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Accumulation of nitrogen oxides in copper-limited cultures of denitrifying bacteria

Abstract—Three strains of heterotrophic denitrifying bacteria were cultured in artificial seawater medium under trace metal clean conditions to investigate their physiological response to changes in copper concentration. Decreasing the copper concentration in cultures of *Pseudomonas stutzeri* and *Paracoccus denitrificans* resulted in accumulation of nitrous oxide (N_2O) gas compared to copper-replete cultures and cessation of growth before complete denitrification to dinitrogen. Correspondingly, the activity of the copper enzyme nitrous oxide reductase, measured for *P. denitrificans* cultures, was high in copper-replete cultures and was below detection in copper-deficient cultures. Addition of copper to copper-deficient cultures following the accumulation of N_2O resulted in resumption of growth and complete consumption of N_2O in solution. Growth of the third strain, WLB20, in copper-deficient medium caused a marked accumulation of nitrite, suggesting that WLB20 has the copper form of nitrite reductase. These observations suggest a role for trace metals in regulating redox cycling of nitrogen and trace gas production in the ocean.

The respiratory decomposition of organic matter from highly productive waters leads to near exhaustion of the available oxygen, triggering the use of alternative oxidants as terminal electron acceptors. Under oxygen-limiting conditions, denitrifying bacteria reduce nitrate (NO_3^-) sequentially to nitrite (NO_2^-), nitric oxide gas (NO), nitrous oxide gas (N_2O), and dinitrogen gas (N_2) (reviewed by Zumft 1997). The global importance of denitrification lies in its distinction as the most important sink for oceanic fixed nitrogen (Codispoti and Christensen 1985). Moreover, denitrification is believed to control oceanic N_2O concentrations, serving either as a net sink of N_2O gas (e.g., Cohen and Gordon 1978; Codispoti and Christensen 1985) or conversely as a source of N_2O (e.g., Yoshida et al. 1989; Law and Owens 1990). Globally, N_2O gas contributes to greenhouse warming with a radiative potential substantially greater than CO_2 on a per molecule basis. Furthermore, N_2O is also involved in catalytic destruction of stratospheric ozone (Jackman et al. 1980).

In spite of the global importance of oceanic denitrification, the processes that regulate it remain poorly understood. The reduction of nitrogen oxides under oxygen limitation is catalyzed by a series of metalloenzymes located in the cell membrane and periplasm of denitrifying bacteria. The respiratory nitrate reductase, which catalyzes the reduction of NO_3^- to NO_2^- , contains iron and molybdenum at its reaction center. The reduction of NO_2^- is in turn facilitated by the respiratory nitrite reductase, of which there are two types present in different organisms, an iron and a copper enzyme. Nitric oxide (NO) gas is respired to N_2O via the nitric oxide reductase, an iron enzyme. And finally, N_2O gas is reduced to dinitrogen by the copper enzyme nitrous oxide reductase. The extensive involvement of copper and iron in the deni-

trification pathway likely imparts to denitrifiers distinctive nutritional requirements for these metals. To investigate these requirements, we studied the physiological response of denitrifying bacteria to reduced copper and iron concentrations. In this paper, we present our observations on the physiology of copper-limited cultures of denitrifiers. Effects of iron limitation on denitrification will be presented elsewhere (Granger and Ward pers. comm.). The data presented here provide insight into a potential regulatory role for copper in oceanic denitrification and nitrous oxide production.

Cultivation of denitrifying bacteria under trace metal clean conditions—Three strains of facultative aerobic heterotrophic denitrifying bacteria were examined: *Pseudomonas stutzeri* (ATCC 14405), generally associated with eutrophic coastal marine environments; *Paracoccus denitrificans* (ATCC 19367), ubiquitous in terrestrial and aquatic systems; and WLB20, a psychrophilic isolate from a hypersaline Antarctic lake (Ward and Priscu 1997). Cells were grown in batch culture using trace metal clean techniques in the artificial seawater medium Aquil (Price et al. 1988/1989) modified for heterotrophic bacterial cultures (Granger and Price 1999). Synthetic ocean water containing $10 \mu\text{mol L}^{-1}$ phosphate and 230 to $250 \mu\text{mol L}^{-1}$ nitrate was purified of trace metals using Chelex 100 ion exchange resin (Bio-Rad Laboratories) following the procedure of Price et al. (1988/1989). Media were first sterilized by microwaving in acid-washed polycarbonate bottles (Keller et al. 1988) and then enriched with trace metals and vitamins (B_{12} , thiamin, and biotin) that were filtered through metal-free $0.2\text{-}\mu\text{m}$ filters (Acrodisc). The organic enrichments (0.2 g bactopectone [Difco] and 0.2 g casein hydrolysate) were purified of trace metals with Chelex 100, then autoclaved before being added to sterile media.

Trace metal additions were buffered with $100 \mu\text{mol L}^{-1}$ ethylenediamine tetraacetic acid (EDTA) so that Fe^{3+} , Mn^{2+} , Zn^{2+} , and Co^{2+} free ion concentrations approximated $10^{-19} \text{ mol L}^{-1}$, $10^{-8.3} \text{ mol L}^{-1}$, $10^{-10.9} \text{ mol L}^{-1}$, and $10^{-10.9} \text{ mol L}^{-1}$, respectively. These activities were computed with the chemical equilibrium program MINEQL (Westall et al. 1976). Total Mo and Se concentrations in the media were $10^{-7} \text{ mol L}^{-1}$ and $10^{-8} \text{ mol L}^{-1}$, respectively. All the above metal concentrations are generally optimal for growth of phytoplankton (Price et al. 1988/1989) and marine bacteria (Granger and Price 1999). Premixed Cu-EDTA (1:1) was added separately at a range of concentrations: $1.2 \mu\text{mol L}^{-1}$, 120 nmol L^{-1} , 11 nmol L^{-1} , and no Cu added. Background concentrations of copper in the no-Cu medium were estimated to be 3 nmol L^{-1} (relative SD = 20%, detection limit = 0.2 nmol L^{-1} ; measurement by ICPMS; Moens et al. 1995).

Cultures were initiated from frozen stocks, acclimated to experimental medium for 10 generations, then inoculated in opaque, trilaminar, polyethylene-lined, gas-tight bags without a headspace. *P. stutzeri* and WLB20 were grown at 12°C

and *P. denitrificans* at 20°C. Medium in the bags was not purged of oxygen, such that initial growth was sustained by respiration of dissolved oxygen. Denitrification was detected subsequently as oxygen concentrations became limiting. Cells in preserved subsamples (4% borate-buffered formalin) were enumerated by flow cytometry: cellular DNA was stained with Cyto green (Molecular Probes), and analysis was performed using a FACSscan (Becton Dickinson) argon laser, with single excitation at 488 nm. Growth rates were calculated from the log-linear relationship of cell density over time as the cells were respiring nitrate, nitrite, and N_2O , respectively.

Nitrite was measured colorimetrically (Parsons et al. 1984). N_2O was analyzed by electron-capture-detector gas chromatography on a Shimadzu mini-2 gas chromatograph, equipped with a 2 m \times 2.2 mm inner diameter Haysep D column (80/100 mesh). The column was maintained at ambient temperature, while the injection port was heated to 50°C and the detector to 250°C. High purity N_2 was used as carrier at a pressure of 1 kg cm^{-2} . Twenty-five milliliters of cell culture were drawn into a plastic syringe and extracted once in an equal volume of N_2 . Dissolved gas in the culture was equilibrated with the N_2 headspace by shaking the syringe for 2 min. The headspace was then injected in an evacuated 10-ml serum vial to store until analysis. A 0.5-ml volume of gas sample was injected in the gas chromatograph for N_2O analysis. The extraction method was standardized with media that were equilibrated with known N_2O concentrations.

Electron transport system (ETS) activity was measured in cell extract concentrated by centrifugation at 10,000 rpm for 15 min and homogenized by sonication (Packard 1971). Activity of the nitrate and nitrous oxide reductases of cell extracts was also measured, following published procedures (Lowe and Evans 1964; Matsubara et al. 1982, respectively). Enzyme activities were normalized to protein concentration (Bradford 1976).

Growth under copper-replete conditions—Cultures of WLB20 and *P. stutzeri* initially grown under aerobic conditions had highest growth rates in medium containing 11 nmol L^{-1} total added copper; higher concentrations (120 nmol L^{-1} and 1.2 $\mu\text{mol L}^{-1}$) effected a reduction in growth rates relative to 11 nmol L^{-1} Cu, although these differences were not significant (data not shown). Significantly reduced growth was, however, apparent in copper-deplete medium (no copper addition). Subsequent experiments were then restricted to two treatments, copper-replete medium (11 nmol L^{-1} Cu added) versus copper-deplete medium (ca. 3 nmol L^{-1} background Cu).

Under denitrifying conditions, copper-replete cultures of *P. stutzeri* reduced nitrate to nitrite in the initial phases of anaerobic growth (Fig. 1a). Nitrite in copper-replete *P. stutzeri* cultures accumulated to 250 $\mu\text{mol L}^{-1}$ from the same initial nitrate concentration. A lag phase of variable length preceded the onset of nitrite reduction. Subsequently, nitrite was completely reduced, as judged by its disappearance from the medium. Initial nitrite consumption was accompanied by transient accumulation of N_2O gas (2 $\mu\text{mol L}^{-1}$ N equivalents = 1 $\mu\text{mol L}^{-1}$ N_2O), after which little N_2O was de-

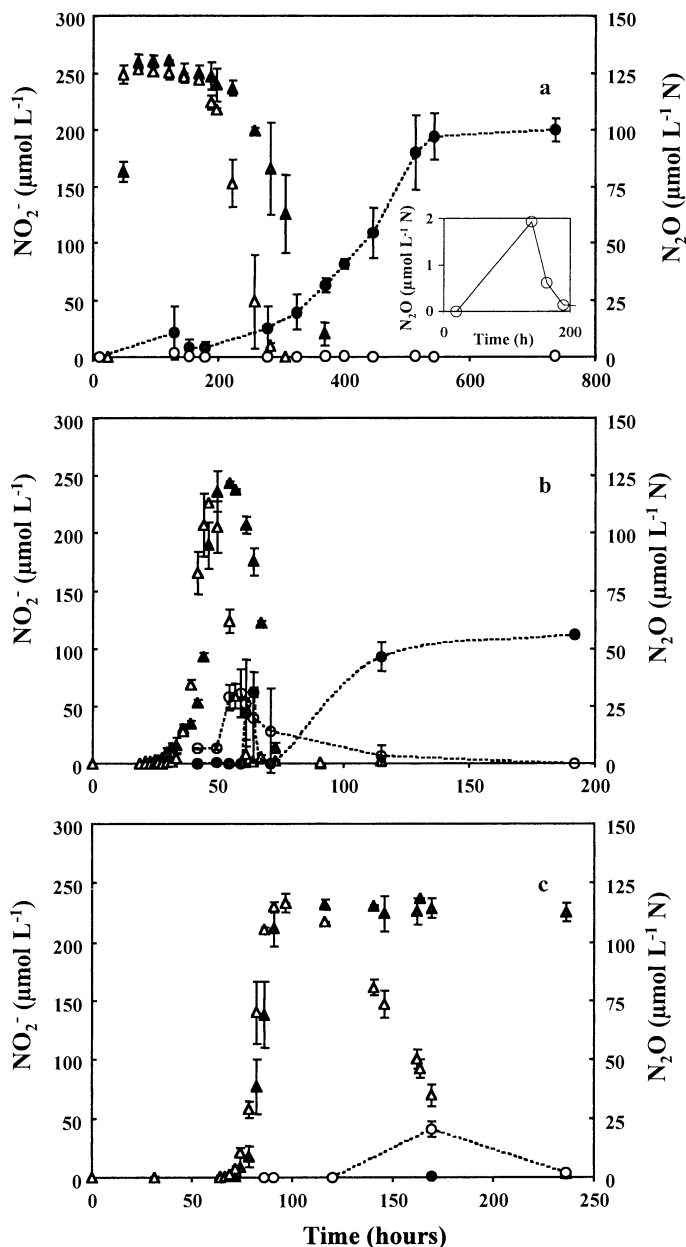


Fig. 1. Effect of copper concentrations on nitrate-dependent growth of (a) *P. stutzeri*, (b) *P. denitrificans*, and (c) WLB20. Concentration of nitrite (triangles) and nitrous oxide (circles) in copper-replete (open symbols) and copper-deplete medium (closed symbols). Error bars are standard deviations of three replicate cultures. N_2O concentrations are plotted as $\mu\text{mol L}^{-1}$ nitrogen equivalents (i.e., there are two N atoms per molecule of N_2O) in order to facilitate comparison to NO_2^- concentrations (only one N atom per NO_2^- molecule). Also note that the origin of the time axis marks the point at which nitrite was first detected in the cultures. Cells had been inoculated a day or two earlier and had presumably grown sufficiently to consume the available oxygen in the culture bags, thence initiating nitrate reduction. The inset in (a) magnifies the portion of the data showing the small transient accumulation of N_2O in the copper-replete *P. stutzeri* culture.

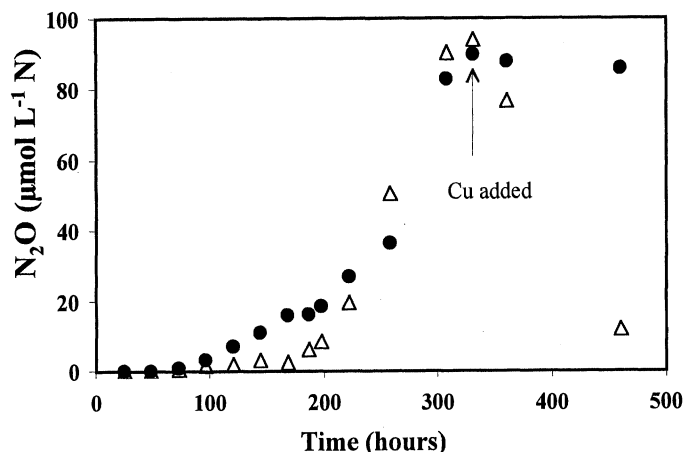


Fig. 2. Disappearance of N_2O following copper addition to copper-deficient *P. stutzeri*. Copper (10 nmol L^{-1}) was added to one (open triangles) of two replicate cultures growing in low copper medium. No copper addition (closed circle).

tected (Fig. 1a). The pattern observed here, namely, quantitative accumulation of nitrite followed by transient accumulation of N_2O , is characteristic for growth of *P. stutzeri* in culture (e.g., Matsubara et al. 1982). We did not measure nitric oxide, the product of nitrite reduction, though it is also generally observed transiently during growth of *P. stutzeri*, preceding the appearance of N_2O . However, this molecule is extremely reactive and thus only accumulates to very low levels in culture.

Growth of *P. denitrificans* in copper-replete medium was similar to that of *P. stutzeri* (Fig. 1b). Nitrite accumulated stoichiometrically from nitrate to a concentration of 250 μmol L^{-1} . Nitrite reduction proceeded almost immediately following the complete consumption of nitrate, in contrast to the variable lag preceding nitrite reduction observed for *P. stutzeri*. The onset of nitrite reduction was closely followed by the emergence of N_2O ($30 \text{ μmol L}^{-1} \text{ N}$ equivalents) to higher concentrations than observed for *P. stutzeri*. N_2O was then gradually depleted.

Growth under copper-limited conditions—The growth patterns of *P. stutzeri* and *P. denitrificans* in low copper

cultures were markedly different from those observed in copper-replete conditions. Decreasing the copper concentration in the growth medium resulted in the permanent accumulation of N_2O gas in cultures of both *P. stutzeri* and *P. denitrificans* (Fig. 1a,b). Approximately 40% of the nitrogen initially provided as nitrate was converted to N_2O for *P. stutzeri* cultures, while upwards of 20% of the nitrogen from nitrate ended up as N_2O for *P. denitrificans*. Continued monitoring of the cultures indicated that N_2O remained in the medium and was not converted to N_2 , the normal end product of denitrification. Addition of 10 nmol L^{-1} copper to copper-deficient cultures of *P. stutzeri* following the accumulation of N_2O resulted in quasicomplete consumption of N_2O in solution (Fig. 2), indicating that N_2O accumulation was caused by the lack of copper.

Copper limitation of the denitrifiers was also manifested in division rates. Growth rates of *P. denitrificans* during respiration of nitrate and nitrite may have been reduced in copper-deficient medium compared to growth in copper-replete medium (Table 1), but the decrease was not statistically significant ($p = 0.19$ for NO_3^- , $p = 0.20$ for NO_2^-). Strikingly, growth on N_2O was completely inhibited by copper deficiency. Enzyme activity measurements of *P. denitrificans* cell extracts reveal that the effect of copper limitation was most pronounced for the terminal N_2O reductase compared to other enzymes (Table 1). While the activity of electron transport systems was only reduced by a third at low copper, N_2O reductase activity was drastically depressed by copper limitation to values below the limit of detection. Lowered N_2O reductase activity likely accounts for cessation of growth on N_2O observed for copper-limited *P. denitrificans*, and, more importantly, for the accumulation of N_2O in cultures. Activity of the denitrifying enzyme nitrate reductase, which has iron and molybdenum rather than copper at its reaction center, showed no discernible difference between copper concentrations (Table 1). This is again in contrast to the observed suppression of nitrous oxide reductase activity, thus emphasizing the targeted effect of copper deficiency on the copper-requiring enzyme N_2O reductase. The suppression of N_2O reductase of denitrifiers under copper deficiency has been reported previously (Matsubara et al. 1982; Iwasaki and Terai 1982). Their observations of denitrifier physiology in

Table 1. Growth rates associated with partial reactions of nitrate, nitrite, and nitrous oxide respiration, as well as electron transport systems (ETS) activity, nitrate reductase activity, and N_2O reductase activity of *P. denitrificans* grown on nitrate in copper-replete and copper-deficient medium.

Treatment	Growth rate ($\text{d}^{-1} \pm \text{SD}$; $n = 3$)			Enzyme activity (nmole substrate reduced $\mu\text{g protein}^{-1} \text{ min}^{-1} \pm \text{SD}$; $n = 6$)		
	NO_3^-	NO_2^-	N_2O^*	ETS†	NO_3^- reductase	N_2O reductase ($\times 10^{-2}$)
Metal replete	2.12 ± 0.16	1.92 ± 0.12	0.52 ± 0.03	0.53 ± 0.02	1.9 ± 0.6	5.3 ± 0.9
Copper deficient	1.93 ± 0.08	1.79 ± 0.03	0.09 ± 0.08	0.34 ± 0.08	1.8 ± 0.7	ND

* Rates quantifying the last stage of growth were computed from the increase in cell number following complete exhaustion of nitrite from the growth medium.

† The substrate for ETS is *p*-iodonitrotetrazolium violet (INT). ETS activity was measured for cells harvested during nitrite reduction. ND = not detectable.

media amended with a copper chelator first confirmed the direct obligate involvement of copper in the reduction of N_2O .

Copper limitation of the third experimental strain resulted in markedly different effects compared to *P. stutzeri* and *P. denitrificans*. Growth of the Antarctic strain WLB20 in copper-deficient medium resulted in permanent accumulation of nitrite (Fig. 1c). Nitrite ($230 \mu\text{mol L}^{-1}$) accumulated stoichiometrically from nitrate in both high and low copper treatments, but complete nitrite consumption was only observed for copper-replete cells. Nitrite remained at $230 \mu\text{mol L}^{-1}$ in copper-limited cultures and was not consumed. N_2O gas was detected transiently in copper-replete cultures ($20 \mu\text{mol L}^{-1}$), as observed for *P. stutzeri* and *P. denitrificans*. However no N_2O was detected in copper-deficient medium, presumably because none of the nitrite was reduced. These results suggest impairment of nitrite reductase activity. The nitrite reductase enzyme of strain WLB20 is yet uncharacterized; our results suggest that it may be the copper form of nitrite reductase. *P. stutzeri* and *P. denitrificans*, in contrast, are known to synthesize the iron form of this enzyme (see Zumft 1997).

Discussion—The results of this study demonstrate the importance of copper in the denitrification pathway. Though copper is known to be toxic to marine phytoplankton at relatively low concentrations (e.g., Brand et al. 1983; Mann et al. 2002), denitrifiers in this study were clearly copper limited. Their general physiological response to reduced copper concentrations is similar to that observed for heterotrophic marine bacteria at low iron concentrations (Tortell et al. 1996), where strains showed decreased growth rates and lower ETS activity in response to iron limitation.

Reduced copper not only affected ETS and growth of the denitrifiers but also clearly and directly inhibited the cells' ability to reduce N_2O , such that growth on N_2O was suppressed in *P. stutzeri* and *P. denitrificans*. The effect of copper limitation on nitrite reduction by WLB20 was equally substantial, though we did not quantify growth or nitrite reductase activity and have not independently established that it contains the copper form of nitrite reductase. Regardless, the suppression of nitrite reduction in WLB20, in addition to the considerable decrease of nitrous oxide reduction in *P. stutzeri* and *P. denitrificans*, can be taken as compelling evidence of the sensitivity of denitrification to the supply of copper.

Ecological implications: The susceptibility of denitrifying bacteria to copper limitation leads to the hypothesis that copper concentrations in low oxygen regions of the ocean may be limiting to denitrifiers and thus contribute to observed N_2O accumulation in denitrifying zones. Existing copper profiles from the Arabian Sea (Danielsson 1980; Saagar et al. 1992) and the eastern tropical North Pacific (Boyle et al. 1977; De Baar et al. 1985), where denitrification is extensive, show surface enrichments in dissolved copper underlain by relatively copper-deplete waters from 250 to 1,000 m depth. These depths are coincident with the O_2 minimum where copper concentrations are as low as 0.5 nmol L^{-1} . A gradual enrichment in dissolved copper is observed beyond

1,000-m depth, which is attributed to remineralization of "mid-depth particles" or diffusion from the sediment (Boyle et al. 1977).

Attempts to draw parallels between field conditions and our culture work are constrained by the lack of existing measurements of copper speciation in denitrifying zones, as well as our lack of knowledge on the bioavailability of various copper pools. The surface water chemistry of copper, which has been investigated in the north Pacific and the Sargasso Sea, is believed to be dominated by complexation to organic molecules (Coale and Bruland 1990; Moffett 1995, respectively). Voltametric measurements of copper speciation in surface water demonstrate that dissolved copper is bound quasiexclusively to organic ligands, while at greater depths (ca. 300 m) nearly 10% of dissolved copper is inorganic (Coale and Bruland 1990). These ligands are typically postulated to mitigate copper toxicity for marine plankton (Mann et al. 2002), such that the potential role of copper ligands in systems where copper demand may be high has not been considered previously. Whether copper ligands constitute a bioavailable pool, or whether accessible copper is restricted to dissolved inorganic species, remains unclear. In any case, if we assume ligand concentrations at the oxygen minimum (around 250–300 m) to be no higher than those measured in the North Pacific and the Sargasso Sea at similar depths, and further assume that such ligands are not bioavailable, the inorganic pool of bioavailable copper (50 pmol L^{-1} , i.e., 10% of 0.5 nmol L^{-1}) still largely exceeds the concentration of inorganic copper that effects limitation in our culture medium (ca. 6 pmol L^{-1} calculated with MINEQL; Westall et al. 1976). This would then suggest that copper concentrations at the oxygen minimum are not limiting to denitrifiers. However the possibility of copper ligands being more abundant in oxygen minimum zones cannot be ruled out.

Factors other than organic complexation may, however, exert yet greater influence on aqueous copper chemistry in suboxic waters. The presence of nanomolar concentrations of sulfides in seawater is postulated to be a large determinant of copper speciation, even in oxygenated waters (Al-Farwati and van den Berg 1999). Measurements of metal-sulfide complexes in the Arabian Sea indicate that copper at the oxygen minimum exists primarily as inert copper-sulfide complexes (Theberge et al. 1997). If copper in the oxygen minimum is indeed chemically unreactive, copper limitation of N_2O reduction in denitrifying zones is entirely plausible. Measurements of copper speciation that take into account the oxygen sensitivity of sulfide complexes should help constrain the reactivity of copper in denitrifying zones and its potential to regulate biological N_2O consumption.

The organisms studied in our laboratory experiments may arguably be construed as fundamentally different from those in oceanic denitrifying zones, since they originate from eutrophic coastal marine waters, soils, and from an Antarctic lake, environments where copper concentrations are high compared to oceanic denitrifying zones. If continually subject to limiting copper, oceanic denitrifiers would undoubtedly have acquired adaptations that increase fitness at lower ambient copper. Analogous adaptations have been reported for marine diatoms with respect to iron, whereby oceanic

strains from low iron habitats have lower cellular requirements for iron than their coastal congeners (Brand et al. 1983; Sunda and Huntsman 1991). Any adaptation of denitrifiers aimed at reducing cellular copper demands for denitrification would nevertheless be constrained by the obligate copper requirement of N_2O reductase. N_2O reductase is the sole enzyme known to catalyze the reduction of N_2O in the ocean, such that the sole sink for N_2O in the ocean is a copper-requiring process. The existence of an alternative form of nitrite reductase that does not require copper makes that step less vulnerable to copper limitation.

Current understanding of the regulation of N_2O distributions in the ocean converges on its well-established link to oxygen. A consistent positive correlation between apparent oxygen use (AOU) and N_2O concentrations justifies the prevailing view that N_2O is produced mainly by nitrification (Yoshinari 1976). However in suboxic and anoxic waters, N_2O generally exhibits a deficit relative to AOI, which is attributed to consumption of N_2O by denitrifiers (e.g., Cohen and Gordon 1978). In other denitrifying regions, however, N_2O is found elevated beyond concentrations expected from AOI (Law and Owens 1990), and N_2O accumulation has been attributed directly to denitrification in both seawater and sediments. The lack of a consistent relationship between N_2O and oxygen concentrations in oxygen minimum zones suggests that secondary controls on N_2O production and/or consumption, beyond the evident regulatory role of oxygen, may be important.

Very low levels of free sulfide can lead to accumulation of nitrous oxide in wastewater treatment, presumably due to sulfide inhibition of various components of the denitrification pathway (Schonharting et al. 1998). Free sulfide was associated with a switch from respiratory denitrification (leading to dinitrogen) to dissimilatory nitrate reduction to ammonia (Brunet and GarciaGil 1996) in freshwater sediments, and the authors suggested direct inhibition of nitrous oxide reductase by sulfide as the reason for the switch. Free sulfide was not detected in the oxygen minimum zone (OMZ) of the Arabian Sea (Theberge et al. 1997), and these authors argued that sulfide was present only in strong and stable complexes, partly associated with copper. Thus free sulfide is not likely to influence the biochemistry of organisms in OMZ environments in the open ocean. Its presence in the form of copper-sulfide complexes, however, could limit the biological availability of both elements.

Thus it is conceivable that large accumulations of N_2O in denitrifying regions could in part be attributed to low bioavailability of copper, as observed in our cultures. Similarly, no entirely satisfactory reason for the main secondary nitrite maximum usually associated with the minimum oxygen concentration in the OMZ has been found. It is possible that copper limitation in the heart of the OMZ is associated with the nitrite accumulation there. Nitrite accumulation and N_2O production by ammonia-oxidizing (nitrifying) bacteria could also be affected by copper limitation because they reduce nitrite via the copper-type nitrite reductase (DiSpirito et al. 1985).

The clear response of the denitrifying bacteria in this study to copper limitation stresses the importance of understanding the potential role of trace metals, not only in oce-

anic denitrification, but also in other reactions of the nitrogen cycle. Key steps in nitrogen cycling are redox reactions carried out by metalloenzymes of denitrifiers, nitrifiers, and nitrogen fixers. The potential for trace metal regulation of these microbial processes raises concern for suggestions that iron fertilization may be a viable approach to enhancing primary production and carbon sequestration in the ocean: before committing to large-scale iron fertilization, it is crucial that we understand the important interactions between trace metals involved in nitrogen cycling, specifically in the production of N_2O gas.

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