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J. Phycol. 12, 172–179 (1976)

STIMULATION OF GROWTH IN *SCENEDESMUS OBLIQUUS* (CHLOROPHYCEAE) BY HUMIC ACIDS UNDER IRON LIMITED CONDITIONS[1,2]

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SUMMARY

Stimulatory effects of humic acids of molecular weight 30,000 or greater on iron-starved Scenedesmus obliquus *(Turp.) Kütz. in association with bacteria were studied by growth and Fe uptake experiments. Humic acids stimulated growth of Fe-starved cells by decreasing the lag phase and extending the growth phase. Humic acids stimulated increased algal growth in medium containing EDTA as well as in medium containing no EDTA, indicating humic acids are not stimulating algal growth under Fe limiting conditions by creating a soluble Fe pool. Humic acids decreased Fe availability to Fe-starved* S. obliquus. *Iron bound to humic acids is unavailable for uptake by Fe-starved cells indicating growth stimulation is not due to chelation effects alone. Stimulation of growth is not a membrane phenomenon as humic acids show the same stimulatory effect when in contact with cells or separated by dialysis membrane. Humic acids also stimulate growth in the dark, with and without aeration, indicating use as a heterotrophic substrate. A photoheterotrophic mechanism is indicated by increased algal growth caused by illuminating cultures containing humic acids but excluding CO_2.*

Key index words: chelation; Chlorophyceae; growth stimulation; heterotrophy; humic acids; iron; Scenedesmus

[1] *Accepted: 27 November 1975.*
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INTRODUCTION

Humic acids are very important in plant growth processes in aquatic systems (46). Dissolved, naturally occurring humic substances stimulate growth of algae and may affect nuisance algal growth in natural waters (14,20,26,32,34,35,46,47,55). Humic acids are considered important in soil and aquatic systems because of their chelation properties (19,22,25,32,45). Of special interest are reactions between humic acids and micronutrients such as Fe, Mg, Cu and Zn (16,29,47). Interactions between humic acids and Fe significantly affect the chemical equilibria of Fe in natural waters (48,49). Under aerobic conditions and alkaline pH, there is almost complete precipitation in natural waters in the absence of organic chelates (11). Shapiro (47) observed many surface waters have higher Fe concentrations than those predicted by ferric hydoxide solubility. Organic chelates may

stimulate algal growth by making relatively insoluble nutrients, such as Fe, more available for uptake (8,23, 43). Shapiro (47) found collodial, organic acid-Fe complexes increased Fe availability to phytoplankton and Lange (26) reported growth stimulation of blue-green algae by fulvic acids was due to organic acids holding Fe in solution, making it available to algae.

The higher molecular weight fraction of naturally occurring colored organic acids is primarily responsible for the chelating capacity in surface waters (48) and is more stimulatory to freshwater and marine phytoplankton (35). The higher molecular weight fraction of dissolved organic acids are more stimulatory to algae, but Stevenson and Ardakani (52) found that humic acids bind metals tightly, making metals unavailable to plants. Larger humic acid molecules bind metals more tightly and are less mobile than the smaller fulvic acid molecules. These findings seem contradictory if algal growth is stimulated by increasing nutrient availability by chelation processes.

This work was conducted to determine if humic acids (nominal molecular weight 30,000 or greater) stimulate growth or affect Fe uptake by Fe-starved *Scenedesmus obliquus* (Turp.) Kütz in the presence of bacteria.

METHODS AND MATERIALS

Humic acids prepared from European sources and supplied in powdered form were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin, (H 1675-2; lot #082091). Aldrich humic acids were dissolved in 0.01 *N* NaOH and reprecipitated with concentrated HCl (pH = 6.6). The brown humic acid precipitate was washed 3 times with 0.05 *N* HCl followed by two 95% ethanol and 4 distilled water washes. Humic acids were dialyzed 24 h against distilled water in a Dow "beaker ultrafilter" with a nominal molecular weight exclusion of 30,000. Purified humic acids had an ash content of 15.9% and 20.0 mg Fe · g^{-1} humic acids.

Scenedesmus obliquus was chosen because *Scenedesmus* species occur in almost every freshwater environment (5) and are common in ponds and lakes with high humic acid content (31). Stock cultures were obtained from Indiana University Culture Collection (No. 1952, isolated by Krauss) (51). Cell density was halved and cells transferred to fresh medium at 7 day intervals to prevent senescence due to cell aging, nutrient depletion and build-up of extracellular by-products and wastes. Single-celled, gram negative, rod-shaped bacteria isolated from a bog lake were present in all cultures. These nonflagellated bacteria occurred free in the medium and adherent to the cells. Because algal-bacterial associations occur as functional units in natural systems (28), an algal-bacterial association was studied as a system. Stock *S. obliquus* cells were maintained in Fe-free medium for 5 days immediately prior to use in experiments to produce Fe-starved cells. Inocula for experimentation were taken from cell suspensions with cell densities of 5×10^5 cells · ml^{-1} and relative growth rate (k') of 0.58. Initial cell densities in uptake and growth studies were about 1×10^5 cells · ml^{-1}, with exact cell densities calculated for each experiment.

Direct microscopic counts of subsamples in counting cells were used to determine algal growth. Fifty random ocular grid fields in a single subsample were counted and cell density data converted to cells · ml^{-1}.

Treatment effects for 2^3 factorials were calculated using Yates' algorythm. Other factorial designs were tested for significant effects by analysis of variance techniques and mean separations accomplished by the Student-Newman-Keuls test.

The culture medium was that of the United States Environmental Protection Agency Algal Assay Procedure (AAP) (2,54). Growth responses of *S. obliquus* to humic acid concentrations were determined as shown in Fig. 1. When present the EDTA concentration was 0.3 mg · l^{-1}. The humic acid concentration used for dialysis and heterotrophic growth studies was 20 mg · l^{-1}. Uptake of Fe by Fe-starved *S. obliquus* was studied, using ^{59}Fe as a tracer in AAP medium without EDTA containing humic acids concentrations in Fig. 3. Media for uptake studies contained 3×10^{-2} mg · l^{-1} stable Fe and 5.56×10^{-10} mg · l^{-1} ^{59}Fe (123 cpm · ml^{-1}), added as carrier-free $^{59}FeCl_3$. Two hundred ml of media was placed in 300 ml erlenmeyer flasks and allowed to react for 24 h before inoculation with Fe-starved cells. Less than 1% Fe was lost by adsorption to glass culture flasks after 24 h. Humic acids reduced ^{59}Fe activity losses to glass due to adsorption. The greater adsorption of ^{59}Fe to glass and membrane filters in the absence of humic acids make estimates of reduction of Fe availability conservative.

Humic acid-bound Fe was produced by reacting stable Fe and ^{59}Fe with humic acids for 24 h and dialyzing the resulting solution for 7 days to remove unbound Fe, yielding a 200 mg · l^{-1} humic acid solution with a specific activity of 6.98×10^4 cpm · mg^{-1} Fe. Ten ml of the humic acid-Fe solution were added to 180.0 ml Fe-free AAP medium (without EDTA) and 10.0 ml Fe-starved cells, producing an Fe activity of 22.5 cpm · ml^{-1}. A control flask was created to estimate adsorption losses by adding 10.0 ml of humic acid-bound Fe solution to 180 ml of Fe-free AAP medium and 10.0 ml distilled water.

Growth experiments were conducted in 300 ml erlenmeyer flasks fitted with 2-hole rubber stoppers. A pasteur pipette inserted through the rubber stopper delivered air which bubbled through the medium and exited through a vent with a porous polyethylene plug to minimize evaporation and prevent foreign matter from entering the flask. Air, supplied at a rate of 20 cm^3 · min^{-1} by piston pumps, passed through activated charcoal and in-line 0.45 μm membrane filters. Flasks were incubated at 23 C in 4035 lx continuous illumination from balanced spectrum "Gro-lux" fluorescent bulbs.

Heterotrophic growth experiments were conducted in 20 ml pyrex vials equipped with rubber diaphragm tops. Dark treatment vials were covered with black tape. Each vial was filled with 15.0 ml AAP medium containing the appropriate humic acid concentration. Air, supplied by piston pumps, passed through a 0.45 μm membrane filter entering each vial by a 2-in 18 gauge hypodermic needle. After bubbling through the medium at a rate of 5 cm^3 · min^{-1}, air escaped through 0.5 in, 21 gauge hypodermic needles. A "Swinnex-13" (Millipore Co., Bedford, Massachusetts) filter unit on the exit vent minimized evaporation and prevented contamination. Flasks were incubated at 23 C in 4035 lx of continuous illumination. Unaerated vials were shaken daily 3 times.

Dialysis experiments were conducted in 300 ml erlenmeyer flasks with 200 ml of EDTA-free AAP medium. Treatment included a control of 200 ml AAP medium alone and one with 100 ml AAP medium inside the dialysis membrane and 100 ml AAP medium outside to determine dialysis membrane effects. Two treatments contained humic acids. One contained 10.0 mg · l^{-1} humic acids in both the 100 ml of AAP medium inside and outside the dialysis membrane, whereas the other had a humic acid concentration of 20.0 mg · l^{-1} in 100 ml AAP inside the dialysis membrane and no humic acids in the 100 ml outside the membrane. Cells were placed in the 100 ml excluded from the dialysis membrane and bubbled with air for 5 days.

To separate algal cells from culture medium, in uptake studies, 20 ml samples were removed at known time intervals

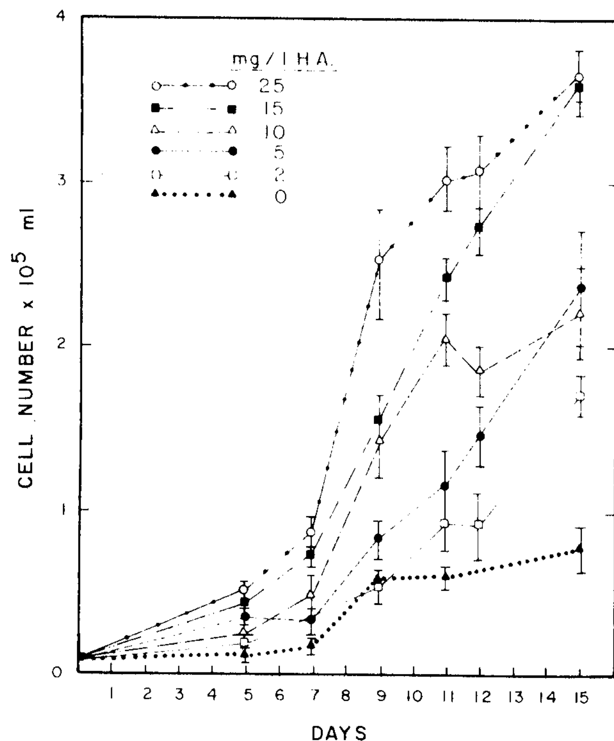

FIG. 1. Effect of humic acids (*H.A.*) on growth of *S. obliquus* (each point represents mean of 4 replications with 95% confidence limits indicated).

and filtered through 5 μm cellulose-ester membrane filters under 5 mm Hg vacuum. Filters and associated algal cells were washed with 50 ml 0.01 N HCl followed by 50 ml distilled water. This procedure removed 98.5% of ^{59}Fe bound to membrane filters. Addition of 10.0 mg · l^{-1} humic acids to an ^{59}Fe solution caused a 97% decrease in Fe adsorption onto cellulose ester filters. ^{59}Fe uptake by Fe-starved *S. obliquus* fixed in 2% formaldehyde was less than 2% that of living cells. Each filter was placed in a 9 dram plastic counting vial and dissolved in 10.0 ml methanol, to maintain constant counting geometry. ^{59}Fe activity was detected with a well type, NaI crystal scintillation detector and corrected for background, which was a constant 5 cpm. Activity was counted for 10 min or 10,000 counts immediately after sampling.

RESULTS AND DISCUSSION

Growth in AAP-humic acid medium. Humic acids significantly increased growth of *Scenedesmus obliquus* in AAP medium (Fig. 1). Although greater algal standing crops were obtained in media containing humic acids, growth rates for all cultures were approximately the same prior to day 7, with relative growth rates (k') ranging from 0.0 to 0.15. This indicates humic acids are able to supply nutrients which become limiting in their absence. Since the stocks were Fe-starved, the nutrient most likely to be limiting was Fe. Preliminary growth experiments proved this to be true. The ability of humic acids to sustain the exponential growth phase and support a greater final standing crop indicates growth stimulation by making Fe available for growth for a longer period of time. Humic acids also shortened and decreased severity of lag phase in population growth. Bozniak (7) similarly found that humic substances decreased the lag period of *Chlorella* grown in batch culture. Decreased lag period may be due to direct cellular stimulation by humic acids or, by making nutrients available sooner. Huntsman and Barber (21) reported that both EDTA and extracellular algal products reduce lag period and concluded this to be due to increased trace metal availability. Increasing concentrations of humic acids above 15.0 mg · l^{-1} does not increase algal growth, since final standing crops in cultures having the 2 highest humic acid concentrations were not significantly different ($P \leqslant 0.90$). Prakash and Rashid (34) found increased growth of marine phytoflagellates with increasing humic acid concentration until a concentration of 35.0 mg · l^{-1} was reached. Humic acids may stimulate algal growth at lower concentrations, but become inhibitory at higher concentrations, due to reduced Fe availability from mass action chelation by humic acids or direct humic acid effects on algal cells. There are many metabolic by-products which are actively secreted and excreted from cells and others which simply leak from cells (15, 57). The presence of some of these extracellular by-products are necessary for the exponential growth of some algal species (15). Humic acids may reduce the initial need for extracellular products for exponential growth and mitigate toxic effects of metabolic by-products that accumulate in batch algal cultures. Reduced lag phase and prolonged growth may be due to chelation effects. Chelated Fe may be more available unless high concentrations of humic acids are present.

Humic acid–EDTA interactions. Humic acids may stimulate algal growth by forming stable complexes and chelates with algal nutrients thereby making them available to algal cells. Thus effects of humic acids on algal growth with and without EDTA, a synthetic chelating agent which stimulates growth of Fe-starved algae by maintaining a soluble Fe pool (45), were compared.

EDTA and humic acids alone caused increased algal growth (Fig. 2). In the absence of EDTA, growth increased with increasing concentrations of humic acids. In the absence of humic acids, EDTA greatly enhanced growth but when humic acids were present in the medium, EDTA increased standing crops only slightly. In combinations, there was greater growth than when each was present singly and analysis of variance showed a significant interaction term for the humic acid and EDTA main effects. The greatest algal standing crop occurred in media containing 25.0 mg · l^{-1} with and without EDTA. EDTA can hold Fe in solution and available to freshwater algae (45) and prevents precipitation of Fe from seawater keeping Fe available to marine

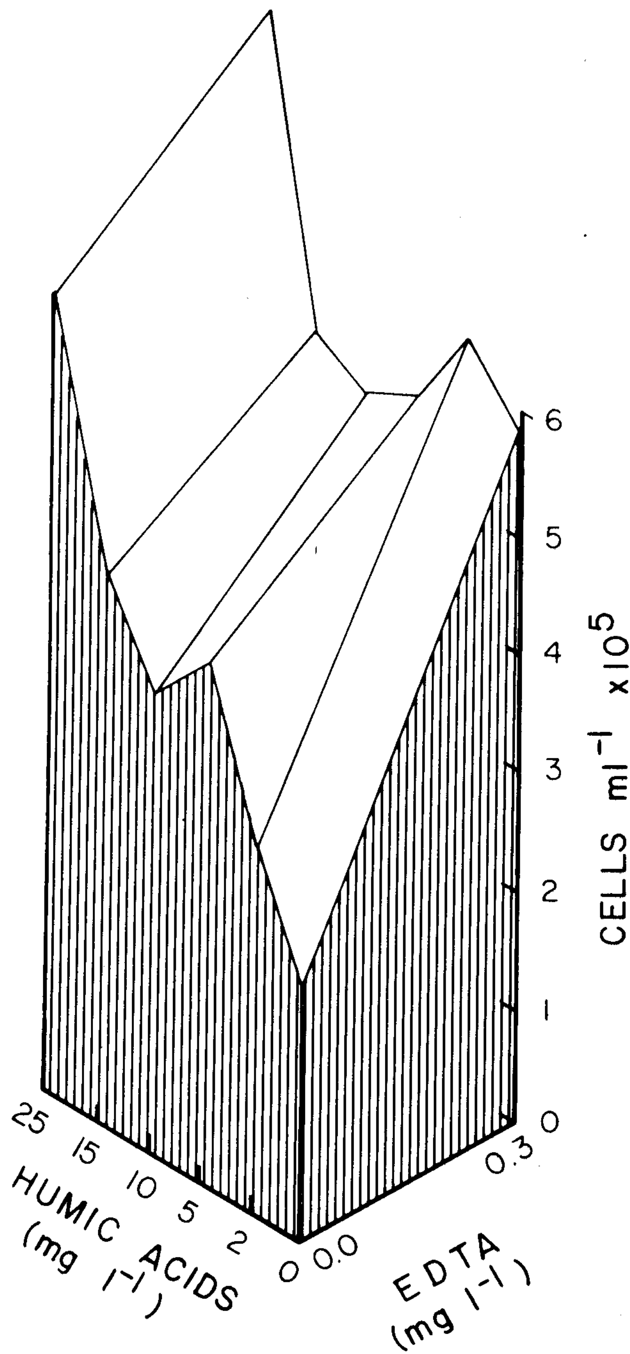

FIG. 2. Standing crops of Fe-starved cells in AAP medium with varying humic acid and EDTA concentrations after 7 days growth.

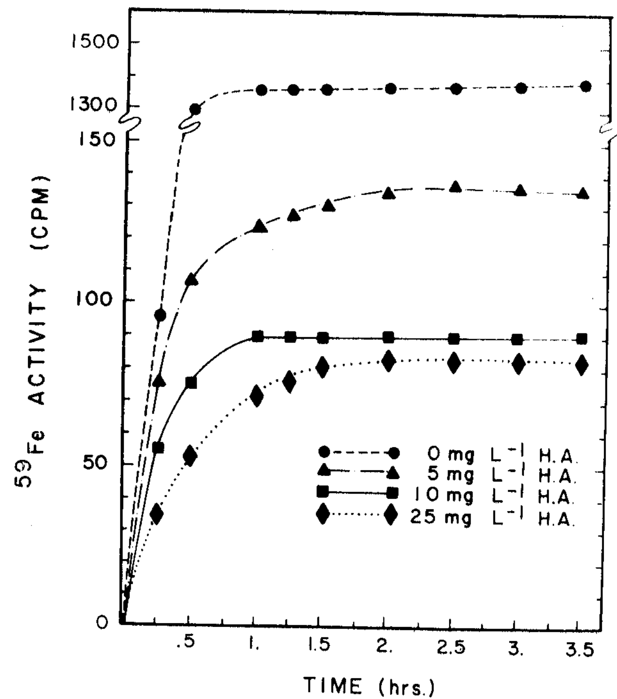

FIG. 3. Effect of humic acids (*H.A.*) on ^{59}Fe uptake by Fe-starved cells.

phytoplankton (30). Similarly, Davies (11) found that EDTA dissolves $Fe(OH)_3 \cdot 2H_2O$ when added to culture media. EDTA stimulates algal growth by chelating Fe and other cationic nutrients, preventing their precipitation, thus maintaining an available pool of cationic nutrients. In the absence of chelating agents, algal growth rate may be limited by rate of Fe release from $Fe(OH)_3$ and $FePO_4 \cdot 2H_2O$. Humic acids enhance algal growth even in the presence of EDTA. This additional growth stimulation is then not due to the creation of an additional available Fe pool but may be due to some mechanism other than chelation alone. The significant interaction term also indicates that 2 separate mechanisms are operating.

^{59}Fe uptake from humic acids. Humic acids decreased Fe uptake by *S. obliquus* (Fig. 3). Not only was there a decrease in the final cellular activity, but also a decrease in the uptake rate in the media containing higher humic acid concentrations. Uptake of ^{59}Fe was complete in about 1 h when humic acids were present but complete after 0.5 h in medium where humic acids were absent (Fig. 3).

When humic acids were reacted with Fe and the resulting solution dialyzed a portion of the Fe was complexed or otherwise bound to the humic acids which did not cross the dialysis membrane. After 72 h there was no significant ^{59}Fe uptake indicating Fe bound to humic acids of molecular weight 30,000 or greater is unavailable to *S. obliquus*.

Results of Fe uptake studies and observed decreases in Fe adsorption by glass and filters, in the presence of humic acids, indicate Fe is tightly bound by humic acids and therefore less available for uptake by *S. obliquus*. Humic acids may compete with terrestrial plant roots for soil Fe resulting in chlorosis (1). Marine phytoflagellates are unable to use chelated Fe (17,23,41). Goldberg (17) demonstrated that the marine diatom *Asterionella japonica* (Cleve & Müller)

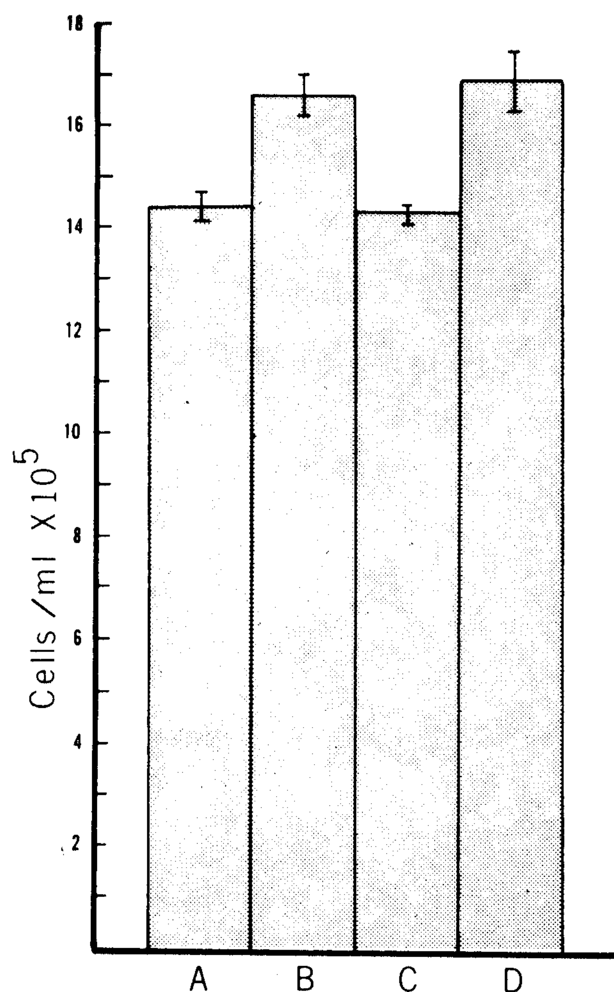

FIG. 4. Cell density after 5 days. A. 200 ml AAP medium, no dialysis membrane. B. 100 ml AAP containing 10.0 mg · l^{-1} humic acids inside and outside dialysis membrane. C. 100 ml AAP without humic acids inside or outside dialysis membrane. D. 100 ml AAP without humic acids outside dialysis membrane; 100 ml AAP containing 20 mg · l^{-1} humic acids inside dialysis membrane.

Gran could not utilize Fe from Fe-humate complexes but Gran (18) showed that growth of another marine diatom, *Skeletonema costatum* (Grev.) Cleve was stimulated by soil extract containing 0.2 μM Fe · l^{-1}. Gran did not determine whether stimulation of growth was due to increased availability of Fe, but theorized that stimulation of diatom growth was due to additional available Fe. Prakash and Rashid (34) demonstrated that Fe-humic acid complexes stimulate the growth of marine phytoflagellates and suggested stimulation was not due to increased Fe availability.

Prakash and Rashid (34) suggest that chelation of trace metals may reduce their toxicity to marine dinoflagellates. Similarly, chelation decreases the toxicity of heavy metals to freshwater algae (55). Certain workers have suggested high concentrations of humic acids may chelate metal ions reducing their availability to algal cells (35,47,50). In this study, Fe was bound tightly by humic acids decreasing availability. The results show that stimulation of *S. obliquus* growth in Fe-limited culture by humic acids was not due to increased Fe availability. Growth of Fe-limited *S. obliquus* increased with increasing humic acid concentrations while Fe availability decreased. These results are not compatible with the hypothesis that humic acids stimulate algal growth in Fe-limited culture by supplying Fe. Hence, stimulatory effects of humic acids on algal cells are probably due to mechanisms other than Fe chelation alone.

Surface membrane effects of humic acids. Prakash and Rashid (34) suggested that growth stimulation of marine phytoplankton by humic acids may be a membrane phenomenon. They reported increased growth rates, yield and uptake of $^{14}CO_2$ by phytoplankton are not entirely attributable to metal chelation. Stimulatory effects of humic acids on bacteria may be due to redox potential effects on cell membranes (9). Waris (55) suggested that humic substances may directly affect the cytoplasmic membrane. Chaminade (10) reported humic acids stimulate growth of violet epidermal cells by allowing mineral transport across the cytoplasmic membrane. Saunders (44) stated that humic acids may stimulate cell membranes of phytoplankton, thereby allowing an influx of bound metal nutrients. Low molecular weight humic acid fractions and fulvic acids may penetrate the cell membranes of phytoplankton (35) but the high molecular weight humic acids (molecular weight 30,000 or greater) probably do not. Prát, *et al.* (38,39) report humic acids cannot penetrate plant cell membranes. Humic substances that penetrate cell membranes were inhibitory to all processes (37).

Media containing humic acids supported greater growth of *S. obliquus* than those without (Fig. 4). The difference in algal densities between control treatments was not significant, indicating the dialysis membrane did not affect algal growth. No significant difference in algal densities between media containing humic acids was demonstrated. Hence, growth stimulation caused by humic acids of this molecular weight was not a membrane phenomenon. Separating humic acids from *S. obliquus* cells prevented direct contact but did not affect growth stimulation in the presence of humic acids which occurred regardless of contact with the algal cells.

The mechanism of Fe uptake by algae is not well-known, but one possibility is equilibrium exchange of cellular Fe with Fe released from Fe precipitates such as $Fe(OH)_3$ which are adherent to the cell membrane. Uptake of Fe by higher plants may involve transport of Fe across the cell membrane by sideramines (40). Although the same mechanism probably does not operate in micro-organisms, the mechanism

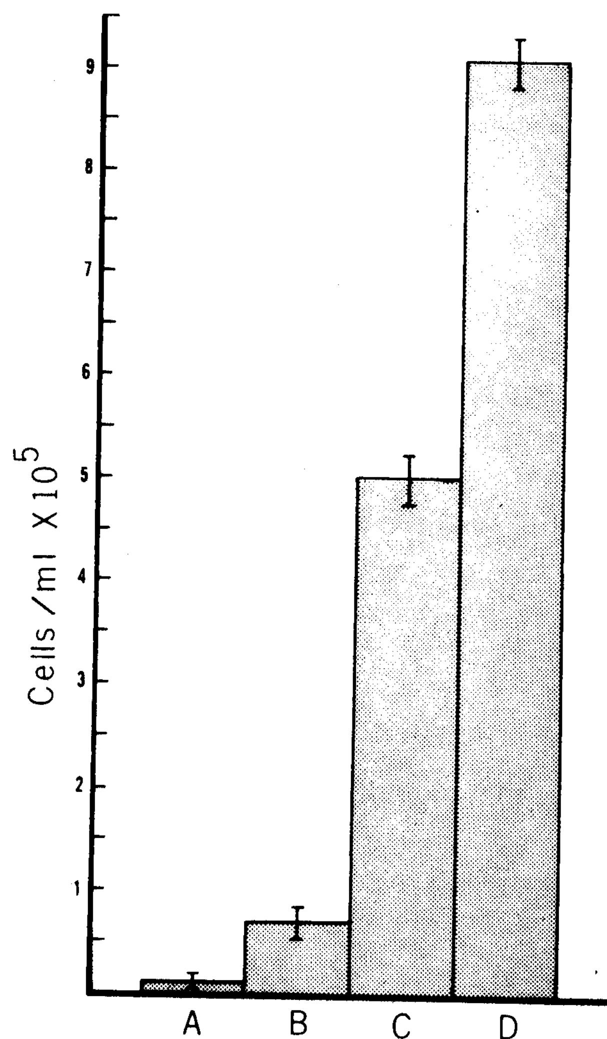

FIG. 5. Cell density after 21 days in aerated cultures (each bar represents mean of 4 replications, with 95% confidence limits indicated). A. dark-grown, no humic acids. B. dark-grown, 20 mg · l⁻¹ humic acids. C. 4035 lx illumination, no humic acids. D. 4035 lx illumination, 20 mg · l⁻¹ humic acids.

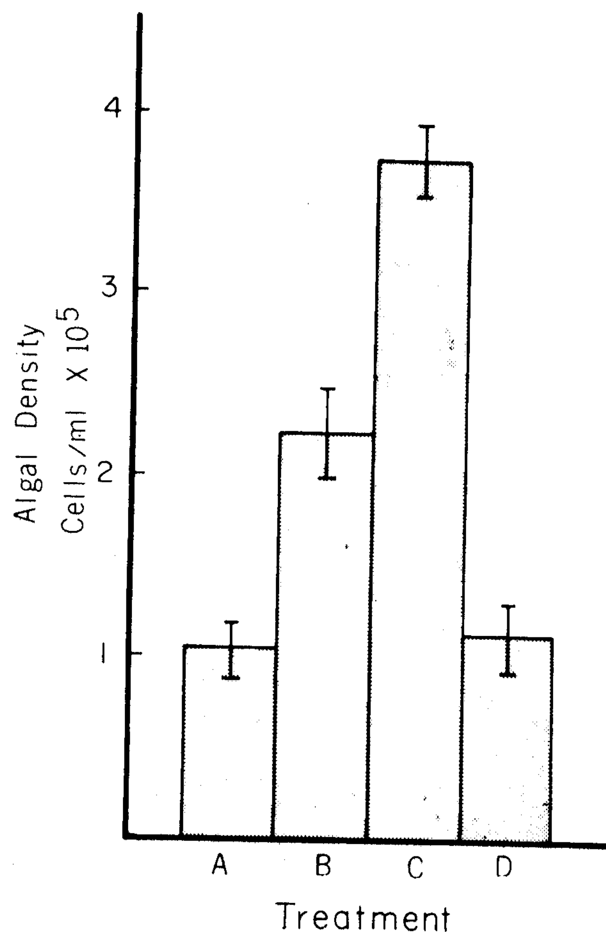

FIG. 6. Cell density after 21 days in vials sealed to exclude CO_2 (each bar represents mean of 4 replications with 95% confidence limits indicated). A. dark-grown, no humic acids. B. dark-grown, 20 mg · l⁻¹ humic acids. C. 4035 lx illumination, 20 mg · l⁻¹ humic acids. D. 4035 lx illumination, no humic acids.

may be similar. Waris (55) suggested that algae may have chelating agents on their surfaces to allow uptake of insoluble Fe compounds. This type of chelate transfer was not the mechanism responsible for algal growth stimulation by humic acids. Stimulation of *S. obliquus* by humic acids occurred even when humic acids were denied contact with cell membranes indicating the stimulatory effect on algal growth was not solely a membrane effect.

Heterotrophic use of humic acids. The genus *Scenedesmus* has actively heterotrophic species, capable of using a variety of C sources. For example, *S. obliquus* can use glucose, cellulose, acetate (53), glycocol and alanine (1) and cellobiose (13) as carbon sources for heterophic growth.

When air was bubbled through cultures, growth of algae cultured in the light was significantly ($P \geq 0.999$) greater than growth of algae cultured in the dark (Fig. 5). When humic acids were present in the media, standing crops in both dark and light cultures were greater ($P \geq 0.95$ and $P \geq 0.999$ respectively) than when humic acids were absent. Cultures grown in dark, without humic acids were not significantly different from the initial cell density. When not bubbled with air, the greatest growth occurred in the medium containing humic acids cultured in the light (Fig. 6). Medium containing humic acids cultured in the dark supported significantly ($P \geq 0.999$) greater growth than the control cultured in the dark but less than medium containing no humic acids cultured in light. The final cell density in medium containing no humic acids incubated in the dark was not significantly greater than the initial cell density. Cell size did not change during either experiment, eliminating the possibility that humic acids stimulated cell division without further production of algal carbon.

Stimulation of algal growth in media containing humic acids may have resulted from direct heterotrophic use of humic acids or their breakdown products from bacterial decomposition. Bacteria may be able to break humic acids into structural components small enough for assimilation by *S. obliquus*. Although humic acids are highly resistant to bacterial degradation (4), bacteria may degrade certain functional groups. DeHaan (12) reported that *Pseudomonas* can use fulvic acids as a carbon source. The bacteria *Azotobacter* sp. is stimulated by 25.0 mg · l^{-1} humic acid solution (9). This may be due to a redox effect on the bacterial cell membranes or nutrient availability. Although the mechanism is not well understood, humic acids stimulate some species of bacteria and this may have an indirect effect on algal growth (28). Reduced C compounds produced by the bacteria may subsequently be used by *S. obliquus*. Bacteria can utilize reduced C sources and release CO_2 which is available to algae (27). Because of the large size of humic acid molecules Prát (36) concluded that stimulation of algal growth by humic acids was not due to their direct use as an algal nutrient.

Algal growth, in medium containing humic acids, was twice as great when cultured in light as when cultured in darkness in unaerated cultures where CO_2 was not available. Light can be used to supply ATP through photophosphorylation and greatly increases heterotrophic growth (13,24). Myers (34) found that *Scenedesmus* sp. growth in glucose increases threefold with illumination of the culture. Although utilization of organic C sources only in the presence of light may not be true heterotrophic growth, it may partially explain the stimulatory effect of humic acids on *S. obliquus*.

A major function of Fe in algal growth is synthesis and maintenance of chlorophyll (10). The heterotrophic use of humic acids may compensate for a reduction in chlorophyll function due to Fe-limitation. Humic acids may function as an Fe sparing factor rather than supplying additional Fe.

ACKNOWLEDGMENTS

This research was conducted at Michigan State University and supported by a North Central Research grant through the Michigan State University Agricultural Experiment Station. Preparation of this manuscript was supported by Contract AT(38-1)-819 between the University of Georgia and the U. S. Energy Research and Development Administration. N. R. Kevern, C. D. McNabb and J. G. Wiener kindly commented on the manuscript.

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