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Effects of Low Concentrations of Bisulfite-Sulfite and Nitrite on Microorganisms

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A wide range of microorganisms was tested to determine their sensitivity to low concentrations of bisulfite-sulfite and nitrite, solubility products of SO_2 and NO_2, respectively. Photosynthesis by blue-green algae (cyanobacteria) was more strongly inhibited by 0.1 mM bisulfite-sulfite and 1 mM nitrite at pH 6.0 than photosynthesis by eucaryotic algae and respiration of bacteria, fungi, and protozoa. At pH 7.7, blue-green algae were still more sensitive to bisulfite-sulfite and nitrite than eucaryotic algae, but the toxicity of bisulfite-sulfite and nitrite decreased as the pH increased. Photosynthesis by *Anabaena flos-aquae* at pH 6.0 was inhibited 25% by a bisulfite-sulfite concentration of 10 μM and 15% by a nitrite concentration of 50 μM. Photosynthesis by the blue-green alga, *Lyngbya* sp., was not exceptionally sensitive to chlorate and thiosulfate. Acetylene-reducing activity of *Beijerinckia indica* was completely inhibited by 0.1 mM bisulfite-sulfite at pH 4.0, the suppression being decreased with increasing pH.

The functioning of aquatic and terrestrial ecosystems requires the activities of a variety of microorganisms, and serious problems could result from the inhibition of an important segment of a natural microbial population by a pervasive pollutant. Sulfur dioxide and NO_x are pervasive pollutants that are anthropogenically produced, primarily from the combustion of fossil fuels. Moreover, the increasing use of coal, particularly high-sulfur coal, could result in a greater amount of SO_2 reaching the atmosphere. Thus, inasmuch as both of these gaseous pollutants might affect the activities of those microorganisms important to the function of terrestrial and aquatic communities, evaluation of the influence of the pollutants is of particular importance.

Many studies have dealt with the interactions between air pollutants and animals and higher plants, but little attention has been given to their effects on the microbial components of natural ecosystems (1). Some investigations, however, have dealt with the influence of SO_2 and NO_x on bacterial survival (7, 12), algal activity (4), and plant pathogens (10, 17, 18).

The present investigation deals with the effects of bisulfite-sulfite and nitrite, which are produced readily in water from SO_2 and NO_2, respectively, on a wide range of microorganisms. This "solubility product" approach is a common means of investigating the effects of volatile pollutants on microorganisms and lichens (1). In this approach, it is assumed that a gaseous pollutant that reacts readily with water affects an organism after interacting with water surrounding or associated with the organism.

MATERIALS AND METHODS

Cultures and maintenance. Algae were obtained from J. M. Kingsbury, Cornell University; protozoa were provided by Ward's Natural Science Establishment, Rochester, N.Y.; and bacteria and fungi were derived from the culture collections of the Laboratory of Soil Microbiology, Cornell University, and the Department of Microbiology and Cell Biology, Pennsylvania State University. The algal cultures were grown and maintained on Bristol solution (2) or VF medium, the modification by G. S. Venkataraman (personal communication) of Fogg medium (5) for blue-green algae (cyanobacteria), which consisted of (per liter): KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; A5 micronutrient solution, 1.0 ml; and Fe-ethylenediaminetetraacetic acid stock solution, 1.0 ml. The pH was adjusted to 7.5. The A5 micronutrient solution consisted of (per liter): boric acid, 2.86 g; $MnCl_2$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.222 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0177 g; and $CuSO_4 \cdot 5H_2O$, 0.29 g. To prepare the Fe-ethylenediaminetetraacetic acid stock solution, 26.1 g of ethylenediaminetetraacetic acid in 268 ml of 1 N KOH was amended with 24.9 g of $FeSO_4$, and the solution was diluted to 1.0 liter with distilled water and aerated overnight.

Beijerinckia indica and *Azotobacter chroococcum* were maintained on the medium (AzB medium) of Peña and Dobereiner (16), and *Agrobacterium tumefaciens* was grown on yeast extract-mannitol agar (YEM) containing 0.1% yeast extract and 0.9% mannitol. All other bacteria and the actinomycetes were

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maintained on slants of nutrient agar amended with 0.1% glucose. Fungi were maintained on potato-dextrose agar slants. *Tetrahymena pyriformis* was cultivated on proteose peptone-yeast extract broth (PPY broth) (15), and the *Colpidium* sp. was maintained on a medium (GPPY medium) composed of (per liter): yeast extract, 5 g; glucose, 2.5 g; and proteose peptone, 10 g. All medium components were obtained from Difco Laboratories (Detroit, Mich.).

Effects on CO_2 fixation by algae. Algae were grown at 25°C on a rotary shaker (170 rpm) with constant illumination in 500-ml Erlenmeyer flasks containing 100 ml of Bristol solution at pH 6.0. When the optical density at 580 nm had reached about 0.2, the cells were collected by centrifugation and resuspended in Bristol solution to an optical density of 0.01 to 0.05, depending on the activity of the culture. Optical densities were measured with a Bausch and Lomb (Rochester, N.Y.) Spectronic 20 spectrophotometer with 1.27-cm tubes. Inhibitors (Na_2SO_3, $NaNO_2$, $KClO_3$, $Na_2S_2O_3$, and KIO_4) in Bristol solution of the desired pH were prepared immediately before use. The reaction mixture consisted of 1.0 ml each of resuspended cells, Bristol solution, and the test inhibitor in test tubes (13 by 120 mm). Test tubes containing the same ingredients were wrapped in aluminum foil to measure activity in the dark. All tubes were then incubated at 25°C for 40 min under 450 μEinsteins of light per m^2 per s, after which 0.25 ml of Bristol solution containing 0.05 μCi of $H^{14}CO_3^-$ with a specific activity of 59 mCi/mmol (New England Nuclear Corp., Boston, Mass.) was added to each tube. The tubes were then sealed with serum stoppers and incubated for an additional 30 min.

The uptake of $^{14}CO_2$ was terminated by the injection of 0.4 ml of 37% formaldehyde into each tube. One-milliliter portions from each tube were then filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass.), and the cells thus retained were washed with 10 ml of 1.0 mM H_2SO_4 to remove residual $^{14}CO_2$. After drying, the filters were placed in scintillation vials containing 10 ml of Bray liquid scintillation cocktail (3), and the radioactivity was counted in a Beckman LS100 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The activity of inhibitor-free cells was determined simultaneously, and the results are expressed as percent activity compared with these controls.

The same procedure was used to determine the effects of bisulfite-sulfite and nitrite on $^{14}CO_2$ uptake by algae grown and tested at a higher pH. In these instances, the concentration of phosphate in Bristol solution was increased to 0.01 M, and the pH was adjusted to 7.7.

Effects on respiration of heterotrophic microorganisms. Cultures of the heterotrophs were grown in 500-ml Erlenmeyer flasks containing 100 ml of medium on a rotary shaker at 28°C until suitable cell densities were obtained. *A. chroococcum* and *B. indica* were grown in the liquid medium of Peña and Dobereiner (16), *A. tumefaciens* was grown in YEM broth, and the other bacteria and actinomycetes were cultured in nutrient broth amended with 0.5% glucose. The fungi were grown in a broth containing 0.5% yeast extract and 1% glucose (YEG broth). The protozoa were grown in stationary culture at 28°C in 500-ml flasks containing 100 ml of PPY broth for *T. pyriformis* and GPPY broth for *Colpidium* sp. The cells were collected by centrifugation, washed with 0.10 M phosphate buffer at pH 6.0, and resuspended. The incubation mixture (total volume, 3.0 ml) consisted of 1.0 ml of cell suspension, 0.5 ml of buffer, and either an additional 1.0 ml of buffer (control) or 1.0 ml of buffer (pH 6.0) containing 3 or 0.3 mM bisulfite-sulfite or 3 mM nitrite. After a 30-min preincubation, 0.5 ml of a 0.10 M solution of substrate was added; the substrates were sucrose for *A. chroococcum* and *B. indica*, mannitol for *A. tumefaciens*, and glucose for all other organisms. The rate of oxygen uptake was measured either manometrically or by means of a biological oxygen monitor (model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio). The results are reported as the percent inhibition of the rate of respiration as compared with cells not exposed to the potential inhibitors.

Effects on acetylene reduction. Blue-green algae were grown in VF medium for 1 week in the light on a rotary shaker. *A. chroococcum* and *B. indica* were grown in the AzB medium for 5 days at 28°C on a rotary shaker. The cells were collected by centrifugation, washed once with 0.10 M phosphate buffer (pH 6.0 or 7.0) or 0.10 M 3,3-dimethylglutaric acid buffer (pH 4.0 or 5.0), and resuspended to an optical density at 600 nm of 1.0. Duplicate 1.0-ml portions of the cell suspensions were placed in 19-ml serum bottles, and 1.0 ml of a 1.0 M sucrose solution was added to the suspensions. The bottles then received 0.10 ml of buffer or 0.10 ml of a buffer containing Na_2SO_3 to give final concentrations of 10, 1.0, and 0.10 mM. The bottles were stoppered and incubated in the dark for 30 min at 28°C, after which acetylene was introduced through the stopper to a final concentration of 10% (vol/vol) in the headspace, and the samples were mixed for 1 min on a Vortex mixer. The bacteria were then incubated at 28°C for 5 h in the dark, and the algal cultures were incubated at 25°C for 5 h in the light. The samples were mixed for 1 min, and 0.2 ml of the headspace was injected into a Perkin-Elmer model 3290B gas chromatograph fitted with a flame ionization detector and a 1-m phenyl isocyanate-Porasil C column. The flow rate of the carrier gas, N_2, was 30 ml/min, and the column temperature was 24°C. The amount of ethylene produced was calculated from standards. The protein content of the cell suspensions was determined after the protein was precipitated with an equal volume of 10% trichloroacetic acid, collected by centrifugation, and redissolved in 2 N NaOH. Protein was determined by the method of Lowry et al. (13).

RESULTS

The photosynthetic activity of all species of blue-green algae studied was almost totally inhibited by 0.1 mM bisulfite-sulfite at pH 6.0 (Table 1). In each instance, the activity was reduced by more than 75% by this bisulfite-sulfite level. The ratio of bisulfite to sulfite at pH 6.0 is 8.1:1. In contrast, the rate of photosynthetic activity by 14 green algae, 2 yellow algae, 1 red alga, and *Euglena gracilis* was reduced

little or not at all by this concentration of bisulfite. Similarly, the nine genera of blue-green algae tested were strongly inhibited by 1.0 mM nitrite, the rate of CO_2 fixation being reduced by 60 to 100%; on the other hand, the activity of nine genera of green algae and *Bumilleria exilis* was little or not at all affected at this nitrite concentration.

The respiratory activity of none of the heterotrophic microorganisms tested was as sensitive to either inhibitor as the photosynthetic activity of the blue-green algae. Thus, the rate of respiration of bacteria and protozoa, as measured by O_2 consumption, was suppressed to only a modest extent, if at all, by 0.10 mM bisulfite-sulfite, a concentration highly toxic to the blue-green algae, and the toxicity was marked only at concentrations of 1.0 mM bisulfite-sulfite (Table 2). Moreover, neither 0.10 nor 1.0 mM bisulfite-sulfite reduced the rate of respiration of *Aspergillus conicus, Saccharomyces cerevisiae,* and *Torulopsis candida.* Similarly, the respiratory rate of bacteria was not affected as adversely by nitrite as was the photosynthetic activity of the blue-green algae. Thus, 1.0 mM nitrite inhibited respiration of *Bacillus circulans, B. megaterium, Enterobacter aerogenes, Micrococcus luteus, Nocardia salmonicolor,* and *Pseudomonas fluorescens* by only 19, 0, 4, 5, 0, and 33%, respectively.

The extreme sensitivity of the CO_2-fixing activity of blue-green algae to both bisulfite-sulfite and nitrite, as compared with other algae, did not result from the fact that the tests were carried out at pH 6.0, a pH that may be below the optimum for many of these algae. Thus, when algae were grown at pH 7.7 and subsequently tested for the effects of bisulfite-sulfite and nitrite at the same pH, the photosynthetic activity of the blue-green algae *Lyngbya* sp., *Anabaena flos-aquae,* and *Oscillatoria* sp. was still more sensitive than that of the green algae *Chlamydomonas reinhardtii, Ankistrodesmus falcatus,* and *Ulothrix fimbriata* (Table 3). A comparison of the results in Tables 1 and 3 shows that well over 10-fold higher concentra-

TABLE 1. *Sensitivity of algal photosynthesis to bisulfite-sulfite and nitrite at pH 6.0*

Microbial group	Genus	Percent inhibition by:	
		0.10 mM HSO_3^-/SO_3^{2-}	1.0 mM NO_2^-
Blue-green algae	*Anacystis nidulans*	90	60
	Lyngbya sp.	79	97
	Anabaena flos-aquae	89	95
	Oscillatoria sp.	87	97
	Schizothrix sp.	96	91
	Synechococcus cedrorum	86	77
	Calothrix anomala	93	100
	Fischerella muscicola	78	99
	Cylindrospermum sp.	86	98
Green algae	*Scenedesmus quadricauda*	0	0
	Ulothrix fimbriata	11	5
	Chlamydomonas reinhardtii	5	1
	Ankistrodesmus falcatus	0	0
	Schizomeris leibleinii	0	12
	Oedogonium foeolarum	10	0
	Staurastrum sp.	8	0
	Draparnaldia pulmosa	1	19
	Gloeocystis vesiculosa	0	0
	Pandorina morum	4	ND[a]
	Pediastrum duplex	29	ND
	Haematococcus droebakensis	1	ND
	Cylindrocapsa involuta	15	ND
	Golenkinia radiata	0	ND
Yellow algae	*Bumilleria exilis*	33	11
	Botrydium becherianum	0	ND
Red algae	*Porphyridium aerugineum*	8	ND
Euglenophyta	*Euglena gracilis*	0	ND

[a] ND, Not determined.

TABLE 2. *Effect of bisulfite-sulfite on respiration rate of bacteria and protozoa*

Species	Percent inhibition by:	
	1.0 mM	0.10 mM
Bacteria		
Agrobacterium tumefaciens	78	12
Arthrobacter oxydans	81	25
Bacillus circulans	66	22
Bacillus megaterium	61	21
Bacillus subtilis	62	11
Beijerinckia indica	77	0
Enterobacter aerogenes	73	0
Micrococcus luteus	61	29
Nocardia salmonicolor	57	0
Pseudomonas fluorescens	86	37
Pseudomonas fragi	71	29
Streptomyces sp.	43	17
Protozoa		
Colpidium sp.	62	13
Tetrahymena pyriformis	66	0

TABLE 3. *Inhibition of $^{14}CO_2$ uptake by algae at pH 7.7 by bisulfite-sulfite and nitrite*

Group	Culture	Percent inhibition of $^{14}CO_2$ uptake by:	
		30 mM NO_2^-	5.0 mM HSO_3^-/SO_3^{2-}
Blue-green algae	*Lyngbya* sp.	84	44
	Anabaena flos-aquae	93	37
	Oscillatoria sp.	82	29
Green algae	*Chlamydomonas reinhardtii*	18	0
	Ankistrodesmus falcatus	10	0
	Ulothrix fimbriata	24	0

tions of both nitrite and bisulfite-sulfite were necessary at pH 7.7 to obtain an inhibition equivalent to that at pH 6.0. Therefore, the toxicity of bisulfite-sulfite and nitrite increased as the pH decreased. As the pH declined from 7.7 to 6.0, the bisulfite-sulfite ratio changed from 0.16:1 to 8.1:1. The rate of bisulfite oxidation to sulfate also rose as the pH increased.

In light of the marked inhibition of photosynthesis by blue-green algae at pH 6.0 by 1.0 mM nitrite and 0.10 mM bisulfite-sulfite, a determination was made of the effect of lower concentrations of these anions on the photosynthetic activity of *A. flos-aquae* at this pH. The influence of bisulfite-sulfite and nitrite levels on the inhibition of $^{14}CO_2$ uptake is depicted in Fig. 1. It is evident that CO_2 fixation by *A. flos-aquae* was inhibited 15% by 50 μM nitrite and 25% by 10 μM bisulfite-sulfite, which were the lowest concentrations tested.

The influence of other redox agents was investigated to determine whether the effects of bisulfite-sulfite and nitrite were simply the result of an alteration of the oxidation-reduction potential of the solution. The uptake of $^{14}CO_2$ by algae in the light was subject to little or no inhibition by the mild redox agents, chlorate and thiosulfate (Table 4), and no marked distinction was evident in the sensitivity of the one blue-green alga and the two green algae to these agents, as was the case with nitrite and bisulfite-sulfite. Periodate was appreciably toxic, but this was expected because of its high oxidation-reduction potential. Thus, the toxicity of bisulfite-sulfite and nitrite apparently did not result from a mere alteration of the oxidation-reduction potential of the solution.

The nitrogenase (i.e., acetylene-reducing) ac-

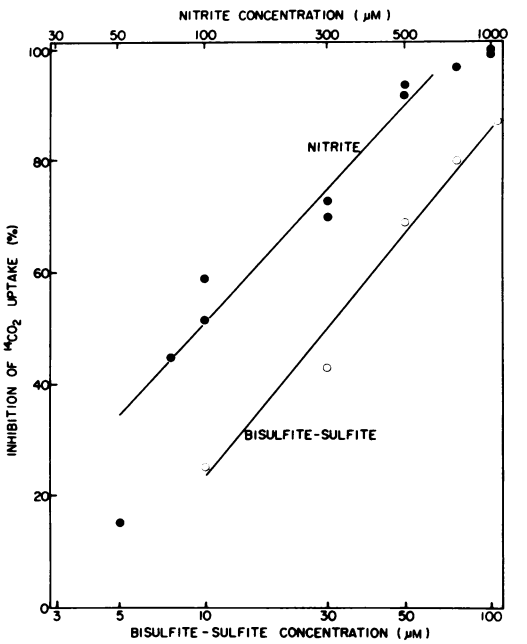

FIG. 1. *Inhibition of $^{14}CO_2$ uptake by Anabaena flos-aquae at pH 6.0 as a function of concentrations of bisulfite-sulfite and nitrite.*

TABLE 4. *Effect of 1 mM chlorate, thiosulfate, and periodate on $^{14}CO_2$ uptake by three algae at pH 6.0*

Culture	Percent inhibition of $^{14}CO_2$ uptake by:		
	ClO_3^-	$S_2O_3^{2-}$	IO_4^-
Lyngbya sp.	0	0	64
Ankistrodesmus falcatus	0	22	21
Chlamydomonas reinhardtii	9	0	82

tivity of two nonsymbiotic nitrogen-fixing bacteria was more sensitive to bisulfite-sulfite than the same enzyme system in the two nitrogen-fixing blue-green algae tested (Table 5). Acetylene reduction by the blue-green algae was rapid even in the dark. Moreover, with *B. indica,* a microorganism with a wide pH tolerance for growth, the toxicity of bisulfite-sulfite increased as the pH decreased, as was shown in the photosynthesis of *A. flos-aquae.* The pH changes altered the bisulfite-sulfite ratios; at pH 7.0, 6.0, and 5.0, the ratios were 0.83:1, 8.1:1, and 81:1, respectively. The concentrations of bisulfite-sulfite that inhibited the nitrogenase activity of both *A. chroococcum* and *B. indica* also reduced the rate of respiration; thus, the respiration of *A. chroococcum* on sucrose was totally inhibited by 10 mM bisulfite-sulfite at pH 6.0, and the respiratory activity of *B. indica* on the same sugar was reduced by 100 and 23% at pH 6.0 by 10 and 1.0 mM bisulfite-sulfite, respectively.

DISCUSSION

The data presented here demonstrate that the blue-green algae, as a group, are exceptionally sensitive to low concentrations of bisulfite-sulfite and nitrite. This sensitivity is pH dependent and is probably not the result of a simple change in the oxidation-reduction potential of the solution.

Concentrations of bisulfite-sulfite in natural waters exposed to an SO_2-polluted atmosphere are not known, and an equilibrium between the SO_2 in the air and bisulfite-sulfite in water may not exist because of the oxidation of bisulfite to sulfate and regular fluctuations in the concentration of SO_2 in the atmosphere. There may be potential environmental significance, however, in the inhibition of the blue-green algae by 0.1 mM bisulfite-sulfite, in that 2.5 mM bisulfite has been reported as the approximate concentration of bisulfite at 25°C in water in equilibrium with a gas phase containing 0.1 μl of SO_2 per liter (11). Moreover, some lichens are inhibited by SO_2 concentrations of 10 nl/liter or less (9), and SO_2 concentrations of 1 μl/liter have been periodically observed in polluted urban areas, such as Chicago (14).

In light of the extreme sensitivity of the blue-green algae to both bisulfite-sulfite and nitrite, they may represent the microbial group affected most adversely in environments exposed to atmospheric SO_2 and NO_x. This potential sensitivity to atmospheric SO_2 may be particularly important because the blue-green algae are important in primary production and nitrogen fixation in lakes and paddy fields (6), some of which are near sites of release of air pollutants. Although a bisulfite-sulfite inhibition of nitrogenase activity in blue-green algae was not observed in the present study, such toxicity has been reported for *Anabaena cylindrica* (8). The lack of inhibition in the present investigation may have resulted from the physiological state of the blue-green algae, since acetylene reduction proceeded at a substantial rate even in the dark. Because photosynthesis was largely or totally inhibited by concentrations of bisulfite-sulfite that did not lower the acetylene-reducing activity of the blue-green algae, it is likely that nitrogen fixation would cease after intracellular energy pools were depleted.

The inhibition of nitrogenase activity of *B. indica* at pH 4.0 by 0.10 mM bisulfite could be of significance, since this acid-tolerant species may be important in nitrogen fixation in acid soils. Moreover, because the toxicity of both bisulfite-sulfite and nitrite increases as pH decreases, the potential adverse effects of air pollution may be of particular concern in regions having soils of naturally low pH or areas having soils whose pH is lowered by acid precipitation.

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TABLE 5. *Effect of bisulfite-sulfite on acetylene-reducing activity of nitrogen-fixing microorganisms*

Species	pH	Ethylene produced (nmol/h per μg of protein)				
		Untreated	0.01 mM[a]	0.1 mM	1.0 mM	10 mM
Anabaena flos-aquae	6.0	333	332	321	316	306
A. flos-aquae	7.0	297	ND[b]	299	305	305
Tolypothrix sp.	6.0	337	339	336	331	312
Tolypothrix sp.	7.0	436	ND	449	441	439
Azotobacter chroococcum	7.0	193	ND	208	186	0
Beijerinckia indica	7.0	325	ND	319	286	10
B. indica	6.0	328	321	318	7	10
B. indica	5.0	215	214	162	0	ND
B. indica	4.0	223	166	0	0	ND

[a] Concentration of HSO_3^-/SO_3^{2-}.

[b] ND, Not determined.

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