

Effects of metals on nitrogen fixation and denitrification in slurries of anoxic saltmarsh sediment*

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ABSTRACT: The effect of metals on nitrogen fixation, as measured by acetylene reduction, and on denitrification, as determined by nitrous oxide accumulation in the presence of acetylene (acetylene blockage), was examined in short-term experiments with saltmarsh sediments. At concentrations of 1000 ppm (weight metal : weight dry sediment), HgCl₂, PbCl₂, CdCl₂, ZnSO₄, CuCl₂, K₂Cr₂O₇, K₂CrO₄, and Na₂MoO₄ decreased acetylene reduction throughout the experiments by more than 30 %. FeCl₃ decreased it to a lesser extent, while NiCl₂ greatly stimulated nitrogenase activity. Initial rate of nitrous oxide production was inhibited by HgCl₂, PbCl₂, NiCl₂, K₂Cr₂O₇, K₂CrO₄, ZnSO₄, CuCl₂, FeCl₃, and CdCl₂, but maximum production was stimulated substantially by PbCl₂, K₂Cr₂O₇, and K₂CrO₄, and somewhat by Na₂MoO₄, ZnSO₄, and CuCl₂. In contrast, NiCl₂ depressed both initial and maximum nitrous oxide production. Lower levels of Ni and Hg (10 and 100 ppm) caused effects that were intermediate between 1000 ppm and controls. We conclude that metal pollution could considerably alter nitrogen dynamics in marine sediments, at least in the short term, and could have repercussions on water-column productivity.

INTRODUCTION

Coastal marine sediments are a major source of combined nitrogen and play an important part in controlling water-column productivity (Nixon, 1981). The abundant nutrients found in the sediments are formed partly by microbial degradation of organic material. For nitrogen, other microbial transformations occur in the sediments: nitrification may occur in aerobic zones (Henriksen et al., 1981), while denitrification (Sorensen, 1978) and nitrogen fixation (Capone, 1983) may be important in anaerobic areas. Most organisms need to take up nitrogen in a combined form and are therefore ultimately dependent on nitrogen-fixing bacteria (Burns and Hardy, 1975). Conversely, *in situ* denitrification is one of the major processes decreasing the supply of available nitrogen. Thus the sediments may act either as a source or a sink of combined nitrogen. Marine sediments are also sites of accumulation of many of the anthropogenic pollutants entering the ocean. Increasing occurrence of heavy metals in sediments has been documented (Williams et al., 1978);

some of the metals of immediate concern are mercury, lead, cadmium, chromium, copper, zinc, and nickel (Greig and McGrath, 1977). The effect of these toxicants on microbial activity, and in particular on nitrogen transformations, is not well known. We report here the results of our recent efforts to assess the effects of several environmentally significant metals on nitrogen fixation and denitrification in anoxic saltmarsh sediments.

MATERIALS AND METHODS

Sediment was obtained in aluminum core tubes of 2.5 cm diameter from *Spartina alterniflora* communities in Flax Pond, a saltmarsh on Long Island, New York. The sediment was saltmarsh peat, with organic content ranging from 10 to 30 % (loss on ignition, Armentano and Woodwell, 1975); although much of this was refractory plant material. Sediment was taken immediately to the laboratory and the top 10 cm of the core were homogenized in a Waring blender with deoxygenated filtered seawater in the ratio 2 : 1 (seawater : sediment, v : v). The resulting slurry was

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screened through a 2 mm sieve. Fifty ml aliquots were pipetted into 125 ml Erlenmeyer flasks, which were immediately stoppered. Throughout setup, the slurry was continuously gassed with oxygen-free nitrogen. The flasks were placed in a water bath and incubated with shaking at 25°C ($\pm 1^\circ$), and all additions and withdrawals were made by syringe through the stoppers.

Nitrogen fixation was assessed by acetylene reduction (Capone and Taylor, 1980), and denitrification potential by the accumulation of nitrous oxide in the presence of acetylene (acetylene blockage, Sorensen, 1978). Acetylene was added to the gas phase of the incubations to final concentration of 20% (v:v). Nitrate was added as potassium nitrate solution; lead, mercury, copper, nickel, cadmium, and iron as the chlorides, zinc as the sulphate, chromium as chromate and dichromate, and molybdenum as molybdate. All additions were kept to 0.5 ml except for PbCl_2 (1 ml). Concentrations were calculated as nmoles ml^{-1} of slurry for nitrate, and as weight of metal (dry weight of sediment) $^{-1}$ for metals. Control flasks were amended with an equal volume of deionized distilled water; 3 replicates of each treatment were run. At the end of the experiment, gas phase volumes were measured for each flask, and the dry weight of sediment was determined after drying at 105°C to constant weight.

Ethylene and nitrous oxide concentrations were measured by gas chromatography on a Perkin-Elmer Sigma 2B gas chromatograph. Samples of 100 μl were withdrawn from the gas phase periodically and injected onto a 3 m, 3 mm O.D., 80 to 100 mesh Porapak-R column. Nitrous oxide was determined by thermal conductivity and ethylene by flame ionization, with detectors connected in series; carrier gas used was helium at 30 ml min^{-1} and oven temperature was 60°C. The detectors were calibrated with gas standards at the same temperature as the samples, in concentrations from 1 ppm to 1%; a standard close to the concentration in the incubation flasks was checked before and after every sample run. Concentrations of ethylene and nitrous oxide in the gas phase were corrected for solubility.

Effects of metals were assessed by comparisons with control flasks. The initial rate in controls was determined by least squares regression of the linear portion of the time course of gas production, excluding the lag period if it existed; the initial rate in the experimental flasks was then taken as the rate over the same period. In the acetylene reduction experiments, ethylene production at 72 h in experimental flasks was also compared with that in control flasks. In the acetylene blockage experiments, the maximum production noted during the experiment was compared between experimental and control flasks. Means of experimental and

control flasks were compared for statistical significance using a t-test.

Concentrations of copper, lead, chromium, and nickel were determined by atomic absorption spectrometry. Ten cm cores from the same site were oven-dried at 105°C, extracted with concentrated HCl-HNO_3 (1:1) for 4 h at 95°C, and the metals determined on a Perkin-Elmer model 403 atomic absorption spectrophotometer.

RESULTS

From experiments on the effects of added nitrate on rates of acetylene reduction and nitrous oxide production (Slater and Capone, in prep.), it was determined that a concentration of 100 μM added nitrate in the slurry would reliably produce large enough amounts of nitrous oxide to be precisely measurable, while never reducing ethylene production by more than 19%. One hundred μM nitrate was therefore added routinely to each incubation flask. Controls with filtered seawater only, with autoclaved homogenates, and without added acetylene were conducted periodically and showed no production of either ethylene or nitrous oxide. Acetylene concentration was routinely monitored and did not decrease during any experiment reported here.

Ethylene production in controls was usually linear from zero time for 15 to 26 h; after this time, the rate of ethylene production slowed down, but in no case did it cease even after 2 wk incubations (Fig. 1).

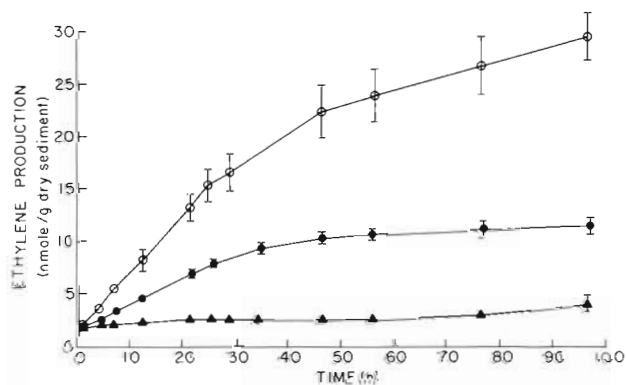


Fig. 1. Ethylene production 26 Jan 1982. Points are means of triplicate samples and error bars are standard errors of means. \circ controls; \bullet 1000 ppm Mo as Na_2MoO_4 ; \blacktriangle 1000 ppm Cr as $\text{K}_2\text{Cr}_2\text{O}_7$.

In controls a lag of 1 to 3 h was observed before nitrous oxide was detected in all but 2 experiments. In all controls, nitrous oxide production rate was constant by 5 h and continued so for 8 to 21 h; there was then a period of 6 to 20 h during which nitrous oxide concentration increased more slowly or remained relatively

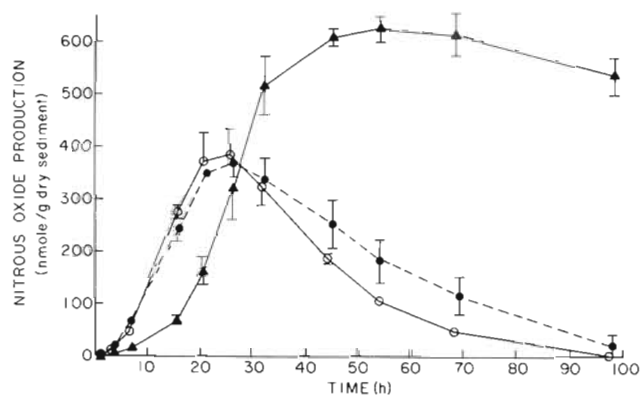


Fig. 2. Nitrous oxide production 23 Feb 1982. Points are means of triplicate samples and error bars are standard errors of means. ○ control; ● 1000 ppm Fe as FeCl_3 ; ▲ 1000 ppm Cr as K_2CrO_4

constant, followed by a decrease in nitrous oxide concentration (Fig. 2). An experiment with chloramphenicol added at $10 \mu\text{g ml}^{-1}$ showed the same time course, as did experiments with 100% acetylene atmosphere (Slater and Capone, in prep.). Addition of nitrate after the nitrous oxide concentration started to decrease had no effect in 3 experiments.

All control and experimental incubations were run in triplicate. The coefficient of variation among triplicates was less than 10% in 76% of cases.

The effects of 1000 ppm Ni, Zn, and Cu (Fig. 3) and of various concentrations of Hg (data not shown) on

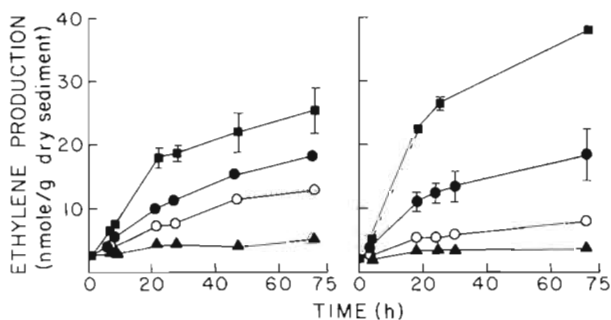


Fig. 3. Ethylene production on 17 Nov 1981 (left) with no added nitrate and on 8 Dec 1982 (right) with $100 \mu\text{M}$ added nitrate. Points are means of triplicate samples and error bars are standard errors of means. ● controls; ○ 1000 ppm Zn; ▲ 1000 ppm Cu; ■ 1000 ppm Ni

ethylene production were similar in the presence and absence of nitrate. That is, when each treatment was compared to the control incubation within the same experiment, both the direction (stimulation or inhibition) and the hierarchy of effects were similar, whether or not nitrate had been added to the sediment.

Initial rate of ethylene production in controls varied from $0.12 \text{ nmole g}^{-1} \text{ h}^{-1}$ to $1.3 \text{ nmole g}^{-1} \text{ h}^{-1}$, and 72 h

production from 6.7 nmole g^{-1} to 36 nmole g^{-1} . For nitrous oxide in controls, initial rate of production was between $5.4 \text{ nmole g}^{-1} \text{ h}^{-1}$ and $36 \text{ nmole g}^{-1} \text{ h}^{-1}$; and maximum production was between 21 nmole g^{-1} and 490 nmole g^{-1} . Experimental incubations were normalized to controls to allow comparisons between experiments on different days (Tables 1 and 2).

Table 1. Summary of effects of metals in various experiments on production of ethylene*

Metal	Ethylene production	
	Initial rate	72 h production
(Ratio of experimental to control incubations)		
Zn (2) 1000 ppm	0.43 ± 0.02 (2)	0.56 ± 0.10 (2)
Cu (3) 1000 ppm	0.20 ± 0.01 (3)	0.15 ± 0.03 (3)
Cr (5) 1000 ppm	0.07 ± 0.02 (5)	0.06 ± 0.02 (5)
Mo (2) 1000 ppm	0.44 ± 0 (2)	0.35 ± 0 (2)
Fe (2) 1000 ppm	0.94 ± 0.16 (1)	0.74 ± 0.05 (1)
Cd (2) 1000 ppm	0.34 ± 0.14 (2)	0.33 ± 0.14 (2)
Ni (1) 10 ppm	1.1	1.2
Ni (1) 100 ppm	2.0 (1)	2.0 (1)
Ni (3) 1000 ppm	2.4 ± 0.64 (3)	2.2 ± 0.46 (2)
Pb (4) 10 ppm	1.2 ± 0.23 (3)	1.2 ± 0.20 (2)
Pb (3) 100 ppm	0.83 ± 0.09 (2)	0.90 ± 0.10 (1)
Pb (4) 1000 ppm	0.70 ± 0.11 (3)	0.64 ± 0.10 (3)
Hg (2) 10 ppm	0.98 ± 0.02	0.95 ± 0.04
Hg (2) 100 ppm	0.89 ± 0	0.88 ± 0.02
Hg (3) 1000 ppm	0.53 ± 0.05 (3)	0.57 ± 0.06 (3)

* Number in parentheses after metal indicates number of experiments; ratio is presented as mean \pm standard error for pooled experiments with number in parentheses indicating number of individual experiments where triplicate dosed flasks differed from controls at or above the 95% confidence level

The effects of all metals examined on ethylene production were the same for both initial rate and final production. Pb, Hg, Cu, Zn, Cr, Mo, and Cd at 1000 ppm all caused marked inhibition, while nickel stimulated nitrogenase activity considerably. Iron caused slight inhibition of both initial rate and 72 h production, although these results were statistically significant in only 1 of the 2 experiments (Table 1).

Most metals examined (Hg, Pb, Ni, Cr, Zn, Cu, Fe, and Cd at 1000 ppm) depressed the initial rate of nitrous oxide production; Mo had no significant effect. Only Ni significantly depressed maximum levels of nitrous oxide; Pb and Cr stimulated maximum nitrous oxide concentration considerably and Mo somewhat; Zn and Cu appeared to stimulate consistently but the results in individual experiments were statistically not significant; the same was true for the depression caused by Fe. Cadmium and Hg were most inconsistent, causing either stimulation or inhibition of max-

Table 2. Summary of effects of metals in various experiments on production of nitrous oxide*

Metal	Nitrous oxide production (Ratio of experimental to control incubations)	
	Initial rate	Maximum production
Zn (1) 1000 ppm	0.58 (1)	1.2
Cu (2) 1000 ppm	0.73 ± 0.17 (1)	1.1 ± 0 (1)
Cr (5) 1000 ppm	0.28 ± 0.07 (5)	1.9 ± 0.11 (5)
Mo (2) 1000 ppm	1.1 ± 0.10	1.2 ± 0 (2)
Fe (2) 1000 ppm	0.74 ± 0.03 (2)	0.94 ± 0
Cd (2) 1000 ppm	0.23 ± 0.07 (2)	1.1 ± 0.25 (2)
Ni (1) 10 ppm	0.89	0.98
Ni (1) 100 ppm	0.81 (1)	0.85 (1)
Ni (2) 1000 ppm	0.14 ± 0.14 (2)	0.35 ± 0.35 (2)
Pb (4) 10 ppm	1.2 ± 0.26 (1)	1.2 ± 0.12 (2)
Pb (3) 100 ppm	1.2 ± 0.16 (2)	1.2 ± 0.12 (2)
Pb (4) 1000 ppm	0.60 ± 0.15 (3)	1.6 ± 0.39 (2)
Hg (1) 10 ppm	0.66	0.63
Hg (1) 100 ppm	0.48	0.62
Hg (2) 1000 ppm	0.49 ± 0.25 (2)	0.97 ± 0.12

* Number in parentheses after metal indicates number of experiments; ratio is presented as mean ± standard error for pooled experiments, with number in parentheses indicating number of individual experiments in which triplicate experimental flasks differed significantly from controls at or above the 95% confidence limit

imum production of nitrous oxide in different experiments (Table 2).

There were no significant differences between Cr added as chromate or as dichromate on either ethylene or nitrous oxide production. In experiments with Ni, Cr, Zn, and Cd, nitrous oxide persisted with little or no decrease in concentration for 6 or more days, in contrast to the disappearance in controls, usually by 72 h. This effect is shown in Fig. 2 for Cr and in Fig. 4 for Ni. Mercury and Pb also caused slight persistence of the nitrous oxide (10 to 20 h over controls). The initial lag before nitrous oxide was detected was increased by 1 to 2 h over controls by Ni, Cr, Cd, Hg, and Pb.

The effects of 10 and 100 ppm of Pb, Hg, and Ni were also examined. For Ni, there was stimulation of ethylene production at all levels, increasing with increasing amounts of nickel, though results at 10 ppm were significant only at the 90% confidence level. Both initial rate and maximum production of nitrous oxide were increasingly inhibited with increasing concentrations of Ni, though again the results at 10 ppm were significant at 90% but not at 95% probability (Fig. 4).

Concentration-dependent inhibition of production of both ethylene (initial rate and 72 h production) and nitrous oxide (initial rate) was noted with 10, 100, and 1000 ppm Hg. The effect of Hg on maximum nitrous oxide production was variable as previously noted,

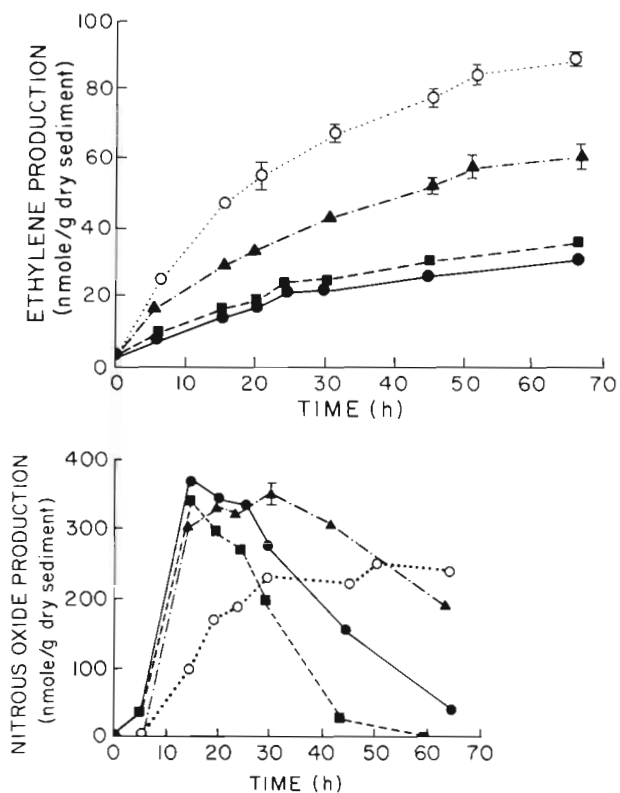


Fig. 4. Effects of Ni on production of ethylene (top) and nitrous oxide (bottom). ● controls; ■ 10 ppm; ▲ 100 ppm; ○ 1000 ppm

sometimes stimulating and sometimes inhibiting. Effects of lower concentrations of Pb were also ambiguous. Ten ppm Pb appeared to stimulate production of both ethylene and nitrous oxide, but the results were not statistically significant in all experiments. One hundred ppm Pb inhibited ethylene production but less than did 1000 ppm; the effect of 100 ppm Pb on nitrous oxide production was to stimulate slightly both initial rate and maximum production.

Representative metal concentrations for the top 10 cm of sediment at the study site were as follows: Cu, 71 ± 6 ppm, Pb, 47 ± 2 ppm, Cr, 30 ± 2 ppm, Ni, 31 ± 5 ppm, where the error is the standard error for 5 replicate samples.

DISCUSSION

Nitrogen is the limiting nutrient in several salt marshes (Valiela and Teal, 1979; DeLaune and Patrick, 1980). The magnitude of bacterial input and output of nitrogen suggests that nitrogen-transforming organisms may be of crucial importance in the overall functioning of the marsh (Haines et al., 1977; Valiela and Teal, 1979). Marshes near developed areas are susceptible to nutrient loading and toxification. It has

been suggested that salt marshes could be used as nitrogen sinks in the presence of nitrate pollution (Van Raalte et al., 1974), and as part of sewage treatment systems (Giblin et al., 1980).

Studies of the effects of metals on nitrogen-transforming bacteria are not extensive. Copper, even at very low concentrations ($5 \mu\text{g l}^{-1}$), has been shown to eliminate nitrogen fixation by cyanobacteria after 3 d of exposure (Horne and Goldman, 1974). However, another study found no correlation between concentrations of Cd, Pb, Zn, and Cu in soils and acetylene reduction activity (Rother et al., 1982). Sewage sludge (with high metal content) decreases the denitrification potential of a saltmarsh and this effect is attributed to some toxic component of the sludge (Sherr and Payne, 1981).

In our study, all the metals screened except Ni caused inhibition of nitrogenase activity as measured by acetylene reduction. The smallest effect was observed with iron, which is already present in the sediments in much higher concentration than the other metals, and in fact greater than the addition (King et al., 1982).

The effects on denitrification were in several cases not statistically significant because of high variability. The variability in any one experiment appeared to be related to the slightly different lag time in each replicate; often the replicates seemed to follow the same time course but were slightly displaced from one another. We have recently determined that this was due to variable residual oxygen concentrations in the flasks (Slater and Capone, in prep.). However, in spite of this variability, for many of these metals (Cr, Pb, Zn, Cu, Mo), it was possible to detect both an initial inhibition and a later increase of denitrification. Nickel, and to a much lesser extent Fe, decreased both initial and maximum nitrous oxide production. Although Fe(III) can act as an electron-acceptor for nitrate-reducing bacteria, this occurs only when nitrate is depleted (Sorensen, 1982), and in any case the effect of Fe here was very small.

Cr, Pb, Zn, Cu, and Mo – the metals that appear to stimulate denitrification – were observed to inhibit sulfate reduction and stimulate methanogenesis by Capone et al. (1983); they speculated that the stimulation of methane production might be caused by release of substrate competition with sulfate respirers. This argument might be extended to explain the increase in denitrification. Denitrifiers can use a much wider array of organic substrates than can sulfate reducers (Nedwell, 1982), and their potential growth yield is greater, but their importance as carbon oxidizers in anoxic sediments is usually limited by lack of nitrate. In these systems, where sufficient nitrate is added, it is possible that the denitrifiers, if metal-resistant themselves,

could have taken advantage of surplus organic substrates resulting from inhibition of sulfate respirers.

For Cr, the inhibition of acetylene reduction and the final stimulation of nitrous oxide production were both large. No difference was observed whether the Cr was added as chromate or as dichromate; in fact the chemical equilibrium between chromate and dichromate depends only on pH. Cr (VI) has been reported to be toxic to *Salmonella typhimurium* whereas Cr (III) is not (Petrilli and Deflora, 1977); when H_2S is present, Cr (VI) is reduced to Cr (III) and then precipitated (Smillie et al., 1981). Here, however, either the Cr (VI) was not reduced or the Cr (III) itself had a marked effect on the nitrogen-cycling bacteria.

Molybdenum caused considerable inhibition of nitrogenase activity. This is in agreement with the results of Nedwell and Aziz (1980), who found that addition of molybdate to saltmarsh sediments reduced acetylene reduction activity. The effect is presumably due to inhibition of sulphate respirers by molybdenum (Oremland and Taylor, 1978). In contrast, molybdenum is known to stimulate nitrogenase activity in legumes (Mulder et al., 1977).

The disappearance of the nitrous oxide measured during the acetylene blockage technique has been noted previously, although on a longer time scale than in these experiments (Yeomans and Beauchamp, 1978; Van Raalte and Patriquin, 1979). The incomplete blockage has been attributed to high sulfide concentrations (Tam and Knowles, 1979), or low nitrate concentrations (Kaspar et al., 1981; Kaspar, 1982). However, the nitrate concentrations in these experiments ($100 \mu\text{M}$) were well above those cited for incomplete blockage by Kaspar et al. (1981) ($10 \mu\text{M}$) and Kaspar (1982) ($1 \mu\text{M}$).

The disappearance of the nitrous oxide may indicate the presence in this sediment of acetylene-resistant denitrifiers, or some other nitrous-oxide consuming process (Slater and Capone, in prep.). The technique is used here as a comparative measure only, and not to assess true denitrification rates. However, some metals (Cr, Ni, Zn, Cd) inhibit the consumption of nitrous oxide, and their effect on nitrous oxide accumulation must therefore be considered as the net result of the effects on production and consumption. In 2 cases (Cr, Zn) the apparent stimulation of nitrous oxide production could be caused solely by an inhibition of consumption. For Ni, however, while N_2O consumption did not occur, production was decreased, and for Cd production was statistically similar to control values. For other metals (Pb, Cu, Mo) no effect on the N_2O -consuming process was apparent, although maximum N_2O production was stimulated. We are currently investigating the phenomenon of nitrous oxide disappearance and the effect on it of various metals.

The results with lower concentrations of the metals were more difficult to interpret; 10 ppm Pb, for instance, may not represent a significant perturbation of a system already containing 47 ppm. However, for Ni, even though the marsh concentration was 31 ppm, an apparent effect was observed even at 10 ppm (with 90 % probability). The effect of all 3 concentrations of Ni on acetylene reduction was opposite in direction from that of any other metal, and Ni was the only metal to depress maximum nitrous oxide production significantly. Nickel has been shown to be required by one methanogen (Diekert et al., 1980); the results of several parallel experiments with 100 and 1000 ppm Ni but without acetylene (data not reported here) showed no evidence of a parallel stimulation of methane production (see also Capone et al., 1983) and did not suggest a link between methanogens and nitrogen fixation.

Precipitation as sulfide or as hydroxy complexes may reduce the availability of the metals to organisms. However, the speciation of metals, and the active form with respect to observed effects on sediment bacteria, is complex and poorly understood in marine sediments. Copper concentrations in a saltmarsh have been related to sulfide formation, but complexes with organic-sulfur ligands are also important (Boulegue et al., 1982). Other work suggests that larger proportions of Pb, Cd, and Cu are present in the dissolved form in an estuarine sediment than consideration of sulfide precipitation would predict (Khalid et al., 1978). Large percentages of Cd and Cu have also been shown to be present in dissolved form in a saltmarsh (Lion and Leckie, 1982). The magnitudes of the effects reported here did not show any correlation with sulfide solubilities (Butler, 1964). Binding to organic matter may be the most important controlling factor (Gadd and Griffiths, 1978); and the effect of this on bioavailability is not simple (Wallace, 1982; Ortner et al., 1983). Other complicating factors include adsorption onto inorganic particles and competition by other ions present in much larger quantities (e.g. Mg and Ca).

In most cases, 1000 ppm of a given metal was used, which may seem a high concentration. However, high levels of metals have been reported from sediments in several areas, for example, up to 1230 ppm Cu and 990 ppm Pb in Raritan Bay, 1530 ppm Zn off California (Greig and McGrath, 1977), and 760 ppm Cr in a Scottish loch (Farmer, 1983). Sewage sludge may contain > 1000 ppm Zn and Fe, and hundreds of ppm Pb and Cu (Sherr and Payne, 1981), and high percentages of these metals are retained by saltmarsh sediments to which the sludge is applied (Giblin et al., 1980). Metals in industrial effluents also accumulate in nearby sediments and cause very high concentrations (Carmody et al., 1973; Ward and Young, 1981).

Metals such as Pb, Cu, Zn, Cr, which inhibit nit-

rogenase activity but apparently increase denitrification, and Ni, which stimulates nitrogenase but inhibits denitrification, affect both the source and the sink of combined nitrogen and would have a double tendency to alter the pool of bound nitrogen. The experiments reported here suggest that elevated metal levels, such as occur in sewage sludge, or are already found in some estuarine sediments, can cause disruption of the natural balance of nitrogen cycling in the sediments, and therefore have the potential to affect the whole water column. The recent recognition of the great importance of benthic activity to the productivity of the overlying water (e.g. Nixon, 1981) suggests the need for concern at the effects of anthropogenic disruption of the sediment system.

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