Biogeochemistry 2: 179–196 (1986)

Sulfate inhibition of molybdate assimilation by planktonic algae and bacteria: some implications for the aquatic nitrogen cycle

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Key words: molybdenum, molybdate, nutrient limitation, phytoplankton

Abstract. Molybdenum is required for both dinitrogen fixation and nitrate assimilation. In oxic waters the primary form of molybdenum is the molybdate anion. Using radioactive [^{99}Mo]Na_2MoO_4 we have shown that the transport of molybdate by a natural assemblage of freshwater phytoplankton is light-dependent and follows typical saturation kinetics. The molybdate anion is strikingly similar to sulfate and we present data to show that sulfate is a competitive inhibitor of molybdate assimilation by planktonic algae and bacteria. The ability of freshwater phytoplankton to transport molybdate is inhibited at sulfate concentrations as low as 5% of those in seawater and at sulfate: molybdate ratios as low as 50 to 100 times lower than those found in seawater. Similarly, the growth of both a freshwater bacterium and a saltwater diatom was inhibited at sulfate: molybdate ratios lower than those in seawater.

The ratio of sulfate to molybdate is 10 to 100 times greater in seawater than in fresh water. This unfavorable sulfate: molybdate ratio may make molybdate less biologically available in the sea. The sulfate: molybdate ratio may explain, in part, the low rates of nitrogen fixation in N-limited salt waters.

Introduction

Molybdenum is an essential component of several key enzymes in nitrogen metabolism. Three processes, dinitrogen fixation, assimilatory nitrate reduction, and denitrification (dissimilatory nitrate reduction), have an absolute requirement for molybdenum (Alexander 1977; Figure 1). In natural, oxic waters the thermodynamically stable form of molybdenum is MoO_4^{2-}, the molybdate anion (Evans et al. 1978). Thus, unlike many other essential trace metals, molybdenum is present as an anion rather than as a cation. Further, the molybdate anion is nearly identical to the sulfate anion (SO_4^{2-}) in terms of charge distribution, effective size (230 vs 240 picometers), structure, and stereochemistry (Cotton and Wilkinson 1972). The extremely close similarity between these two anions may make enzymatic discrimination between them rather poor.

We have hypothesized that the availability of molybdenum to organisms in natural waters may be low because of an inhibition of molybdate transport by sulfate (Howarth and Cole 1985). The biological availability of molybdate in seawater may be particularly low because of high sulfate. This low

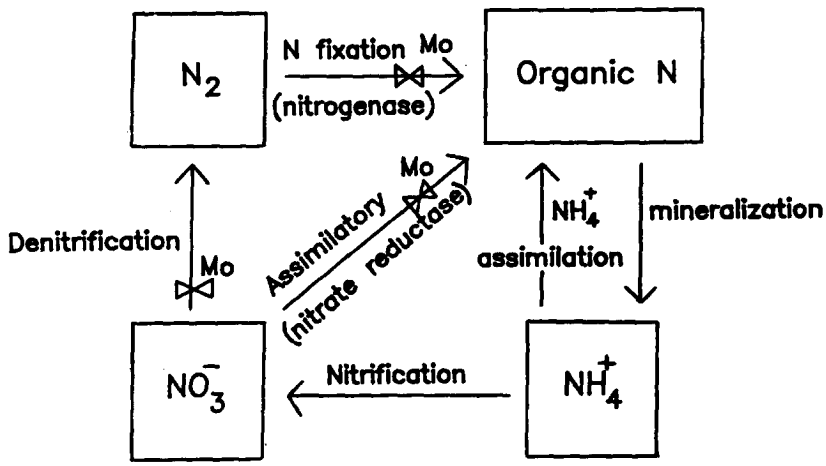

Figure 1. Diagram of the nitrogen cycle illustrating the importance of molybdenum. Major forms of nitrogen are shown in boxes: major fluxes are designated with arrows. The large X's indicate processes which require molybdenum. A molybdenum requirement for nitrification has been reported by Finstein and Delwiche (1965), but that requirement may not be absolute.

availability, coupled with the absolute requirement by molybdenum in nitrogen fixation, may explain in part why net primary production in marine systems tends to be limited by the supply of N while net primary production in most freshwaters tends to be limited by P (Boutros et al. 1982, Boynton et al. 1982, Ryther and Dunstant 1971, Schindler 1977, Vince and Valiela 1973; see, Howarth and Cole 1985).

Sulfate is the second most abundant anion in seawater and averages 28 mM. In fresh waters sulfate is much more variable but usually ranges between 0.02 and 0.6 mM (Wetzel 1975). Although the concentration of molybdate is also greater in seawater than in average freshwater (Evans et al. 1978), the sulfate:molybdate ratio is much higher in seawater than in freshwater. Thus, processes requiring molybdenum may occur more slowly (or at greater energetic expense) in seawater than in freshwater.

In an earlier paper we presented some preliminary data suggesting that sulfate can inhibit molybdate uptake by phytoplankton and that the ratio of sulfate:molybdate can affect rates of nitrogen fixation and nitrate assimilation (Howarth and Cole 1985). In this paper we present further data on the effect of sulfate on molybdate assimilation and on the effect of the sulfate:molybdate ratio on nitrate assimilation.

Methods

Measurement of dissolved molybdenum

Water samples were taken by peristaltic pump (all plastic parts) directly into acid-washed polypropylene or polyethylene bottles.

The molybdenum in a 2-liter water sample was co-precipitated with MnO_2 by the method of Bachmann and Goldman (1964) and concentrated by filtration through 3-μm pore size Nuclepore polycarbonate filters (47-mm diameter). The retained floc was later redissolved in 10 ml of 3 N hydrochloric acid and 1% hydroxylamine. Aliquots of the redissolved extract were analyzed for molybdenum by graphite-furnace atomic absorption spectroscopy on a Perkin-Elmer model 5000 spectrophotometer equipped with an HGA-400 graphite furnace. Samples were dried at 120 °C for 20 seconds, charred at 1500 °C for 15 seconds, and then atomized at 2700 °C for 6 seconds (zero ramp time). Molybdenum was read at 313.3 nm. This procedure gives a 200-fold concentration factor, allowing measurement of ambient molybdenum concentrations of 0.5 nM and above. Standards were prepared from dilute Na_2MoO_4 solutions in deionized water that were precipitated, extracted and analyzed exactly as were the samples.

Molybdenum uptake experiments

The assimilation of molybdate was measured as the incorporation of radioactive $[^{99}Mo]MoO_4^{2-}$ into particulate matter in incubation experiments in Mirror Lake, NH. Radioactive ^{99}Mo was purchased from New England Nuclear as $[^{99}Mo]Na_2MoO_4$ at a specific activity of 80 mCi/g Mo. The initial stock was diluted with deionized water and filtered twice through 0.22-μm pore Millipore filters to remove particulate contaminants. Aliquots of the diluted solution were added to lake water in either 30 ml or 240 ml sterile polycarbonate flasks which were incubated in situ.

We varied a number of parameters in these experiments, including time, light intensity, and the concentrations of molybdate, sulfate, and chloride. Incubation times ranged from 15 minutes to 30 h in time-course experiments and the molybdate concentrations ranged from 2.7 to 440 nM (final concentration).

The incubations were terminated by adding unlabeled Na_2MoO_4 to give a final concentration of 10 μM, about 25-fold greater than the largest addition of labeled molybdate. Time-zero controls run in parallel, had a concentration of unlabeled molybdate of 10 μM at the start of the experiment. After termination the samples were held in a cold (4 °C) dark cooler and processed rapidly, within 30 minutes. The entire contents of each flask was filtered through a 25-mm Gelman A/E filter which had been pretreated by soaking for 72 h in 1 μM Na_2MoO_4. This soaking procedure was adopted to decrease sorption of ^{1}abled molybdate on the filter. The flasks were then rinsed with 20 ml of deionized water and shaken vigorously to remove particles from the walls; this rinse water was then passed through the same filter. Finally, two, 2-ml aliquots of 1 μM Na_2MoO_4 were passed through each filter, followed by a rinse of 3 ml of deionized water. To process these samples rapidly, we used a manifold which allowed 14 samples to be filtered simultaneously.

The amount of radioactivity retained on each filter was measured by

liquid scintillation counting on a Packard model 2000 counter. The scintillation cocktail was 2:1 toluene:2-methoxyethanol plus 7 g PPO per liter. Quenching was checked with the channels ratio method; within a given experiment, quenching was identical between samples. To determine that the retained radioactivity was in fact ^{99}Mo and not some other radionuclide contaminant of the stock, we measured the decay rate of both the samples on the filters (e.g. of the assimilated portion) and of the ^{99}Mo solutions we added. In both cases the calculated half-lives were within 1% of the 66.7 h half-life published for ^{99}Mo (Weast et al. 1983).

Growth Experiments

Molybdenum is required for the assimilation of nitrate but not for the assimilation of ammonium. To test our hypothesis that sulfate affects the availability of molybdate, we subjected test organisms to a range of sulfate:molybdate ratios and measured their growth rates with nitrate as the sole nitrogen source in comparison to growth rates with ammonium as the sole nitrogen source. The ammonium treatment served as a control; thus, if the growth rate on ammonium were affected by the sulfate:molybdate ratio, the effect would have to be ascribed to something other than inhibition of molybdate transport.

Algal cultures

Two cultures of marine diatoms, *Thalassiosira pseudonana* and *Cyclotella sp*, were obtained from Dr R.R.L. Guillard and were maintained in modified F/4 media (Guillard 1975) with citrate as the chelator (no EDTA) and a molybdate concentration of 0.01 μM; *Cyclotella* was grown at a salinity of 6 ppt, *Thalasiosira* at 32 ppt. The cells were transferred at low inoculum to artifical seawater media in which total salinity, sulfate, chloride, and molybdate could be varied (Table 1). In our first experiments were merely increased the concentration of sulfate or chloride but made no attempt to maintain a constant ionic balance in the media. In later experiments we kept either total moles of Na^+ or ionic strength constant (using NaCl) but we varied either the Na_2MoO_4 or Na_2SO_4 concentration to change the sulfate concentration and sulfate:molybdate ratio. This way we could determine the effect of molybdate or sulfate on growth in a medium of ionic strength similar to that of seawater.

A culture of freshwater, planktonic microalga, *Selenastrum sp.*, was obtained initially from Carolina Biological Supplies; we maintained it in artificial media with low molybdate and with nitrate as the sole nitrogen source (Table 1). This culture was used in experiments similar to those described above.

Growth was assayed by measuring in vivo chlorophyll-a fluorescence with a Turner Designs model 10-000 R fluorometer.

Table 1. Chemical composition of the media used in the growth experiments. A – artificial saltwater media; B – artificial freshwater media. For the freshwater bacterial experiments we used the artificial freshwater media with the addition of dextrose at a final concentration of 150 mg/liter. In the experiments, we varied NaCl and Na_2SO_4 to change the sulfate concentration without altering the total ionic strength. The saltwater media shown here (A) is configured for brackish water at about 6 ppt salinity as in the *Cyclotella* experiment; salinity can be varied by increasing the concentration of all major constituents in the left column or by separately increasing NaCl or Na_2SO_4. Molybdate was added as Na_2MoO_4 as explained in the text

A. Artificial salt mater media				
Major elements	Final concentration (mM)		Minor elements	Final concentration (μM)
NaCl		82.6	$CuSO_4$	0.027
$MgCl_2$		10.6	$ZnCl_2$	0.047
$CaCl_2$		2.08	$CoCl_2$	0.032
KCl		1.9	$MnCl_2$	0.55
Na_2SO_4		0.36	ferric citrate	1.8 mg/liter
$NaHCO_3$	(to maintain	3.0)	citric acid	10.0 mg/liter
$NaNO_3$ or NH_4Cl		0.18		
Na_2HPO_4		0.03		
Na_2SiO_3		0.2		
B. Freshwater media				
$MgSO_4$		0.37	$CuSO_4$	0.04
Na_2SiO_3		0.20	$ZnCl_2$	0.11
$CaCl_2$		0.08	$CoCl_2$	0.42
$NaHCO_3$	(to maintain	0.25)	Citric acid	30.0
$NaNO_3$ or NH_4Cl		0.18	Na-EDTA	1.0
K_2HPO_4		0.03	Vitamins	(B_{12}, biotin, thiamine trace)

Bacterial cultures. An unidentified heterotrophic pseudomonad was isolated from freshwater on the Cary Arboretum and maintained in completely mineral media with dextrose as the sole carbon source and nitrate as the sole nitrogen source (Table 1). The organism was inoculated into media in which the ionic strength was kept constant but the NaCl and Na_2SO_4 concentrations were varied to provide a wide range of sulfate concentrations (Table 1). The growth of the bacterium was measured by epifluorescent microscopy using the acridine orange method of Hobbie et al. (1977) at 1000 x magnification. Again, we compared the effect on growth when ammonium was the sole nitrogen source as a control for the case when nitrate was the sole nitrogen source.

Results

Assimilation of ^{99}Mo in lake water

The conversion from dissolved molybdate to particulate molybdenum showed saturation-type kinetics that fit the Michaelis-Menten model (Figure 2-A). In Figure 2-B the data are plotted following Wright and Hobbie (1966). Plotted this way, the slope is equal to the reciprocal of Vmax, and calculated Vmax would be 270 pmol $liter^{-1}$ h^{-1}. When plotted as a standard Lineweaver-Burke

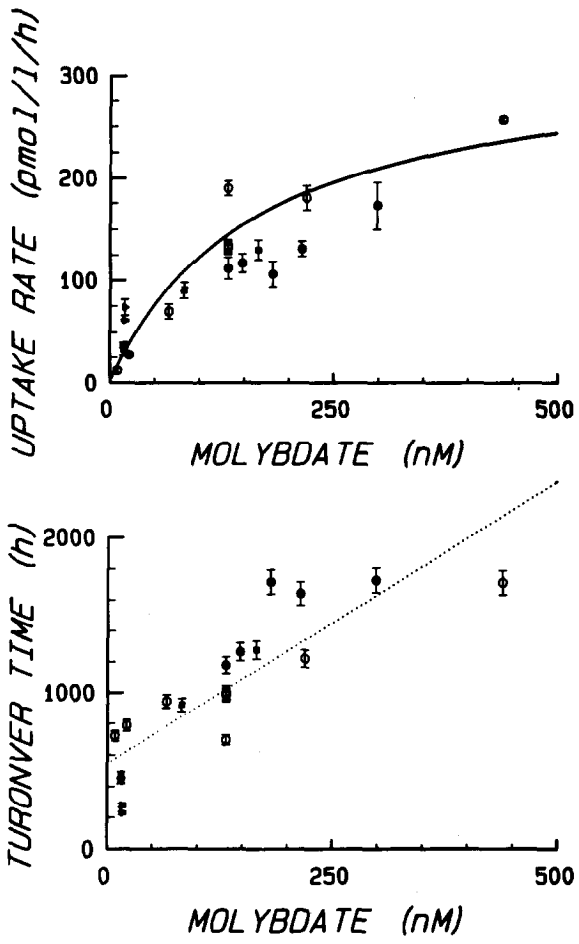

Figure 2. Effect of added molybdate concentration on conversion from dissolved molybdate to particulate molybdenum in experiments with ^{99}Mo. Labeled molybdate was added as $[^{99}Mo]Na_2MoO_4$ to water from Mirror Lake (8-m depth) at a variety of concentrations, and the rate of molybdate uptake was measured. Each symbol represents an experiment done on a different date during August 1984; for each experiment mean and standard deviation are shown for triplicate incubations.

(A) Rate of uptake plotted against the molybdate concentration. The line represents the best fit of the equation:

$$v = Vmax^{*}S/(Km + S)$$

The parameters Vmax (270 pmol liter^{-1} h^{-1}) and Km (160 nM) were obtained from a least squares regression of the reciprocal of rate against the reciprocal of molybdate concentration (Lineweaver-Burke plot).

(B) Data are plotted after Wright and Hobbie (1966) such that the slope is inversely proportional to Vmax and the Y-intercept is the turnover time at the ambient molybdate concentration. The regression line is:

$$Y = 3.62X + 545; \quad r^2 = 0.73, n = 19.$$

The regression is significant at $p < 0.001$.

plot (e.g. double reciprocal plot) the regression is also significant ($r^2 = 0.76$; $n = 19$) and calculated Vmax is 323 pmol liter^{-1} h^{-1}.

The conversion from dissolved molybdate to particulate molybdenum required time, and essentially no conversion took place in time-zero controls (Figure 3, Table 2). This result gives us some confidence that the uptake we are measuring is not a rapid abiotic sorption process. Further, the time course experiment suggested that the rate of uptake was more rapid in the light than in the dark (Figure 3). We investigated this suggestion more directly in a light and dark bottle experiment (Table 2). The rate of conversion was clearly light-dependent and correlated well with the assimilation of inorganic carbon suggesting that the transport of molybdate required energy or otherwise depended upon photosynthesis (Table 2). In this experiment, roughly 17 nmoles of molybdate were assimilated for every μmole of inorganic carbon. As the added molybdate concentration in this experiment was 0.017 μM, about 50 times greater than ambient levels, the ratio of 17 nmole Mo per μmole C must be considered an overestimate. The actual ratio of uptake at ambient levels would be about 0.3 nmole Mo per μmole C. Based on this uptake ratio the molybdenum content of Mirror Lake plankton would be about 10 nmol per gram dry weight (1 ppm). This is close to the average value for freshwater

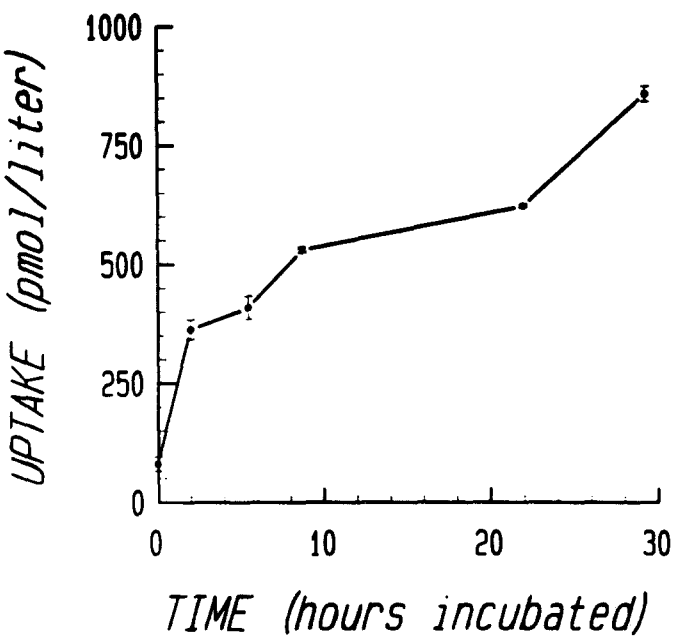

Figure 3. Time course of the uptake of [^{99}Mo]Na_2MoO_4 in Mirror Lake. Labeled molybdate was added at a final concentration of 0.017 μM in clear, polycarbonate bottles filled with water from 8-m depth and incubated in situ. The amount of ^{99}Mo in particulate material (after rinsing with cold Mo; see text) was measured over time. The data are corrected for decay of ^{99}Mo during the experiment. The zero hour is 0800 hours.

Table 2. Effect of light on the assimilation of $[^{99}Mo]Na_2MoO_4$ in Mirror Lake water. Water from 8 m was incubated for 8.5 hours in either clear or opaque polycarbonate bottles and assimilation of both ^{99}Mo and of $[1^4C]NaHCO_3^-$ were measured. Time-zero controls are shown for both ^{14}C and ^{99}Mo uptake

Treatment	Number of trials	^{14}C-uptake μmoles C liter^{-1} h^{-1}		^{99}Mo uptake pmol Mo liter^{-1} h^{-1}	
		mean	sd	mean	sd
Time zero	3	0	0	0.79	0.18
Dark bottles	5	0.02	0.01	6.4	1.3
Light bottles	8	2.02	0.2	33.7	6.9

phytoplankton of 2 to 40 nmol per gram dry weight (0.2–4 ppm; Evans et al. 1978).

The calculated turnover time for molybdate at the ambient lake water concentrations ranged from more than 1000 days in 2-m water, where the chlorophyll concentration was low ($< 1\,\mu$g chlorophyll-a/liter) to less than 25 days in 8-m water where the chlorophyll concentration was higher ($30\,\mu$g chlorophyll-a liter^{-1}). For 8-m water the Vmax averaged 320 pmol liter^{-1} h^{-1} (calculated from both Wright-Hobbie plots and Lineweaver-Burke plots), and the apparent Km averaged about 160 nM (Figure 2). The ambient concentrations of molybdate at 2 and 8 m were about 100 and 300 pmol liter^{-1}, roughly 1000 fold lower than the calculated Km concentration.

In summary of these results, the conversion from dissolved molybdate to particulate molybdenum required time, appeared to follow saturation-type kinetics, and occurred more rapidly in the light than in the dark. Taken together these results suggest that this conversion is a biological process that requires energy or net growth.

Inhibition of molybdate uptake

Sulfate inhibited the conversion from dissolved molybdate to particulate molybdenum in lake water at sulfate concentrations as low as $200\,\mu$M (Figure 4). At concentrations equal to inhibitory levels of sulfate, chloride did not inhibit molybdate uptake indicating that the effect was not due to increased Na or salt (Figure 4). We then varied both the sulfate and molybdate concentrations to produce a series of kinetic inhibition plots (Figure 5). Sulfate had a large effect on the apparent Km and turnover time but a much smaller and inconsistent effect on Vmax. The addition of a near-saturating concentration of molybdate of about $0.2\,\mu$M essentially reversed the kinetic inhibition caused by the sulfate (Figure 5). Thus, sulfate appears to act as a competitive inhibitor of molybdate uptake. From the intersection of lines A and B in the Dixon plot (Figure 5) the calculated constant of inhibition (K_i) is about $560\,\mu$M. It is noteworthy that lines A, B and C do not intersect exactly at a common point. This suggests that the competitive model may be imperfect for these data.

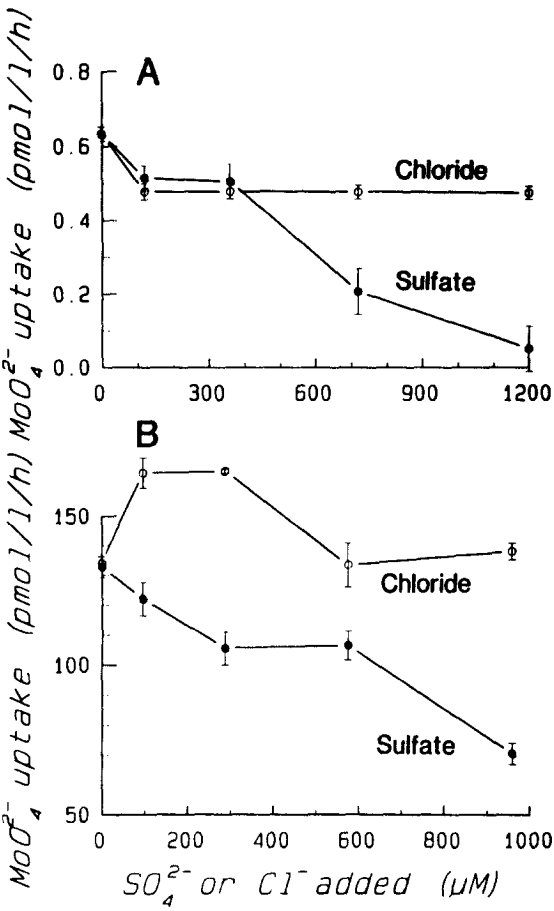

Figure 4. Effect of sulfate or chloride on assimilation of [^{99}Mo]Na_2MoO_4 in Mirror Lake water from (A)-2-m depth and (B)-8-m depth. Labeled molybdate was added at a final concentration of 0.017 μM at 2 m and 0.132 μM at 8 m and incubated in situ for 6.5 h. Mean and standard deviations of triplicate incubations are shown for each point. Open circles represent additions of NaCl; solid circles represent additions of Na_2SO_4.

Growth experiments

Freshwater bacteria

We varied the molybdate concentration in artificial media from 0 to 2.08 μM and found that the growth rate of a freshwater pseudomonad (with nitrate as the sole nitrogen source) was a hyperbolic function of the molybdate concentration under these conditions. We then grew this isolate in media in which the ionic strength was kept at 0.008 and the molybdate concentration was held at 0.02 μM but the sulfate concentration was varied from 0.03 to 4 mM. When nitrate was the sole nitrogen source, the growth rate was depressed

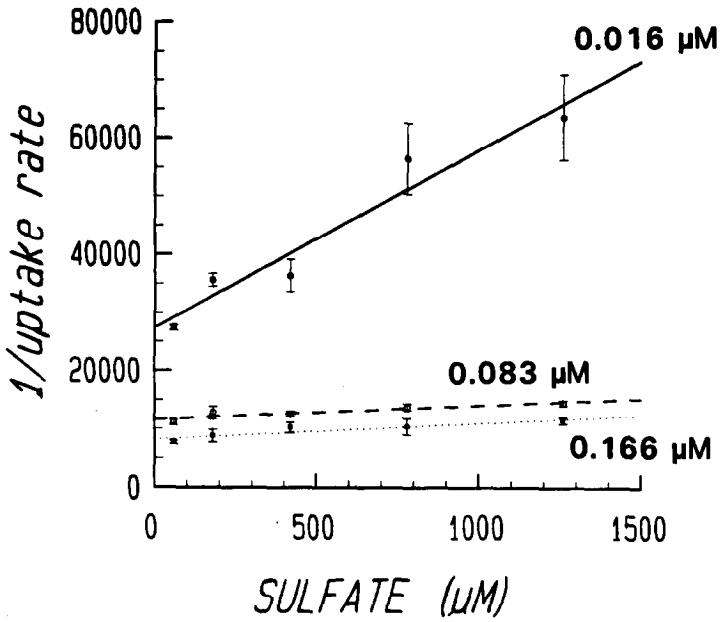

Figure 5. Kinetic plots (Dixon plots) in which both the Na_2SO_4 and [^{99}Mo]Na_2MoO_4 concentrations were varied. Five levels of sulfate were added to water from 8-m depth along with three levels of molybdate. Concentrations of inhibitor (sulfate) is plotted on the X-axis and the reciprocal of the uptake rate is plotted on the Y axis (1/(µmol liter^{-1} h^{-1}); mean and standard deviation for triplicate incubations shown). Each regression line is for a different molybdate concentration. Plotted this way, the common X-intercept gives a measure of the inhibition constant, Ki.

(A) 0.016 µM; $y = 30.5X + 27399$; $r^2 = 0.93$;

(B) 0.083 µM; $y = 2.93X + 11600$; $r^2 = 0.86$;

(C) 0.167 µM; $y = 2.84X + 8164$; $r^2 = 0.86$.

Each regression is significant at $p < 0.01$.

as the sulfate concentration was increased; when ammonium was the sole nitrogen source, sulfate had no consistent effect on the growth rate (Figure 6). Thus, altering the sulfate : molybdate ratio did not affect ammonium metabolism but had a pronounced effect on nitrate metabolism. The cause of this effect is presumably the inhibitory effect of sulfate on molybdate uptake.

Marine microalgae

We used a marine diatom, *Thalassiosira pseudonanna* in a similar experiment and obtained similar results. *Thalassiosira* was maintained in nitrogen-limited batch cultures in artificial media. We did not maintain constant ionic strength but allowed the ionic strength to increase as the sulfate concentration was increased from 0.34 mM to 35 mM. The NaCl concentration was kept at 0.02 µM. In this case the increase in Na_2SO_4 caused an increase in the ionic

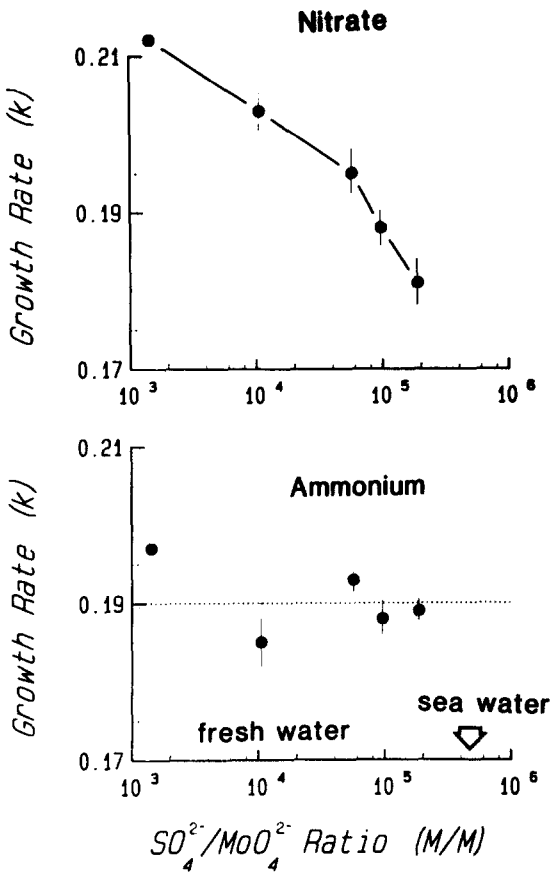

Figure 6. Effect of molybdate concentration and sulfate: molybdate ratio on the growth rate of a freshwater pseudomonad. At constant ionic strength (0.008) and molybdate (0.02 μM) concentration, the sulfate concentration was varied. The growth media is outlined in Table 1. Growth rate is plotted as a function of the log of the sulfate: molybdate ratio for growth with nitrate as the sole N-source (A) and (B) growth with ammonium as the sole N-source. Mean and standard error for quintuplicate incubations are shown for each point.

strength of about 23%. When nitrate was the sole nitrogen source, both the growth rate and final yield of *Thalassiosira* were decreased as the sulfate concentration was increased. When ammonium was the sole nitrogen source, however, neither the growth rate nor the final yield showed any trend with sulfate concentration (Figure 7 and Table 3). Again, sulfate appeared to interfere with nitrate metabolism but not with ammonium metabolism, an effect that is consistent with our hypothesis.

In another series of experiments, high sulfate concentrations induced a long lag phase for a brackish marine *Cyclotella. sp.* grown on nitrate but had no effect on growth on ammonium (Figure 8). However, in this experiment,

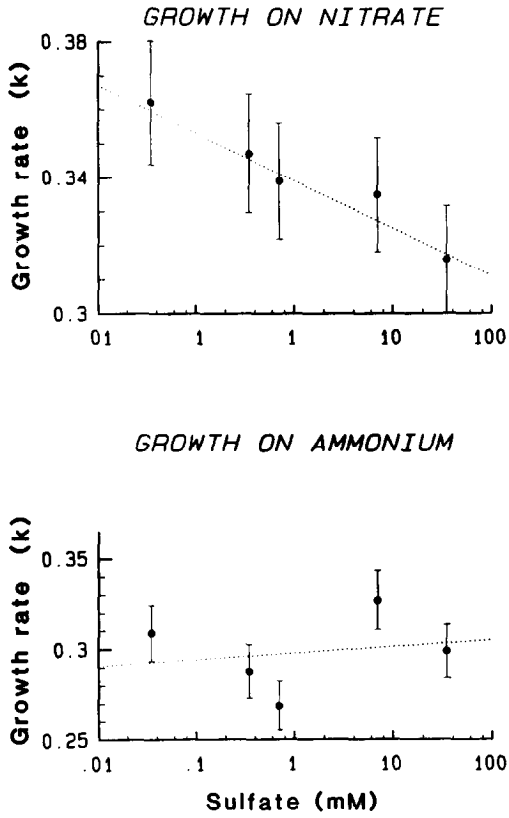

Figure 7. Effect of varying sulfate:molybdate ratio on the growth rate of a salt-water diatom, *Thalassiosira pseudonana.* A) nitrate is the sole N-source; B) ammonium is the sole N-source. The growth media is outlined in Table 1. Mean and standard errors are shown. Further data for this experiment are presented in Table 3. Regression lines:
(A) $Y = -0.086X + 2.03$; $r^2 = 0.95$ significant at $p < 0.01$;
(B) regression not significant.

neither the growth rate per se nor the yield were affected by sulfate with either nitrate or ammonium as the nitrogen source (Figure 8).

Freshwater microalgae

We maintained a culture of *Selenastrum sp.* in artificial media (Table 1) and subjected it to varying molybdate levels in an attempt to induce molybdenum limitation prior to trying a sulfate experiment. After 12 successive transfers into low molybdate (0.01 μM) media with nitrate as the sole nitrogen source, we increased, stepwise, the molybdate concentration from 0 to 0.1 μM and assayed the growth rate. Surprisingly, the increased molybdate had no discernable effect on the growth phase or lag phase for *Selenastrum.* Thus, *Selenastrum* did not respond to molybdate the way our other test organisms did. Perhaps *Selenastrum* has very low requirements for molybdenum or

Table 3. Growth of a marine diatom, *Thalassiosira*, in artificial media (Table 1) as a function of the sulfate concentration. The concentration of molybdate was held constant at 0.02 μM. The data for both doubling time and final yield are shown for triplicate incubations for a 14-day period and all replicates were pooled. a.f.u. = arbitrary fluorescence unit and is proportional to total chlorophyll. A) Nitrate was the sole N source. Regressions of log [Sulfate] against either growth rate or yield are both significant at $p < 0.01$. B) Ammonium was the sole N source. Neither regression is significant for growth on ammonium

A. Growth on nitrate				
Sulfate	Doubling time (day)		Final yield (a.f.u.)	
(mM)	Mean	s.d.	Mean	s.d.
0.035	1.91	0.61	1367	58
0.35	1.99	0.63	1000	100
0.71	2.04	0.62	983	29
7.06	2.07	0.62	833	57
35.0	2.19	0.69	493	103
Regressions: $r^2 = 0.95$	doubling time = 0.086 (log [sulfate]) + 203;			
	yield = − 264 (log [sulfate]) + 952; $r^2 = 0.95$			
B. Growth on ammonium				
Sulfate	Doubling time (days)		Final yield (a.f.u.)	
(mM)	Mean	s.d.	Mean	s.d.
0.035	2.24	0.72	650	327
0.35	2.41	0.79	536	238
0.71	2.58	0.83	280	0.58
7.06	2.11	0.68	733	58
35.0	2.32	0.74	490	147
Regressions:	doubling time = − 0.027 (log [sulfate]) + 2.3; $r^2 = 0.03$			
y	yield = − 6.1 (log [sulfate]) + 538; $r^2 = 0.002$			

exceedingly good luxury uptake capabilities. Another possibility is that some other trace metal or co-factor was limiting growth. We repeated the experiment with EDTA – chelated trace metals and found a further surprise. Under these conditions, again with nitrate as the sole nitrogen source, increasing the molybdate concentration over the same range had a small but significant inhibitory effect on both the growth rate and yield of *Selenastrum* (Table 4). We do not yet have an explanation for these results.

Discussion

Little is known in general about molybdate uptake and even less about sulfate inhibition of that uptake. Of the few published studies the most comprehensive is that of Elliot and Mortenson (1975) who studied the transport of molybdate by a nitrogen-fixing strain of *Clostridium pasteurianum*. Unfortunately, *Clostridium* is an obligate anaerobe that is usually isolated from, anoxic wet soils or muds. In these reducing environments molybate is not likely to be the major form of molybdenum, and it is not clear which chemical

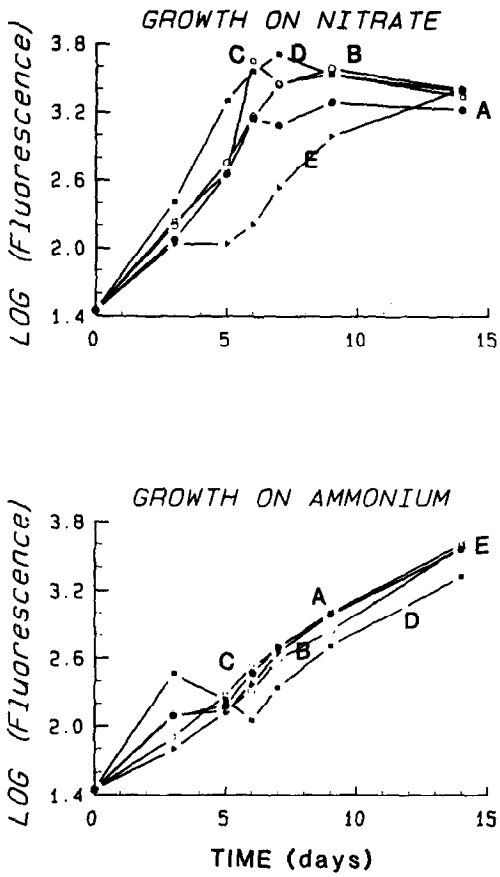

Figure 8. Effect of varying the sulfate:molybdate ratio on the growth of a saltwater diatom, *Cyclotella sp* in brackish media (Table 1). Both the ionic strength (0.587) and molybdate concentration were kept constant but the sulfate concentration was varied broadly. Lines represent added sulfate levels of A; (solid circles) 0.3 mM, B; (open circles) 1.5 mM, C; (open squares) 7.5 mM, D; (solid squares) 37.5 mM, and E (solid triangles) 75 mM. Upper panel – Nitrate was the sole N source. Lower panel – Ammonium was the sole N source. In each case triplicate incubations were analyzed at each point. The average coefficient of variation was 22%; the variance is not shown to simplify the figure. Note that there is an indication of increased lag at high sulfate on nitrate but no effect on ammonium.

form of molybdenum would normally be transported by the organism. Nevertheless, there are some salient features of that study which are useful for comparison here. Elliott and Mortenson (1975) determined that *Clostridium pasteurianum* transported molybdate against a concentration gradient, and that this transport had clear pH and temperature optima, and that transport required energy. They found an apparent Km of 48 μM for molybdate transport.

Table 4. The growth of a freshwater alga, *Selenastrum* as a function of molybdate concentration in artificial media with EDTA-chelated micronutrients (Table 1). Note that the doubling time increased (e.g. growth rate slowed) as the molybdate concentration was increased. Also, yield decreased as molybdate increased

Molybdate (μM)	Doubling time (days)		Final yield (a.f.u.)	
	Mean	s.d.	Mean	s.d.
0.001	2.24	0.29	21.3	1.2
0.021	2.48	0.09	19.0	2.6
0.104	2.60	0.38	17.8	3.5
0.52	2.66	0.08	16.3	0.58
2.08	3.03	0.55	13.3	0.58

Regressions: doubling time = 0.21 (log [molybdate]) + 2.84; $r^2 = 0.90$

yield = $-$ 2.56 (log [molybdate]) + 15; $r^2 = 0.944$

We calculate that the apparent Km for the same process in Mirror Lake plankton was 160 nM, roughly 300 fold lower than for the *Clostridium* culture. As the concentration of molybdate in Mirror Lake is very low (100 to 300 pM), it is not surprising that the Km is lower than for *Clostridium*, which inhabits sediments and soils and had probably been kept in rich laboratory media for many generations. It is noteworthy, however, that the Mirror Lake plankton are living at molybdate concentrations about three orders of magnitude lower than their apparent Km concentration.

In Mirror Lake the Vmax for molybdate transport averaged about 300 pmol liter^{-1} h^{-1} at 8 m. If we assume that only phytoplankton had assimilated molybdate the Vmax would be about 0.02 nmol (g dry weight)$^{-1}$) min^{-1}. The Vmax for the *Clostridium* culture (at 37 °C) was 55 nmol (g dry weight)$^{-1}$) min^{-1}, 2500 times greater than for Mirror Lake plankton (Elliot and Mortenson 1975). Thus, *Clostridium* in culture has a much greater capacity for molybdate uptake than does Mirror Lake plankton, which again is not surprising considering the difference in environments.

Inhibition of transport by sulfate

Our hypothesis about N limitation in seawater requires that sulfate inhibits molybdate uptake (Howarth and Cole 1985). In Mirror Lake added sulfate inhibited molybdate uptake at concentrations lower than 5% of seawater sulfate levels and at sulfate: molybdate ratios two orders of magnitude lower than that ratio in seawater. In Mirror Lake sulfate appeared to affect the apparent Km (or Ki) and turnover time but had little effect on Vmax; the inhibitory effect of sulfate could be reversed by adding more molybdate.

An inhibitory effect of sulfate on molybdate transport was first demonstrated by Stout and Meagher (1948) for tomato plants in solution culture. This effect was later demonstrated for *Clostridium* (Elliot and Mortenson 1975), for duodenal loops of the sheep (Huising and Matrone 1975), and for everted sacs of the tissue of the small intestine of the rat (Cardin and Mason

1976). In agreement with our results, Elliott and Mortenson (1975) demonstrated that sulfate was a competitive inhibitor of molybdate transport for *Clostridium* and calculated an apparent constant of inhibition (K_i) of 30 μM.

Our data, although limited, suggests that the inhibitory effect of sulfate on molybdate transport may be important in natural systems. In this regard Elliot and Mortenson (1975) found significant inhibition of molybdate transport by *Clostridium* at sulfate:molybdate ratios as low as 2 or 4 mole/mole. We observed inhibition of molybdate transport in Mirror Lake at sulfate:molybdate ratios near 4000 mole/mole. In seawater the average sulfate:molybdate ratio is 265000 mole/mole. If molybdate metabolism by marine organisms is similar to that of *Clostridium* or Mirror Lake plankton (which may not be the case and remains to be shown directly), it is probable that molybdate transport is severely limited in the sea. Our growth experiments with marine diatoms tend to support this idea.

In our growth experiments, with both freshwater and marine organisms in culture, increasing the sulfate:molybdate ratio lowered growth (or increased lag time before exponential growth began) if nitrate was the sole nitrogen source but not if ammonium was the sole nitrogen source. Because molybdenum is required for nitrate reductase but not for ammonium metabolism, we believe that increased sulfate lowered the biological availability of molybdate in these experiments. In some of our growth experiments, we were unable to demonstrate an affect of changing the sulfate:molybdate ratio, however, and sometimes were also unable to force molybdenum-limitation on our cultures. We have also seen highly variable and thus far inexplicable results when natural fresh or brackish waters were fertilized with molybdate or sulfate (A. Hagstrom, personal communication). Clearly both nitrogen and molybdenum nutrition of planktonic algae is complex, and factors such as luxury uptake of molybdate and toxicity of high levels of molybdate may require some modification of our original hypothesis (Howarth and Cole 1985).

In shallow-water systems such as some lakes, or bays reduced molybdenum from the sediments may be available to the phytoplankton; the uptake of reduced forms of molybdenum should not be inhibited by sulfate (Howarth and Cole 1985). In this regard it is interesting that the highest rates of nitrogen-fixation in marine systems occurs in productive benthic environments (Capone and Carpenter 1982) where a supply of reduced molybdenum from sediments is most likely. In some of our growth experiments (not shown here), in which we used natural phytoplankton communities from coastal water (Vineyard Sound, MA), an increased sulfate concentration, (at constant ionic strength), resulted in decreased growth on either nitrate or ammonium. In these cases we suspect that the inhibitory effect was caused by the change in Ca^{2+} and Mg^{2+} activity which was, in turn, caused by the increased sulfate (R. Marino, unpublished data). These complexities made the problem experimentally more tractible in dilute, low sulfate waters which is why we have done much

of our work, thus far, in freshwater. Both our data and the literature indicate that sulfate inhibits molybdate transport by organisms which inhabit low sulfate waters; it is possible that organisms which inhabit high sulfate waters have a different mechanism for molybdate transport. We are investigating this possibility now.

A number of hypotheses have been advanced to explain why the sea is nitrogen limited. We believe that no one of these completely or satisfactorily explains the phenomenon. Nixon et al. (1980) argued that high rates of denitrification in estuaries deplete N stocks relative to P and results in N limitation. This argument explains only a possible mechanism for how seawater becomes N-depleted. The argument does not explain why nitrogen fixation fails to overcome this deficit as originally predicted by Redfield (1958; but see Doremus 1982; Smith 1984). Further, while denitrification rates in estuaries are high, these rates are also high in productive freshwater. In fact, available evidence suggests that these rates are highest in freshwaters (Knowles 1982).

We believe that N-limitation in the ocean is the result of low rates of nitrogen-fixation and that some extrinsic factor limits the rate of fixation. Clearly, nitrogen-fixation does occur in the ocean but does so at low rates or in specialized environments (Codispoti 1985, Martinez et al. 1983, Capone and Carpenter 1982). Whatever factor limits fixation must, therefore, limit the rate and not eliminate the process. Our hypothesis, that sulfate is a competitive inhibitor of molybdate uptake identifies one possible rate-limiting factor.

Acknowledgements

Financial support was provided by NSF Ecosystem Studies Program (grant #BSR-83-05176), by the Institute of Ecosystem Studies of the NYBG, and by The Ecosystems Center, Marine Biological Laboratory, Woods Hole. This paper was part of a symposium, 'The Nitrogen Cycle: Central Issues for Ecosystems Research and Management' held at the annual meeting of the Ecological Society of America in Minneapolis, Minn. on 30 June 1985.

We thank N.F. Caraco and G.E. Likens for comments throughout this work and on this ms. R.R.L. Guillard advised us and provided algal cultures and S. Merkel assisted with some of the growth experiments. G.E. Likens and F. Manheim provided us with the use of atomic absorption and plasma emmission equipment without which we could not have done this work.

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