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Free Ammonia Inhibition of Algal Photosynthesis in Intensive Cultures†

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The effect of free NH_3 inhibition on short-term photosynthesis was investigated in three microalgal species: the freshwater chlorophyte *Scenedesmus obliquus*, the marine diatom *Phaeodactylum tricornutum* and the marine chlorophyte *Dunaliella tertiolecta*. By performing a series of assays at various concentrations of added NH_4Cl and culture pH, we demonstrated that the inhibitory compound was free NH_3 and that pH played no role in determining the magnitude of inhibition, other than in establishing the degree of dissociation of nontoxic NH_4^+ to toxic NH_3. When corrections were made for pH, all three species displayed the same sigmoidal response curve to free NH_3 concentration; 1.2 mM NH_3 led to 50% reduction in photoassimilation of ^{14}C. Based on literature values, some marine phytoplankton appear to be significantly more sensitive to free NH_3 than were the test species, which are noted for their excellent growth characteristics. However, the combination of low algal biomass and strong pH buffering commonly found in most marine and many freshwater environments probably limits the possibilities for NH_3 toxicity to low alkalinity freshwaters and intensive algal cultures in which NH_4^+ is the main source of N. Such conditions occur commonly in algal wastewater treatment systems.

Although ammonia is thought to be an excellent source of nitrogen for algal growth (13), virtually no distinction is made between ionic NH_4^+ and free NH_3 as N sources, and little is known about which chemical species is the substrate for assimilation or how the actual transport into the cell occurs (15). Part of this problem arises because the equilibration between NH_4^+ and NH_3 via the reaction

$$NH_4^+ + OH^- \rightleftarrows NH_3 + H_2O \qquad pK_a = 9.25\ (25°C) \qquad (1)$$

is so rapid that it is impossible to distinguish on the basis of pH which form of N actually is taken up. On the other hand, the uncoupling effect of ammonia on photosynthetic processes in isolated chloroplasts is a well-established phenomenon (3, 5). Inhibitory effects of ammonia on algal growth and photosynthesis have been occasionally reported (1, 2, 14, 16, 17). However, difficulties in separating the effect of pH on the ratio of NH_3 to NH_4^+ from numerous other adverse effects on growth that are related to increasing pH have plagued most researchers. Moreover, virtually no quantitative data exist to relate free NH_3 concentration calculated for pH to the inhibition of algal metabolism. In this study, by varying both the pH and the addition of NH_4Cl, we examined the effect of NH_3 concentration on carbon assimilation in the freshwater alga *Scenedesmus obliquus* and two marine algae, *Phaedodactylum tricornutum* and *Dunaliella tertiolecta*.

MATERIALS AND METHODS

Cultures of the marine species *Phaeodactylum tricornutum* (TFX-1) Bohlin and *Dunaliella tertiolecta* (Dun) Butcher were obtained from the culture collection of R. R. L. Guillard at the Woods Hole Oceanographic Institution, and the freshwater chlorophyte *Scenedesmus obliquus* (Turpin) Kützing Kutz was obtained from the laboratory of M. Gibbs at Brandeis University.

Each species was first grown to steady state at different pH values in continuous cultures maintained at a dilution rate of 0.5 day^{-1}. Medium for the marine species was synthetic seawater containing 400 mM NaCl, 20 mM $MgCl_2$, 20 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 0.8 mM KBr, 0.2 mM H_3BO_3, 2 mM $NaHCO_3$, 5 mM $NaNO_3$, 0.5 mM KH_2PO_4, and vitamins and trace metals plus the sodium iron salt of EDTA in a twofold dilution of the amount specified in *f*-medium (10). The freshwater medium contained 0.4 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.2 mM $CaCl_2$, 2 mM $NaHCO_3$, 12 mM $NaNO_3$, 1 mM KH_2PO_4, and the same concentrations of EDTA and trace metals as found in the seawater medium.

The continuous culture apparatus (a bank of six 0.5-liter cultures), the culturing protocols, and the experimental analyses were virtually identical to those de-

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scribed previously (9). The cultures were grown under continuous illumination with cool white fluorescent light (3,000 J of visible light per m^2 per min) and temperature control (20°C for the marine algae and 25°C for *S. obliquus*); they were mixed with magnetic bar stirring.

Culture pH was maintained at various levels in the range 8.0 to 9.5 with a pH-stat system described previously (7a). Fluctuations in pH did not exceed ±0.1 units from the set value. Upon reaching steady state, samples were withdrawn from each culture and used for a series of short-term incubations to measure photoassimilation of inorganic carbon during exposure to various concentrations of NH_4Cl.

Appropriate buffers were added to the assay chambers during the incubation to maintain constant pH that was similar to the pH of the steady-state culture. The buffers employed were HEPPS (Research Organics, Cleveland, Ohio) for pH 8.0, BICINE (Sigma Chemical Co., St. Louis, Mo.) for pH 8.5, and AMPSO (Research Organics) for pH 9.0 to 9.5. All buffers were added to obtain a final concentration of 10 to 20 mM, depending on algal biomass concentration in the assay (biomass levels were varied in some experiments to determine whether the degree of NH_3 toxicity was dependent on algal concentration). We were unable to carry out incubations at pH values above 9.5 because the only appropriate buffers, CAPS (Sigma, pK_a = 10.4) and CHES (Sigma, pK_a = 9.3), were toxic to all three test species. In each experiment an unbuffered control sample was used to test for buffer toxicity. Variations in pH during incubations did not exceed ±0.05 units. The experimental assays, in final volume of 40 ml, were placed in 150-ml, water-jacketed, glass incubation vessels (light intensity and temperature were identical to steady-state cultures), and $NaH^{14}CO_3$ (New England Nuclear Corp., Boston, Mass.) was added to give a final radioisotope concentration of about 0.05 μCi/μmol of the total dissolved inorganic carbon. During the incubation the contents were gently mixed with small magnetic stirring bars. Samples (1 ml) were harvested every 10 min for the first hour of the assay, and a final sample was taken at 90 min. The samples were transferred to a scintillation vial containing 2 ml of methanol (acidified with 5% acetic acid); the contents were evaporated to dryness under an infrared lamp and resuspended in 1 ml of distilled water, and 10 ml of scintillation fluid (Handifluor) was added. Radioactivity was measured by liquid scintillation counting on a Beckman LS-100C instrument. Algal particulate carbon was analyzed on precombusted filters (Whatman GF/C) with a Perkin Elmer 240 elemental analyzer. Total dissolved inorganic carbon was measured on a Dohrmann PR-1 analyzer (7).

The effect of NH_3 concentrations on algal photosynthesis was determined by comparing the linear regression analyses of the time course uptake curve. Linear ^{14}C uptake during the incubation period was a prerequisite for making this determination. Concentrations of NH_3 were determined from the pH and the ionization coefficient for equation 1, corrected for temperature and salinity (4, 6).

Most of the experiments were performed with *S. obliquus* and *P. tricornutum* in the pH range 8.0 to 9.5 and with NH_4Cl additions varying from 0.5 to 16 mM. One set of experiments at pH 9.0 and with NH_4Cl additions ranging from 2 to 8 mM was performed with *D. tertiolecta*.

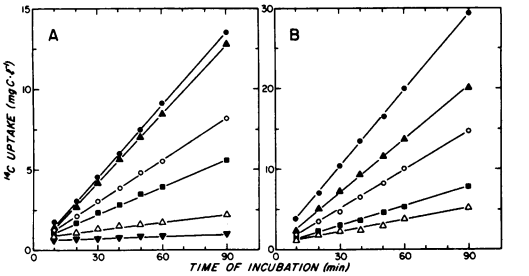

FIG. 1. Time course uptake of $^{14}CO_2$ by *S. obliquus* exposed to various additions of NH_4Cl. (A) pH 8.9; ●, no added NH_4Cl (control); ▲, 2 mM NH_4Cl; ○, 3 mM NH_4Cl; ■, 4 mM NH_4Cl; △, 6 mM NH_4Cl; ▼, 8 mM NH_4Cl. (B) pH 8.4; ●, no added NH_4Cl (control); ▲, 8 mM NH_4Cl; ○, 10 mM NH_4Cl; ■, 12 mM NH_4Cl; △, 14 mM NH_4Cl.

RESULTS

Linearity of ^{14}C uptake. We observed linearity of ^{14}C uptake in each 90-min incubation regardless of algal species, pH, or concentration of added NH_4Cl (Fig. 1). However, as exemplified by the results of the *S. obliquus* assays at pH 8.9 (Fig. 1A) and pH 8.4 (Fig. 1B), uptake rates of ^{14}C at each pH and biomass level decreased systematically with increasing concentration of added NH_4Cl.

Effect of pH on NH_4Cl inhibition. By comparing the ^{14}C uptake rates at a given pH for increased concentrations of added NH_4Cl with the rate for no NH_4Cl addition (ratio of V to V_{max}), we observed a dramatic effect of pH on the inhibition of ^{14}C uptake by NH_4Cl addition for *S. obliquus* (Fig. 2). For example, exposure

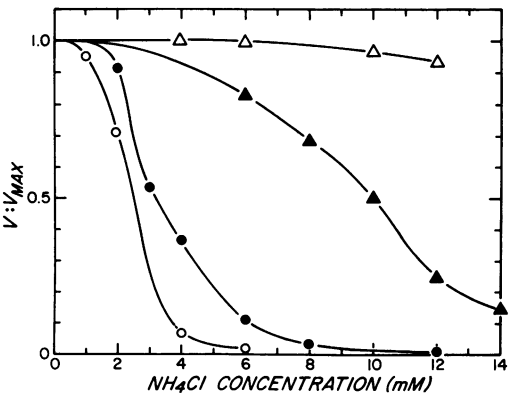

FIG. 2. Effect of added NH_4Cl concentration on the ratio of V to V_{max} for *S. obliquus* incubated for 90 min at various pHs. V_{max} is the rate of $^{14}CO_2$ in the absence of added NH_4Cl. Symbols: △, pH 8.0; ▲, pH 8.4; ●, pH 8.9; ○, pH 9.4.

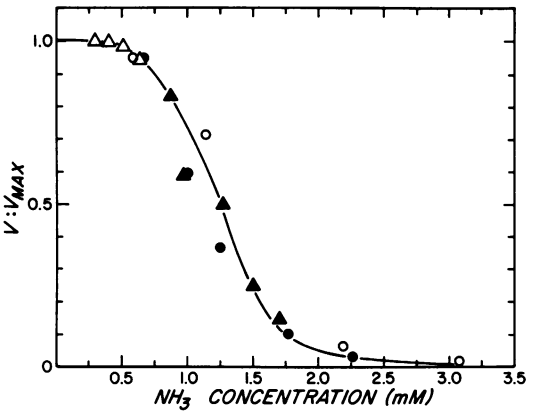

FIG. 3. Effect of calculated free NH_3 concentration on the ratio of V to V_{max} for *S. obliquus* incubated for 90 min at various pHs. Free NH_3 concentration was determined from NH_4Cl and pH data in Fig. 2. Symbols: △, pH 8.0; ▲, pH 8.4; ●, pH 8.9; ○, pH 9.4.

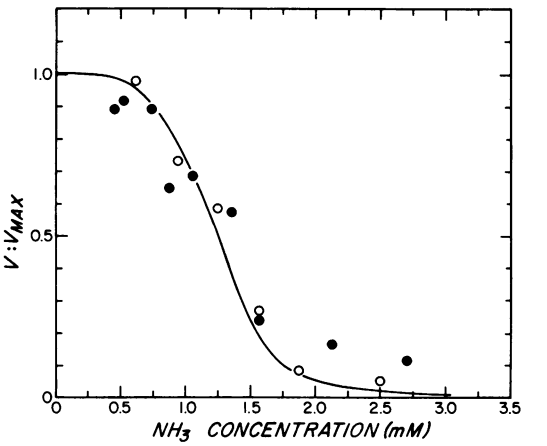

FIG. 4. Effect of calculated free NH_3 concentration on the ratio of V to V_{max} for test algae incubated for 90 min at various pHs. Solid curve is inhibition curve for *S. obliquus* transposed from Fig. 3. Symbols: ●, *P. tricornutum*; ○, *D. tertiolecta*.

to 10 mM NH_4Cl at pH 8.0 led to a slight reduction in the ratio of V to V_{max} to 0.95, but this same NH_4Cl concentration at pH 8.4 resulted in a dramatic decrease in the ratio of V to V_{max} to 0.50. To reduce the ratio of V to V_{max} to 0.50 at pH 8.9 required only 3.0 mM NH_4Cl (Fig.2).

NH_3 inhibition. When the relative uptake results shown in Fig. 2 were plotted as a function of free NH_3 concentration calculated from culture pH rather than as the concentration of added NH_4Cl, a single curve relating the ratio of V to V_{max} with NH_3 concentration in the assay chamber was formed (Fig. 3; each point in Fig. 3 represents one point from Fig. 2; the same symbols are used). The curve represented a modified threshold response in which exposure up to ~0.5 mM NH_3 caused no inhibition of ^{14}C uptake, but between 0.5 and 2.0 mM NH_3 suppression of ^{14}C uptake increased dramatically so that the ratio of V to V_{max} was <0.05 at free NH_3 concentrations >2.0 mM.

The inhibitory effect of NH_3 on the carbon uptake rates of both marine algae, *P. tricornutum* and *D. tertiolecta*, seemed to follow an identical response curve as found for *S. obliquus*. As seen in Fig. 4, the relative uptake data for the marine species fit the response curve generated from the experiments with *S. obliquus* very well.

We were unable to discern an effect of algal biomass concentration on ammonia photosynthetic inhibition expressed as a reduction in the ratio of V to V_{max}. Doubling the biomass concentration of *S. obliquus* at either pH 8.4 or 8.9 did not change the amount of NH_3 necessary to reduce the ratio of V to V_{max} to levels of 0.9, 0.5, or 0.1 (Table 1).

DISCUSSION

Although free NH_3 generally is believed to act as an inhibitor of algal photosynthesis, to date most of the evidence to support this conclusion has been circumstantial. Lack of precise pH control, together with difficulties in distinguishing between responses to NH_4^+ and NH_3 on the basis of pH and in separating other pH-related effects from those involved with the dissociation of NH_4^+ (equation 1), has prevented a clear interpretation of NH_3 toxicity data.

One of the possible effects of high pH on algal photosynthesis is the reduction of free CO_2 in water (Y. A., manuscript in preparation). To eliminate such an interpretation of our results, we did not compare absolute carbon uptake rates but only relative uptake rates (V:V_{max}),

TABLE 1. Effect of algal biomass concentration at two pH values on NH_3 concentration required to reduce ^{14}C uptake rate with NH_4Cl addition relative to maximum uptake rate without NH_4Cl addition for cultures of *S. obliquus*[a]

Ratio of V to V_{max}	NH_3 conc. (mM)			
	pH 8.4		pH 8.9	
	78 mg of PC per liter	158 mg of PC per liter	84 mg of PC per liter	153 mg of PC per liter
0.9	0.4	0.6	0.7	0.7
0.5	1.4	1.3	1.0	1.2
0.1	1.9	1.9	1.8	N.M.[b]

[a] Units: algal biomass concentration, milligrams of algal particulate carbon per liter; NH_3 concentration, millimolar; ^{14}C uptake rate with NH_4Cl addition, V; uptake rate without NH_4Cl addition, V_{max}.

[b] N.M., Not measured.

where V_{max} was determined for each pH value tested from the control assay (without NH_4Cl addition). This way we were able to demonstrate that NH_3 inhibition of algal photosynthesis is not a pH-dependent phenomenon, but rather the dissociation of NH_4^+ as a function of pH is the main determinant of how much NH_3 is available to inhibit photosynthesis.

The biochemical basis for NH_3 inhibition of algal photosynthesis remains unclear. The best evidence points toward NH_3 uncoupling of electron transport in photosystem II by the breakdown of proton gradients necessary to drive photophosphorylation (5) or by inhibition via NH_3 competition with H_2O in oxidation reactions leading to O_2 evolution (18), or both. In either case free NH_3 molecules are believed to be the reactive chemical species, a conclusion substantiated by Fig. 3 and 4. Although beyond the scope of this work, the sigmoidal shape of the NH_3 inhibition curve (Fig. 3) is analogous to the type of inhibition curve that results from allosteric interactions between inhibitors and enzymes (12) and thus indicates a complex mode of inhibition. The form of inhibition, however, does not appear to be related to the biomass present or more appropriately, to total cell surface area (Table 1) as has been demonstrated for mercury, chlorine, and chloramine toxicity in phytoplankton (8, 11). Mercury toxicity in phytoplankton involves initial toxicant accumulation through absorptive mechanisms on cell surfaces (11), whereas chlorine and chloramine inhibition is through total oxidation of organic matter (8). Hence, it is not surprising that the inhibitory effects of some toxicants are biomass-dependent and that there is no biomass effect on NH_3 toxicity which involves passive transport of NH_3 molecules across cell membranes succeeded by complex enzyme inhibition reactions that are concentration-dependent.

A major difficulty in comparing our NH_3 toxicity results with similar data is that in most previous studies either pH was not rigorously regulated or no attempt was made to measure inhibitory effects on the basis of free NH_3 concentration (estimated from added ammonium salt and culture pH), or both. In addition, many of the assay protocols, including ours, deviate widely from each other, particularly the duration of exposure to NH_4Cl and the light intensity used during the incubation. For example, we observed linear ^{14}C uptake during relatively short (90-min) incubation periods in response to various additions of NH_4Cl (Fig. 1). However, Abeliovich and Azov (1) have found that longer (5-h) exposure to a given NH_4Cl concentration at a fixed pH led to significant reductions in photosynthesis, thus leading them to suggest that exposure time is a critical factor in defining

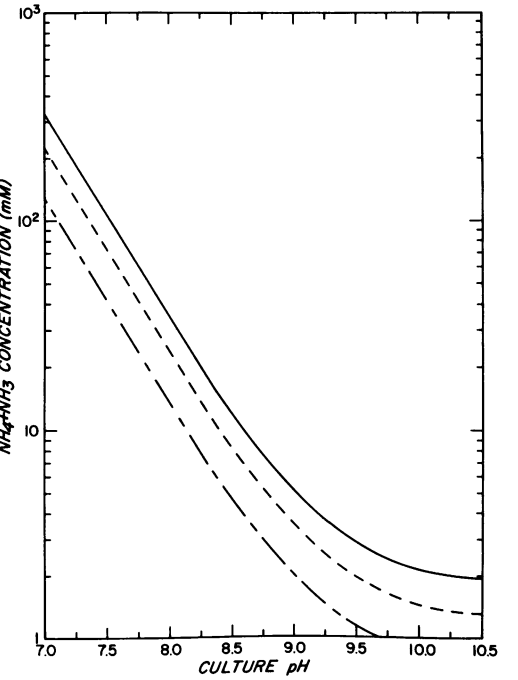

FIG. 5. Effect of culture pH on the total ammonia concentration ($NH_4^+ + NH_3$) required to inhibit photosynthesis of test algae grown at 25°C by 10% (broken curve), 50% (dashed line), and 90% (solid line) level of inhibition as determined by the free NH_3 (data in Fig. 4) required to cause these levels of toxicity.

relationships between NH_3 concentration and inhibition of photosynthesis. Similarly, whereas we used only one light intensity, Admiraal (2) has found a magnifying effect of increasing light intensity on NH_3 toxicity. If this is true, then NH_3 toxicity to microalgae in outdoor conditions, in which both light intensity and exposure duration are unregulated, could be far more severe than represented by the summary NH_3 toxicity curves shown in Fig. 5. In this figure the concentrations of total ammonia ($NH_4^+ + NH_3$) required to produce the 10, 50, and 90% levels of inhibition determined from Fig. 4 are plotted as a function of pH.

It is surprising that the two marine and one freshwater test species which belong to different taxonomic orders are inhibited in a similar fashion by free NH_3 (Fig. 4); yet it is difficult to extrapolate these results into a general model of NH_3 toxicity in phytoplankton without more data. A major problem, as mentioned, is the difficulty of comparing our results with the limited NH_3 toxicity data available. However, from our estimates of the free NH_3 concentration necessary to inhibit algal metabolism in previous studies (based on reported pH and added ammonium salts), it appears that some marine phyto-

plankton are far more sensitive to NH_3 than were the three test species in our study. For example, photosynthesis in a variety of marine diatoms was severely inhibited at free NH_3 concentrations in the range 0.05 to 0.2 mM (2, 14). In the case of the dinoflagellate *Gymnodinium splendens*, exposure to only about 10 μM free NH_3 was lethal (17).

In most natural waters, particularly the marine environment, the combination of low total ammonia ($NH_4^+ + NH_3$) and relatively neutral pH prevents the development of NH_3 toxicity to phytoplankton. However, in both low carbonate alkalinity freshwaters and intensive microalgal cultures, in which NH_4^+ is the major form of N and bicarbonate is the main source of inorganic carbon, high pH can be expected concomitant with NH_3 toxicity.

The possibilities for extreme NH_3 inhibition are most likely in algal wastewater treatment systems in which millimolar concentrations of total ammonia are typically present in the influent domestic wastewater. For example, when the pH rises to 9.5 and the temperature is 20 to 25°C, only 2 and 3 mM total ammonia will lead to 50 and 90% reductions in photosynthesis, respectively (Fig. 5). Moreover, because the equilibrium in equation 1 is shifted towards increased NH_3 formation with increasing temperature, the problem of NH_3 toxicity in outdoor ponds is magnified considerably during the summer. For example, at 25°C only one-third of the total ammonia is required to produce the same free NH_3 as at 10°C.

Our results clearly demonstrate that the role of pH in NH_3 inhibition of algal metabolism is to establish the concentrations of free NH_3 relative to NH_4^+ via equation 1. Hence, for most situations, NH_3 toxicity will only become important at high pH. For example, according to the summary inhibition curves of Fig. 5, to achieve 50% reduction in algal photosynthesis, more than 15 times less total ammonia is required at pH 10 as is necessary at pH 8. For algal mass culture operation, NH_3 toxicity is a distinct possibility when NH_4^+ and HCO_3^- are major nutrient sources. To avoid such conditions, pH control or substitution of ammonium salts with another suitable N source (e.g., NO_3^- or urea compounds), or both, are required. When algal systems are used for wastewater treatment and the source of N cannot be regulated, pH control will be the only effective method to avoid NH_3 toxicity.

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