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Effect of Sulfide on Nitrogen Fixation in a Stream Sediment-Water System

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Nitrogen fixation (C_2H_2 reduction) in a sediment-water system was studied under anaerobic incubation conditions. Sodium sulfide at low concentrations stimulated activity, with a twofold increase in C_2H_4 production occurring in the presence of 8 μmol of S^{2-} per ml of stream water. Sodium sulfide at concentrations of 16 μmol of S^{2-} per ml or greater inhibited nitrogen fixation, with 64 μmol of S^{2-} per ml being completely inhibitory. Sulfide at levels of 16 μmol/ml or above inhibited CO_2 production, and the degree of inhibition increased with increasing concentration of sulfide. Titanium (III) citrate (used to modify Eh levels) stimulated both nitrogen fixation and CO_2 production, but could not duplicate, at any concentration tested, the twofold increase in nitrogen fixation caused by 8 μmol of S^{2-} per ml. Sulfide additions caused pH changes in the sediment, and when the sediment was adjusted and maintained at pH 7.0 all concentrations of sulfide inhibited nitrogen fixation activity. From considerations of the redox equilibria of H_2, H_2S, and other sulfur species at various pH values, it appeared that H_2S was the toxic entity and that HS^- was less toxic. The observed stimulation of activity was apparently due to a pH change coupled with the concurrent production of HS^- from H_2S.

Sulfide has been shown to inhibit nitrous oxide reduction during denitrification both in soil (17) and in pure cultures of denitrifiers (14, 17), although the inhibitory effect of sulfide on soil nitrification is much weaker than that of other volatile sulfur compounds such as carbon disulfide (2). In addition, the presence of both molybdate and sulfide inhibited hydrogen production by *Ruminococcus albus* and *Ruminococcus bromei* (19). On the other hand, it has been reported that in *Oscillatoria limnetica* and in the *Chlorobiaceae*, anoxygenic photosynthesis proceeds optimally in the presence of 0.7 to 5 and 4 to 8 mM S^{2-}, respectively (6, 12). Nitrogen fixation by cyanobacteria has been observed in anaerobic sediments (9), and sulfide may serve as an electron donor for nitrogen fixation in the dark (15). Also, sulfide has been reported to relieve the acetylene inhibition of nitrous oxide reduction in soil; the optimum concentration of sulfide for the relief of inhibition was 8 μmol/g of soil (17).

Sulfide production usually occurs at neutral pH values and at low redox potentials (3). In fact, it is considered to be one of the predominant volatile sulfur compounds in tidal salt marsh soil (5), estuaries (8), flooded rice soil (4), and marine sediments (7, 11, 13). High concentrations of H_2S production (8.85 μmol/ml) at 1 cm below the sediment surface in a small lagoon have been reported (7).

Although a relation between sulfate reduction and nitrogen fixation in soil has been reported (4), the effect of sulfide on nitrogen fixation has not previously been studied. This is particularly important in assessing nitrogen fixation in sulfidic habitats such as coastal marine and some freshwater sediments.

MATERIALS AND METHODS

Sediment and water samples were obtained from a stream near Floradale, Ontario, Canada. Sediment was taken with a sediment core sampler. Both sediment and stream water samples were stored at 4°C in the dark before use. A detailed description of the characteristics of the sediment and stream water samples was reported previously (18).

The sediment was extruded from the plexiglass cores, and the samples (0- to 10-cm depth) were carefully mixed. To each 50-ml Erlenmeyer flask, 10 g (fresh weight) of the sediment and 10 ml of the stream water were added. When sterile controls were required, flasks containing the sediment and stream water were autoclaved at 121°C for 1 h on 2 consecutive days. All flasks were capped with sterile serum stoppers and evacuated and back-filled three times with helium (He). Autoclave-sterilized glucose solution (0.2 ml of a 5% [wt/vol] solution) was added to each flask. Sodium sulfide and titanium (III) citrate

TABLE 1. Eh values of the sediment-water system in the presence of various concentrations of sulfide and titanium citrate

Concn of reductant (μmol/ml of stream water)	Eh (mV)	
	Sulfide	Titanium citrate
0	−165	−165
2	−205	−212
4	−225	−220
8	−290	−222
16	−360	−225
32	−405	−228
64	−430	−275
128	−480	−322

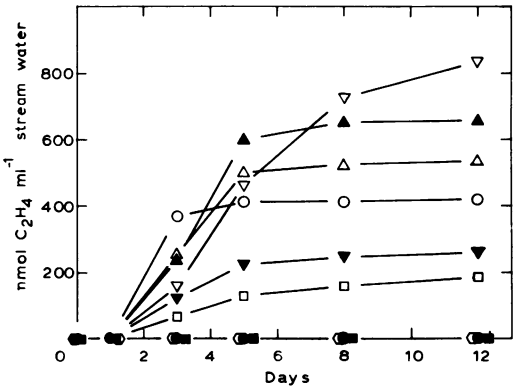

FIG. 1. Production of C_2H_4 from C_2H_2 by the sediment-water system. The sediment was supplemented with glucose and the following concentrations of Na_2S: 0 (○), 2 (△), 4 (▲), 8 (▽) 16 (▼), 32 (□), 64 (■), and 128 (○) μmol/ml. In the sterile controls, flasks contained sterile stream water and sterile sediment plus 128 μmol of S^{2-} per ml (●). Non-significantly different values ($P = 0.05$) for different treatments on the same day of sampling have been placed horizontally on the same line.

solutions were prepared under an He atmosphere. Titanium (III) citrate solution was adjusted to pH 7.0 by using sodium carbonate solution as described by Zehnder and Wuhrmann (20). As required, 0.2-ml portions of either sodium sulfide or titanium (III) citrate solution (concentrations expressed in micromoles per milliliter of stream water and indicated in the text) were filter-sterilized before addition to the flasks with a sterile syringe. For measuring the initial redox potentials (Eh values) in each treatment, reductants in a parallel set of flasks were allowed to equilibrate with the sediment-water for 1 h in the dark at 20°C. Flasks were uncapped, and Eh values were measured by the procedures of Jones and Pickard (10). The Eh values for various treatments are presented in Table 1.

In another series of flasks, the pH of the sediment was monitored for 12 days after the addition of the various sodium sulfide concentrations. The amount of 1 N HCl or 1 N NaOH required to maintain a pH of 7.0 was measured, and this amount was added to another set of flasks which were thereby maintained at pH 7.0 for the 12-day duration of the experiment. All flasks were incubated in the dark at 20°C.

For analysis of acetylene reduction activity, high-purity C_2H_2 to give 0.1 atm (10.13 kPa) was injected into the flasks after an equivalent amount of gas phase had been removed. At appropriate intervals, gas-phase samples were subjected to gas chromatographic analysis as previously described (18). Portions (0.2 ml) of the gas phase from each flask were withdrawn with a syringe equipped with a Mininert valve (Precision Sampling Corp., Baton Rouge, La.) and analyzed for CO_2, C_2H_4, and C_2H_2 by gas chromatography. All gas chromatographic data are corrected for leakage and solubility and are expressed in nanomoles (C_2H_4) or micromoles (CO_2) per milliliter of stream water. All significant differences ($P = 0.05$, $n = 3$) were calculated according to the Student-Newman-Keuls multiple-range test.

RESULTS AND DISCUSSION

The presence of Na_2S at concentrations from 2 to 8 μmol/ml of stream water stimulated nitrogen fixation (C_2H_2 reduction) by the sediment-water system (Fig. 1). Indeed, a twofold increase in total C_2H_4 produced compared with the controls was observed when the sulfide concentration was 8 μmol/ml. On the other hand, Na_2S concentrations of 16 to 32 μmol/ml inhibited nitrogen fixation, with total inhibition occurring at 64 μmol/ml. Ethylene production was not observed in the sterile controls, indicating that there was no chemical reaction between sediment, sulfide, and C_2H_2. In addition, carbon dioxide production was inhibited by sulfide at concentrations from 8 to 128 μmol/ml (Fig. 2). The degree of inhibition increased as the concentration of sulfide increased, and complete inhibition of CO_2 production occurred at 128 μmol/ml.

The presence of sulfide may have enhanced the nitrogen fixation by lowering the redox potential of the system, by providing additional reducing power or energy for nitrogenase activity, by changing the pH or other environmental factors, or by combinations of these mechanisms.

To investigate whether lowered redox potential alone could account for the twofold increase in nitrogen fixation promoted by 8 μmol of S^{2-} per ml, titanium (III) citrate solution (a strong reducing agent) was used at molar concentrations equal to those of the sulfide. During the preparation of titanium (III) citrate solution, an excess amount of citrate was used to ensure complete reaction with titanium (III) ions, thus resulting in some free citrate in the solution. Some part of the microbial populations in the sediment could have utilized this free citrate as a source of carbon or energy. The addition of titanium (III) citrate to the sediment-water system stimulated nitrogen fixation (Fig. 3). Nitro-

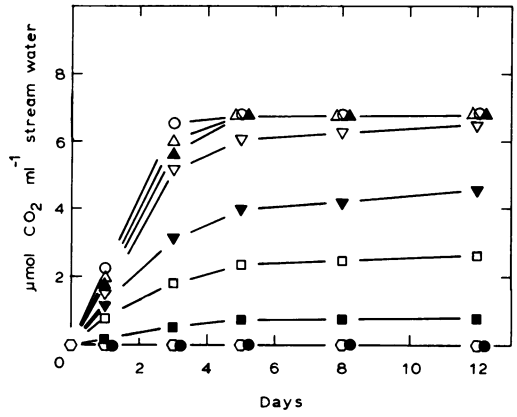

FIG. 2. Production of CO_2 by the sediment-water system in the presence of various concentrations of Na_2S. Treatments and symbols are the same as in Fig. 1. Non-significantly different values (P = 0.05) for different treatments on the same day of sampling have been placed horizontally on the same line.

gen-fixing activity increased as the concentration of titanium (III) citrate increased. An almost 1.5-fold increase in C_2H_4 production occurred when the concentration of titanium (III) citrate was 64 μmol/ml (Fig. 3). Further increase in titanium (III) citrate concentration to 128 μmol/ml caused a small decrease in C_2H_4 production. In the sterile control flasks, C_2H_4 production did not occur, indicating that there was no chemical reaction between sediment, titanium (III) citrate, and C_2H_2. The production of CO_2 increased as the concentrations of titanium (III) citrate increased, suggesting that some of the citrate was metabolized to CO_2 (Fig. 4). Evidently, the lowering of the redox potential by titanium (III) and the provision of an extra carbon or energy source from the citrate could not reproduce the twofold increase in nitrogen fixation promoted by sulfide.

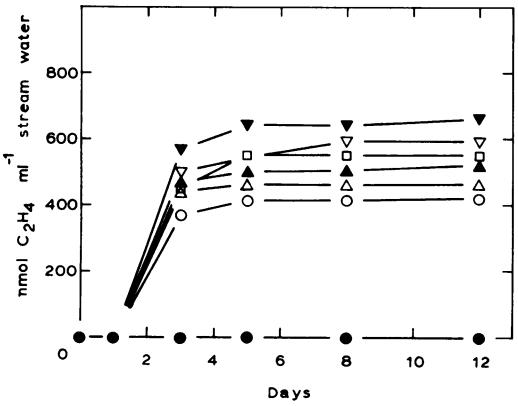

FIG. 3. Production of C_2H_4 from C_2H_2 by the sediment-water system. The system was supplemented with glucose and the following concentrations of titanium (III) citrate: 0 (○), 2 and 4 (△), 8 (▲), 16 and 32 (▽), 64 (▼), and 128 (□) μmol/ml. In the sterile controls, flasks contained sterile sediment and water plus titanium (III) citrate at 128 μmol/ml (●). Non-significantly different values (P = 0.05) for different treatments on the same day of sampling have been placed horizontally on the same line.

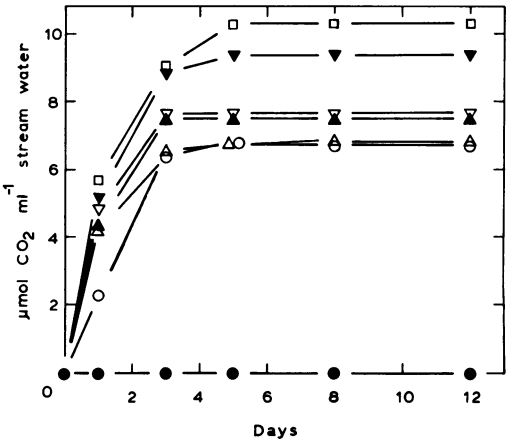

FIG. 4. Production of CO_2 by the sediment-water system. The sediment was supplemented with glucose and the following concentrations of titanium (III) citrate: 0 and 2 (○), 4 and 8 (△), 16 (▲), 32 (▽), 64 (▼), and 128 (□) μmol/ml. In the sterile controls, flasks contained sterile sediment and water plus titanium (III) citrate at 128 μmol/ml (●). Non-significantly different values (P = 0.05) for different treatments on the same day of sampling have been placed horizontally on the same line.

Sulfide addition caused an increase in the pH of the sediment. A concentration of 2 μmol/ml resulted in a transient increase in pH to 8.1 for a period of 2 days, whereas concentrations of 8, 32, and 128 μmol/ml caused the pH to increase to 8.7, 10.3, and 12.9, respectively. With these higher levels, the sediment remained above pH 8.0 for 8, 12, and >12 days, respectively. When the pH of the sediment was maintained at 7 for the duration of the experiment (Fig. 5), no stimulation of nitrogen fixation was caused by the low sulfide levels. All concentrations of sulfide led to decreased C_2H_2 reduction activity in the sediment. At pH levels greater than 7 and at Eh values more reducing than −0.20 V, HS^- is the predominant species present. At lower pH levels H_2S predominates (1, 16). Rapid interconversions between HS^- and H_2S are probable in aqueous systems even though the oxidation-reduction kinetics of sulfur species are complex and are influenced by trace catalysis due to metals, anions, and organic compounds (16). Even though the concentrations of the various sulfur species under various Eh and pH condi-

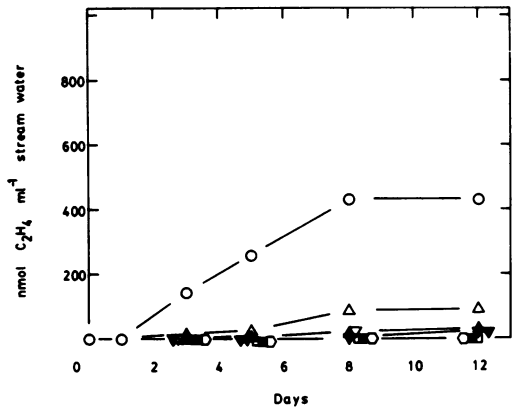

FIG. 5. Production of C_2H_4 from C_2H_2 by the sediment-water system maintained at pH 7.0 throughout the incubation in the presence of the following concentrations of Na_2S: 0 (○), 2 (△), 4 (▲), 8 (▽), 16 (▼), 32 (□), 64 (■), and 128 (○) μmol/ml. Non-significantly different values ($P = 0.05$) for different treatments on the same day have been placed horizontally on the same line.

tions are usually calculated at equilibrium, that equilibrium will be rapidly approached in sediment-water systems.

The effect of sulfide was twofold; it changed the pH of the sediment and was also toxic (as H_2S) at low concentrations at pH 7. Low concentrations of sulfide caused only a small, temporary change in the pH of the sediment (from 6.8 to 8.0 for 2 days). Under those conditions, the C_2H_2 reduction activity was stimulated. When the pH of the sediment was maintained at 7, the sulfide was predominantly in the form of H_2S, instead of HS^-, and was toxic at all tested concentrations.

The results obtained are consistent with the following hypothesis. The toxic form of reduced sulfur in sediment systems is H_2S (in terms of nitrogen fixation inhibition). The HS^- ion is relatively nontoxic and is predominant at pH values above 7 under anaerobic conditions. The addition of sulfide causes an increase in pH, thereby promoting the formation of the "nontoxic" HS^- species, but also inhibiting C_2H_2 reduction activity when it raises pH levels to above 8.5. The apparent stimulation of nitrogen fixation activity caused by low sulfide concentrations is due to it causing only a small pH increase, but this changes the predominant reduced sulfur species from H_2S to HS^-. This prevents toxic effects from being manifested and may also cause the stimulation by changing the pH to more favorable levels for nitrogen fixation activity. The small degree of stimulation noticed with titanium citrate addition may also indicate that lowered Eh values caused by sulfide additions may promote nitrogen fixation activity when toxic effects are eliminated.

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LITERATURE CITED

1. **Berner, R. A.** 1971. Principles of chemical sedimentology. McGraw-Hill Book Co., New York.
2. **Bremner, J. M., and L. G. Bundy.** 1974. Inhibition of nitrification in soils by volatile sulfur compounds. Soil Biol. Biochem. **6**:161–165.
3. **Connell, W. E., and W. H. Patrick.** 1968. Sulfate reduction in soil: effects of redox potential and pH. Science **159**:86–87.
4. **Durbin, K. J., and I. Watanabe.** 1980. Sulfate-reducing bacteria and nitrogen fixation in flooded rice soil. Soil Biol. Biochem. **12**:11–14.
5. **Farwell, S. O., A. E. Sherwood, M. R. Pack, and D. F. Adams.** 1979. Sulfur compounds volatilized from soils at different moisture contents. Soil Biol. Biochem. **11**:411–415.
6. **Garlick, S., A. Oren, and E. Padan.** 1977. Occurrence of facultative anoxygenic photosynthesis among filamentous and unicellular cyanobacteria. J. Bacteriol. **129**:623–629.
7. **Hansen, M. H., K. Ingvorsen, and B. B. Jørgensen.** 1978. Mechanisms of hydrogen sulfide release from coastal marine sediments to the atmosphere. Limnol. Oceanogr. **23**:68–76.
8. **Kellogg, W. W., R. D. Cadle, E. R. Allen, A. L. Lazrus, and E. A. Martell.** 1972. The sulfur cycle. Science **175**:587–596.
9. **Kjeldsen, C. K., and A. E. Walsby.** 1977. Nitrogenase activity in a marine blue-green algal mud flat community. J. Phycol. Suppl. **13**:36.
10. **Jones, G. A., and M. D. Pickard.** 1980. Effect of titanium (III) citrate as reducing agent on growth of rumen bacteria. Appl. Environ. Microbiol. **39**:1144–1147.
11. **Jørgensen, B. B.** 1977. The sulfur cycle of a coastal sediment (Limfjorden, Denmark). Limnol. Oceanogr. **22**:814–832.
12. **Pfennig, N.** 1975. The phototrophic bacteria and their role in the sulfur cycle. Plant Soil. **43**:1–16.
13. **Sørensen, J., B. B. Jørgensen, and N. P. Revsbech.** 1979. A comparison of oxygen, nitrate and sulfate respiration in coastal marine sediments. Microb. Ecol. **5**:105–115.
14. **Sørensen, J., J. M. Tiedje, and R. B. Firestone.** 1980. Inhibition by sulfide of nitric and nitrous oxide reduction by denitrifying *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **39**:105–108.
15. **Stewart, W. D. P., and M. Lex.** 1970. Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. Arch. Microbiol. **73**:250–260.
16. **Stumm, W. and J. J. Morgan.** 1981. Aquatic chemistry, 2nd ed., Wiley-Interscience, New York.
17. **Tam, T.-Y., and R. Knowles.** 1979. Effects of sulfide and acetylene on nitrous oxide reduction by soil and by *Pseudomonas aeruginosa*. Can. J. Microbiol. **25**:1133–1138.
18. **Tam, T.-Y., C. I. Mayfield, and W. E. Inniss.** 1981. Nitrogen fixation and methane metabolism in a stream sediment-water system amended with leaf material. Can. J. Microbiol. **27**:511–516.
19. **Wolin, M. J., and T. L. Miller.** 1980. Molybdate and sulfide inhibit H_2 evolution and increase formate production from glucose by *Ruminococcus albus*. Arch. Microbiol. **124**:137–142.
20. **Zehnder, A. J. B., and K. Wuhrmann.** 1976. Titanium (III) citrate as a non-toxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science **194**:1165–1166.