–14.7 after correction for carbonate complexation; Xue and Sigg 1993) and estuaries (pCu' 11.7–14.2 after correction for carbonate complexation; Shank et al. 2004) by researchers using CLE-CSV.

Despite significant organic complexation of Cu in Linsley Pond, Cu' remained above levels found to induce Cu limitation in the laboratory experiments with *P. denitrificans*. It is therefore unlikely that Cu would limit the activity of denitrifiers in Linsley Pond. Furthermore,

incubations of Linsley Pond water with added Cu-binding ligands did not result in notable N₂O accumulation during denitrification until pCu' was raised above 21.7. Additions of 100 μmol L⁻¹ EDTA ($K_{\text{CuEDTA}} = 10^{20.5}$) or 10 μmol L⁻¹ oxine ($\beta_{\text{Cu(8-HQ)}_2} = 10^{23.56}$) were calculated to raise pCu' above 15.5, but this did not alter N₂O processing. Only an extremely high concentration (100 μmol L⁻¹) of oxine effected significant N₂O accumulation. However, even in this treatment N₂O was completely reduced within 24 h. It therefore appears that N₂O utilization can be slowed but not completely inhibited at extremely low inorganic Cu levels in natural systems. The Cu-addition incubations (Nos. 7 and 8) also indicate that Cu availability influences kinetics, as peak N₂O levels were lowest in the Cu-amended bags. Thus natural Cu-binding ligands can at most slow the rate of N₂O utilization. It is also possible that N₂O in Linsley Pond is reduced through a separate abiotic pathway.

These results suggest that native bacteria are able to either access organically bound Cu or lower their Cu requirements in the face of reduced availability. There is evidence that organically bound metals may be taken up by plankton. Studies with Fe-limited phytoplankton have demonstrated the ability of some species to access Fe bound to strong organic ligands (Hutchins et al. 1999). Eukaryotic phytoplankton are able to separate Fe from the ligands with a ferric chelate reductase system (Maldonado and Price 2001), while prokaryotes directly accumulate Fe bound to siderophores (Granger and Price 1999). Methane-oxidizing bacteria have recently been found to produce a siderophore-like compound for Cu acquisition (Kim et al. 2004), and it would be logical to assume that other bacteria with Cu requirements do the same. Denitrifying bacteria may also be able to lower their Cu quotas in the face of low availability. Phytoplankton adapted to low-Fe waters are able to decrease their physiological Fe requirements by substituting non-Fe proteins for Fe metalloproteins (LaRoche et al. 1996), modifying their biochemical (physiological) architecture (Strzepek and Harrison 2004), and reducing cell size (Sunda et al. 1991). Denitrifying bacteria may have evolved similar mechanisms. There are multiple forms of nitrite reductase incorporating either Cu or Fe as a cofactor (Zumft 1997). Some bacteria may be able to switch to the Fe form to reduce Cu requirements, leaving more available for synthesis of nitrous oxide reductase.

The apparent difference in the abilities of *P. denitrificans* and Linsley Pond bacteria to obtain Cu could be an artifact of the low levels of total dissolved Cu used in the laboratory work. The laboratory cultures may have grown themselves into Cu limitation by significantly reducing the concentration of total dissolved Cu in the media below those used for the speciation calculations. Although Cu²⁺ concentrations were buffered with EDTA, total Cu levels were below 1 nmol L⁻¹ in cultures where elevated N₂O accumulation was observed. At such low total Cu concentrations, cells may accumulate much of the Cu in the surrounding water, growing themselves into limitation. The Cu demand of the cells was estimated by assuming a moderate cell density of 3 × 10⁷ cells mL⁻¹ (corresponding to the measured A₆₆₀ of 0.03), a C content of

2.6 fmol cell⁻¹, and a Cu:C ratio of 4 × 10⁻⁶ (Sunda and Huntsman 1995). The calculated Cu demand of 0.3 nmol L⁻¹ would lower total dissolved Cu in the media by 30% or more. In this situation, total dissolved Cu rather than Cu²⁺ or Cu' may be a better predictor of limitation. Since dissolved Cu concentrations are typically higher than 1 nmol L⁻¹ in most natural waters and bacterial densities lower than in laboratory cultures, such an effect is unlikely to occur in the field.

Interpretation of the incubation results may also be complicated by the potential for Cu-oxine complexes to be passively accumulated by bacteria. It is known that lipophilic Cu complexes such as Cu-oxine can diffuse across cell membranes, introducing an alternate mechanism for metal uptake (Phinney and Bruland 1997). Uptake of Cu-oxine complexes by Linsley bacteria during the incubations might explain the lower N₂O accumulation in the 10 μmol L⁻¹ oxine treatment. However, we did observe Cu limitation in two separate experiments with 100 μmol L⁻¹ oxine, so Cu accumulated via this mechanism appears unavailable for insertion into nitrous oxide reductase. Copper taken into cells as oxine complexes might also exert toxic effects, but we did not observe oxine toxicity—even at 100 μmol L⁻¹—as evidenced by the complete denitrification of NO₃⁻ and N₂O in all treatments.

N₂O accumulation in Linsley Pond—To determine the role of Cu in N₂O accumulation and utilization in Linsley Pond, it is also necessary to determine the processes contributing to the production and removal of N₂O. Nitrous oxide can be produced by several processes other than denitrification, including nitrification and assimilatory nitrate reduction. Profiles of nitrogen species, taken together with measurements of O₂ and MBRS, indicate that all three processes contributed to N₂O dynamics in Linsley Pond, although nitrification seems to be the most consistent source. Throughout the field sampling program a N₂O peak was present above the oxic/suboxic interface. This peak likely occurred because of nitrification, as O₂ concentrations >10 μmol L⁻¹ favor this process over denitrification (Cohen and Gordon 1978; Codispoti et al. 2001). Profiles of ammonium and nitrate also indicate that nitrification was occurring at the depth of the upper N₂O peak, with ammonium being consumed and nitrate produced. The depth of nitrification rose in the water column throughout the spring, reaching 5 m by the last sampling date (29 June). The N₂O produced by nitrification was available to mix with overlying waters and diffuse to the atmosphere.

Underlying the depth of nitrification was a suboxic zone in which denitrification occurred, resulting in both N₂O consumption and production at various dates. Denitrification is favored at low oxygen concentrations, and nitrate was depleted within this zone. Additionally, nitrite, a marker for denitrification in aquatic systems (Yoh et al. 1983; Codispoti and Christensen 1985), was found to peak at these depths. On three of the sampling days, N₂O was depleted in the suboxic waters, indicating removal by denitrification. Between 17 May and 13 June, however, a secondary N₂O peak was measured in the suboxic zone.

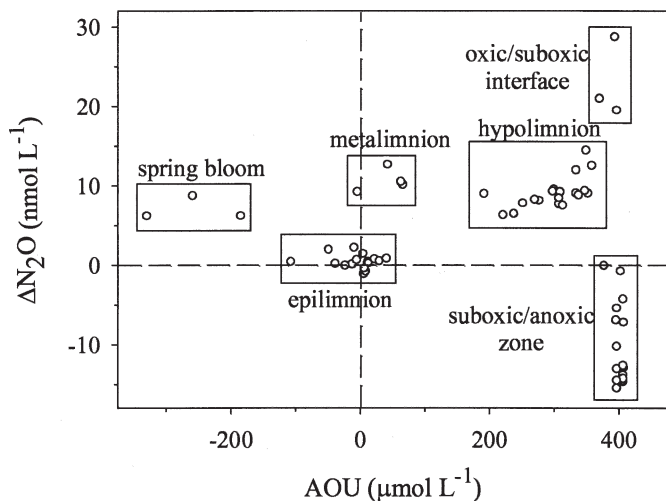


Fig. 7. Plot of ΔN_2O ($[N_2O]_{\text{measured}} - [N_2O]_{\text{saturated}}$) versus apparent oxygen utilization ($[O_2]_{\text{saturated}} - [O_2]_{\text{measured}}$) for Linsley Pond. Zones of specific vertical structure are delineated with boxes, as are the samples collected from the epilimnion during the spring bloom.

These dates correspond with the arrival of measurable MBRS in the underlying water column of Linsley Pond. Nitrous oxide was absent in the anoxic waters underlying the suboxic zone on all dates, indicating complete consumption by denitrification. Thus, the processing of N_2O during denitrification in Linsley Pond showed a shift over the spring season, switching from full N_2O utilization to accumulation for 3 weeks and then back to full utilization. The same trend was observed in Linsley Pond the previous year (2004), when a N_2O peak centered on 8 m briefly appeared for approximately 2 weeks below a longer-lived N_2O peak at 5 m that had persisted until July (data not shown). Multiple N_2O peaks have been observed in several marine and freshwater environments characterized by low O_2 concentrations. This feature is present in the Eastern Tropical North Pacific (Cohen and Gordon 1979) and the Arabian Sea (Naqvi et al. 1998), as well as in other temperate lakes (Mengis et al. 1997). Previous studies have also concluded that the upper peak is a result of nitrification and the lower peak a result of denitrification. The two peaks were always separated by a sampling depth with lower N_2O , suggesting that denitrification does not contribute N_2O to the upper nitrification peak or the overlying epilimnion. Thus, denitrification is not a significant source of N_2O to the atmosphere.

Nitrous oxide was also produced in the epilimnion during the spring phytoplankton bloom. This can be most clearly observed by plotting ΔN_2O ($[N_2O]_{\text{measured}} - [N_2O]_{\text{saturated}}$) against apparent oxygen utilization (AOU: $[O_2]_{\text{saturated}} - [O_2]_{\text{measured}}$), as shown in Fig. 7. This figure shows several distinct processes occurring at various depths over the course of the growing season. Most samples collected from the epilimnion were at equilibrium with atmospheric O_2 and N_2O . However, N_2O and O_2 supersaturation co-occurred during the 3 weeks of the spring bloom (18 April to 2 May). This indicates that N_2O was

produced directly by phytoplankton through assimilatory nitrate reduction. Certain phytoplankton are known to be capable of N_2O production (Weathers 1984), and Oudot et al. (1990) observed a similar process occurring in the Atlantic Ocean off the coast of Africa. Nitrous oxide can also be produced by denitrifying bacteria attached to the outer surfaces of algae (Law et al. 1993). But during our study the depth of maximum Chl *a* concentration was often offset from the O_2 max by 1 m, suggesting that N_2O production correlated with maximum primary production and not biomass. Thus attached denitrifiers are probably only a minor source, if any, of N_2O at these points.

Role of Cu bioavailability in N_2O accumulation—By combining the incubation experiments, Cu speciation measurements, and nitrogen cycling data, it is apparent that Cu can exert at most a weak influence on N_2O production in Linsley Pond. Our results indicate that most of the N_2O in the surface waters is produced by either assimilatory nitrate reduction or nitrification. The former process does not involve Cu, and the latter process requires Cu only in specific instances: most N_2O produced during nitrification is generated by either the chemical reduction of NO_2^- by NH_2OH or the oxidation of NH_2OH by O_2 (Stuven et al. 1992). Ironically, abiotic NH_2OH oxidation may be catalyzed by the presence of Cu(II) (Anderson 1964), but the importance of this mechanism in the environment is unknown.

It is more likely that temporal variations in dissolved oxygen and redox conditions are controlling N_2O yield during denitrification in Linsley Pond. Nitrous oxide production by denitrifying bacteria increases during transitions between suboxic and anoxic conditions, most likely as a result of differential inhibition of nitrite and nitrous oxide reductases by ambient O_2 (Otte et al. 1996). Nitrous oxide production returns to ambient (low) levels as the cells acclimate to the new conditions, but future changes will again elevate N_2O . Such a situation can easily be envisioned in Linsley Pond, where mixing or diffusion of undetectable concentrations of reduced sulfur to the depth of denitrification might have resulted in altered O_2 , redox potentials, and N_2O yield. Nitrous oxide production would drop as the cells acclimate to the new conditions, resulting in a relatively short-lived peak such as we observed. It is also possible that the disappearance of the N_2O peak after 3 weeks resulted from a shift in the composition of the bacteria community. Nevertheless, the greatly lowered production of N_2O in incubations where Cu was supplemented (Nos. 7 and 8) provides at least suggestive evidence that Cu speciation may limit denitrification even in Linsley Pond. Also, conclusions drawn for Linsley Pond may not be directly transferable to lakes exhibiting large peaks of (presumably) denitrification-derived N_2O (Mengis et al. 1997), which deserve further study.

The incubations of the unfiltered pond water with added ligands and Cu do show that Cu speciation may influence the kinetics of N_2O reduction during denitrification. However, these effects were short-lived, and only seen when Cu speciation was dramatically altered through the addition of synthetic ligands. The inorganic Cu concentra-

tions at which Cu limitation was induced were far lower than those measured in the natural environment or shown to cause limitation in laboratory cultures. When N₂O accumulation was observed, these effects lasted less than 24 h. This responsiveness can again be seen in the short-lived N₂O within the suboxic zone at Linsley Pond. If the production of N₂O between 17 May and 13 June was caused by Cu complexation to strong reduced-S ligands diffusing up from the underlying water, then the bacteria were able to adapt to the new conditions and resume utilization of N₂O rapidly.

Further work is needed to clarify the bioavailability of Cu bound to reduced-S compounds in low-oxygen environments. Denitrification typically occurs in suboxic—not anoxic—waters, so reduced-S compounds can only influence N₂O accumulation if they are stable in the presence of oxygen. We did not specifically measure Cu-sulfide complexes in this study, but any kinetically inert Cu species in oxic waters would have been included in the CLE-CSV measurements, at least as part of the broader group of strong organic copper ligands. The ligands that were measured in this manner did not appear to cause notable N₂O accumulation. It appears that denitrifying bacteria have evolved mechanisms for coping with extremely low concentrations of inorganic Cu.

The ability of denitrifiers to function in the presence of apparently extraordinarily low levels of inorganic Cu is a surprising result that has a number of possible explanations, each one of which has important ramifications worthy of further study. The bacteria may reduce their cellular requirement for Cu by adaptation (e.g., decreasing average cell size), strategic use of scarce supplies (shifting Cu away from other physiological functions), or by substituting another metal for Cu. Some of the bacteria present in natural communities may be able to directly use bound Cu, either by direct reduction at the cell membrane or with the use of a competing biotic ligand. This result has important consequences for existing understanding of metal–organismal interaction, such as the free-ion activity or biotic ligand models. Finally, our knowledge of Cu speciation may be imperfect as a consequence of failure of current measurement technology (voltammetry), unexpectedly slow ligand exchange, unsuspected competition between metals, or other causes. Again, any of these mechanisms would reflect an important gap in our understanding of metal uptake by plankton and deserve further analysis.

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