THE EFFECTS OF HUMIC ACIDS ON THE GROWTH OF AND UPTAKE OF IRON AND PHOSPHORUS BY THE GREEN ALGA SCENEDESMUS OBLIQUUS (TURP) KUTZ

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ABSTRACT

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UPTAKE OF IRON AND PHOSPHATE BY
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By

John Paul Gra[illegible]

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In this study the effects of humic acids of molecular weight 32,000 or greater, on the growth of Scenedesmus obliquus (Türp) Kütz, were studied in batch cultures. The uptake of Fe and PO_4 from culture media containing humic acids was also studied, using ^{59}Fe and $^{32}PO_4$ as tracers.

Fe and PO_4 were found to have concentration dependent negative effects on each other. This supposed precipitation effect was mitigated by the presence of humic acids in the medium.

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By

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Naturally occurring colored organic acids have long been known to affect the growth of microorganisms as well as vascular plants. It has been suggested that the effects of humic acids on the growth of plant cells may be due to their chelation properties. Humic acids are thought to be able to chelate divalent and trivalent cations such as Fe and prevent them from precipitating, thus making them available to plants.

In this study the effects of humic acids of molecular weight 30,000 or greater, on the growth of Scenedesmus obliquus (Türp) Kütz, were studied in batch cultures. The uptake of Fe and PO_4 from culture media containing humic acids was also studied, using ^{59}Fe and $^{32}PO_4$ as tracers.

Fe and PO_4 were found to have concentration dependent negative effects on each other. This supposed precipitation effect was mitigated by the presence of humic acids in the medium.

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Humic acids stimulated increased growth of S. obliquus over controls grown in AAP culture medium (Algal Assay Procedure, United States Environmental Protection Agency), with a concentration of 25.0 mg/l humic acids causing the greatest growth response. The presence of humic acids increased the maximum relative growth rates, maximum standing crops, final standing crops, and decreased the length of the lag phases of the S. obliquus cultures.

The addition of humic acids to AAP medium caused algal growth responses similar to those caused by EDTA addition, with the addition of humic acids and EDTA in conjunction causing an even greater growth response than when either humic acids or EDTA was added alone.

Fe-starved S. obliquus cells gave similar growth responses to the addition of Fe or humic acids. The addition of humic acids and Fe in conjunction caused the greatest growth enhancement. This effect was greater in the presence of 0.9 mg/l PO_4 than in the presence of only 0.5 mg/l PO_4.

The addition of Ca to algal culture media caused an increase in early growth of S. obliquus cultures when humic acids were present at a concentration of 10.0 mg/l or less. In the presence of a humic acid concentration of 10.0 mg/l or greater, the increased growth due to Ca was very small. After the cultures reached the stationary growth phase, there was no difference in growth due to the addition of Ca.

There was a slight decrease in the total uptake of $^{32}PO_4$ from AAP media by S. obliquus in the presence of humic acids. Humic acids greatly reduced the total uptake of ^{59}Fe from AAP medium. The presence of 5.0 mg/l humic acids reduced the Fe uptake by more than a factor of 10, with increased concentrations of humic acids causing proportionally smaller decreases. Fe-starved S. obliquus cells were found to be saturated with Fe in less than 10 min at all humic acid concentrations. Fe was tightly bound to the humic acids studied and S. obliquus was unable to obtain this bound Fe.

The presence of 1.0 mg/l PO_4 in the medium caused a decrease in the uptake of Fe over that observed when PO_4 was absent from the medium. This effect was more pronounced when humic acids were not present in the medium.

Increasing the Fe concentration from 0.03 mg/l to 1.03 mg/l caused an increase in the Fe uptake by Fe-starved S. obliquus greatly in the absence of humic acids and to a lesser extent in the presence of various concentrations of humic acids. The addition of more Fe that could be bound by the humic acids did not overcome the effect of the humic acids.

A concentration of 40.0 mg/l Ca and a pH range of 4.0-10.0 had very little effect on the uptake of Fe by Fe-starved S. obliquus from culture media containing humic acids,

although the addition of Ca did increase the uptake of Fe in the absence of humic acids.

Humic acids were found to support heterotrophic growth of S. obliquus in non-axenic cultures, with the response enhanced in cultures grown in the light and humic acids did not have to come in contact with the algal cells to cause a stimulation.

Culture media made from filtered bog water gave similar results to those observed when humic acids were added to media made with distilled water and the addition of the purified humic acids of 30,000 molecular weight or greater to media made with bog water caused a further growth enhancement, but addition of Fe to the bog water medium did not cause an increased growth response.

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By

John Paul Giesy, Jr.

A DISSERTATION

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Michigan State University
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DEDICATION

To my wife, Susan

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INTRODUCTION

> It is the care we bestow on apparently trifling, unattractive and very troublesome minutiae which determines the result (Bullock, 1935).

Natural aquatic environments are very complex, with many organic compounds affecting the availability of inorganic nutrients to algae. One of the most important groups of naturally occurring organic compounds are the humic acids. These colored organic compounds are major constituents of soil and sediment organic matter (Otsuki and Hanya, 1966) and are of world-wide distribution in soil and aquatic systems (Schnitzer and Khan, 1972). The physical and chemical features of these compounds are described in Appendix II. Because of their ubiquity, humic acids are involved in the chemical processes of nearly all surface waters. Some areas, such as bogs and marshes, are noted for their high humic acid content. Although some humic acids may be of autochonous origin in aquatic systems, most of the humic acids are allochthonous, originating in the soil. Large amounts of organic matter are eroded and leached from soils and washed into surface waters each year (Yentsch and Reichert, 1962).

Recent interest in the cultural and natural eutrophication of surface waters has increased the urgency of understanding the role of naturally occurring organics in the availability of inorganic algal nutrients. An understanding of the cycling of these inorganic nutrients and their interactions with dissolved organic acids is essential if key algal nutrients are to be controlled.

Humic acids are very important in plant growth processes in soil and aquatic systems (Schnitzer and Khan, 1972) and have long been known to stimulate terrestrial plant growth (Hajduhovic and Ulrich, 1965). Dissolved, naturally occurring humic substances have also been reported to stimulate growth in some species of algae and may play an important role in the nuisance growth of algae in natural waters (Horner et al., 1934; Flaig and Otto, 1951; Shapiro, 1957; Prakash and Rashid, 1968; Martin et al., 1971; Schnitzer and Khan, 1972; Prakash et al., 1973).

Early work by Allen (1919) found that humic acids stimulated growth in the bacterium Azotobacter sp. and the alga Chroococcum sp. Other early studies found that humic acids were stimulatory to the aquatic angiosperm, Lemna minor (Ashby, 1929; Clark, 1930, 1931).

Shapiro (1957) demonstrated humic acids extracted from lake water could stimulate growth of Scenedesmus quadricauda, Chlamydomonas sp. and Haematococcus sp. at concentrations

between 5 and 50 mg/l. Scenedesmus obliquus and S. biguga-tus have also been found to be stimulated by humic acids (Prát, 1955).

In his work with blue-green algae, Lange (1970) found the fulvic acid fraction of the dissolved organic acids to stimulate the growth of Anabaena circinalis, Gloeotrichia echinulata, Microcystis aeruginosa and Nostoc muscorum. In the marine environment it is thought that humic acids are important in stimulating the red-tide dinoflagellate Gymnodinium breve (Wilson and Collier, 1955; Prakash and Rashid, 1968, 1969; Martin, 1971).

Humic acids stimulate increased respiration in cereal roots at a concentration of 0.01% (Smidova, 1960) and have been observed to affect the cell division and elongation of plants (Oata and Tsudzuki, 1971). Humic acids have also been shown to accelerate the penetration of alkali salts into plant cells (Heinrich, 1966) and stimulate nitrogen fix-ation in Azotobacter sp. (Iswaran, 1960). Humic acids may also be taken up by plants and translocated within the plant (Aso and Sakai, 1963). This observation has increased specu-lation that humic acids may act in the same way as plant growth substances (Schnitzer and Khan, 1972).

The large polyphenolic humic acids have long been thought to be important in soil and aquatic systems because of their chelation properties (Harvey, 1937b; Hutchinson,

1957; Kawaguchi and Kyuma, 1959; Schelske et al., 1962; Mun et al., 1966; Martin et al., 1971). Of special interest are the reactions between humic acids and micronutrients such as Fe, Mg, Cu and Zn (Leclerc and Beaujean, 1955a, 1955b; Shapiro, 1957; Gjessing, 1964).

Interactions between humic acids and Fe have been shown to be significant in the chemical equilibria of natural waters (Shapiro, 1966a, 1966b). Shapiro (1957) also found that many surface waters had higher Fe concentrations than would be predicted by the solubility of ferric hydroxide ($Fe(OH)_3$). With a solubility product of approximately 10^{-39}, $Fe(OH)_3$ is the most insoluble Fe precipitate and the inorganic species that controls the free Fe concentrations in aerobic surface waters. In a study of Linsley Pond, Shapiro (1957) found that colored, dissolved, organic acids were able to keep Fe from precipitating even when the pH was raised to 13.2. In the absence of organic chelates, under aerobic conditions and alkaline pH, there was almost complete precipitation of Fe in natural waters (Davies, 1970). Studies of the surface waters of Northern Europe showed that most of the Fe in the water column existed as soluble chelates (Gjessing, 1964).

Iron (Fe) and phosphorus (P) have both been shown to be important in algal nutrition and may be limiting to algal growth and reproduction in some natural waters (Menzel and

Ryther, 1961; Mackenthun, 1968; Fitzgerald, 1970; Goldman, 1972; Lee, 1973; Hutchinson, 1973). The P and Fe of natural waters can exist in many forms and their chemistry is complex. Some of the forms are available for uptake and use by algal cells while some are not (Lee, 1973). Because of this complexity of nutrient chemistry, the availability of nutrients to algae may vary spatially and temporally. If the eutrophication process is to be elucidated, the complex interactions between naturally occurring organic molecules with inorganic nutrients and their availability to algal cells must be studied.

Nutrient limitation is considered to be the most critical factor determining the amount of growth in the epilimnion of thermally stratified lakes (O'Brien, 1972). It has been postulated that organic chelates may stimulate algal growth by making normally insoluble nutrients, such as Fe, more available for uptake than they would be in the absence of the chelating substances (Burk et al., 1932a; Provasoli and Pinter, 1959; Johnson, 1964). Shapiro (1957) found that colloidal, organic acid-Fe complexes could make Fe more available to phytoplankton and Lange (1970) reported that the stimulation of blue-green algae by fulvic acids was due to the ability of the organic acids to hold Fe in solution and make it available for uptake by algae.

Early workers in algal culture found that many species of algae grew better if soil or soil extract was added to the

culture medium. Then it was found that synthetic chelating agents could be used for the same end. Synthetic organic chelating agents have been found to enhance algal growth during *in situ* field experiments as well as in laboratory batch cultures (Schelske *et al.*, 1962). Knezek and Maier (1971) found that the synthetic chelating agent, EDDHA (ethylenediamine di-o-hydroxyphenylacetic acid) promoted cell division in *Euglena gracilis* and EDTA (ethylene-diamine-tetraacetic acid) has been used in many algal culture media, including the AAP (Algal Assay Procedure, United States Environmental Protection Agency) medium used in these studies. Most of the synthetic media used presently for the culture of algae contain a chelating agent (Stein, 1973). This is especially true of artificial sea water media. The fact that synthetic chelating agents seem to replace soil extracts in these culture media indicates that both may be responsible for enhancing algal growth by the same mechanism.

Shapiro (1966b) found that the higher molecular weight fraction of the naturally occurring colored organic acids was responsible for most of the chelating capacity in surface waters. The larger molecular weight fraction has also been found to be more stimulatory to freshwater as well as marine phytoplankton (Prakash *et al.*, 1973). The larger molecular weight fractions of dissolved organic acids seem to be more stimulatory to algae, but Stevenson and Ardakani (1972) found

that humic acids bind metals very tightly and thought that the metals bound to the humic acids were unavailable for uptake by plants. The larger humic acid molecules were found to bind metals more tightly and were less mobile than the smaller fulvic acid molecules. These two findings seem to be contradictory, if in fact humic acids are stimulatory to algal growth by making nutrients available by chelation processes. The low ionic strength and low productivity of dystrophic bog waters may, among other things, be due to the binding of metals to humic acids, where they are unavailable to algae.

The hypothesis upon which this work was based was that high molecular weight (30,000 or greater) humic acids bind Fe tightly and make it unavailable to algal cells. To test this hypothesis, sets of experiments were designed to determine the effects of humic acids on the uptake of Fe and Po_4 by Scenedesmus obliquus (Türp) Kütz as well as their effects on growth. These experiments tested the ability of humic acids of molecular weight 30,000 or greater to stimulate growth of S. obliquus and if so to identify the stimulating mechanism.

The effect of humic acids on algal growth in the presence of Fe and PO_4 and Fe and PO_4 uptake were studied in conjunction because of the possible antagonistic effects of Fe and PO_4 on each other via precipitation as strengite

($FePO_4.2H_2O$). Although generally not the controlling factor in most natural surface waters, this compound is fairly insoluble (pK_{sp}=25) and removal of Fe and PO_4 from solution by this mechanism can effectively reduce the availability of these nutrients. Fe is routinely used in tertiary sewage treatment systems to remove soluble PO_4 from water and PO_4 is often added to drinking water to remove undesirable concentrations of Fe.

Initial studies of the growth responses of S. obliquus to various concentrations of Fe and PO_4 were also studied and reported as baseline information before the effects of humic acids on the growth of S. obliquus were studied.

A description of the experimental alga as well as the experimental and maintenance culture methods are reported in Appendix I. The humic acid characterization and purification methods and a description of the humic acids used in this study may be found in Appendix II.

Appendix III contains the results of a study of various ion exchange methods for removing humic acids from water.

The pH records, relative growth rates and generation times for each experiment are reported in Appendices IV and V respectively.

Fe AND P DYNAMICS

Fe and Phosphorus (P) are both essential for growth and reproduction of plants. Phosphorus is used in proteins and energy transfer systems, while Fe is a constituent of enzymes and respiratory cytochromes. Fe is also needed for syntheses that maintain chlorophyll content (Oborn, 1960; Karali and Price, 1963; Bowen, 1966).

The genus Chlorella requires a minimum concentration of $1.8X10^{-5}$ M Fe when growing autotrophically, but may require as much as $1.8X10^{-4}$ M Fe in dense cultures (Eyster, 1962). Heterotrophic growth by Scenedesmus sp. requires an Fe concentration of only $1X10^{-9}$ M to grow and reproduce (O'Kelly, 1968), although the optimum Fe concentration for autotrophic growth of this genus was reported to be 0.05 mg/l Fe in the absence of chelating agents (Eyster, 1967). The level of Fe required for growth in the genus Scenedesmus is related to the extent of hydrogenase synthesis. Below a certain species specific cellular Fe level the algae can no longer divide (Davies, 1970). Before the effect of humic acids on the PO_4 and Fe nutrition of S. obliquus could be studied, the limiting levels as well as the algal growth responses to various levels of these nutrients had to be established for the bioassay system being used (Appendix I).

Stock Scenedesmus obliquus cells from cultures growing actively in AAP medium (Algal Assay Procedure, Environmental Protection Agency) showed no variation in their response to Fe concentrations ranging from $1X10^{-3}$ mg/l Fe to 1.0 mg/l Fe. This is not surprising since by luxury nutrient uptake, many algae are able to accumulate excess nutrients in their cells when these nutrients are available (Fitzgerald and Lee, 1971; Lee, 1971; Fitzgerald, 1972). This carry-over of nutrients allows algae to grow and divide normally for several generations when placed in a medium deficient in the stored nutrient.

The AAP medium was developed to minimize the carry-over of nutrients which can interfere with the testing of nutrient limitation by batch bioassays. The AAP medium has an Fe concentration of about $6X10^{-8}$ M which should be limiting to algal growth (Anon., 1971). The cellular build-up of Fe may be due to stock S. obliquus cells being regularly transferred to fresh culture medium. These transfers may enable the algal cells to store excess Fe, even though the Fe level in the medium is very low. To avoid the carry-over problem, a stock of Fe-starved S. obliquus cells was grown in Fe-free culture medium (Appendix I).

Fe Limiting Levels

The Fe-starved stock S. obliquus did show a response to varied Fe concentrations (Figure 1 and Table 1).

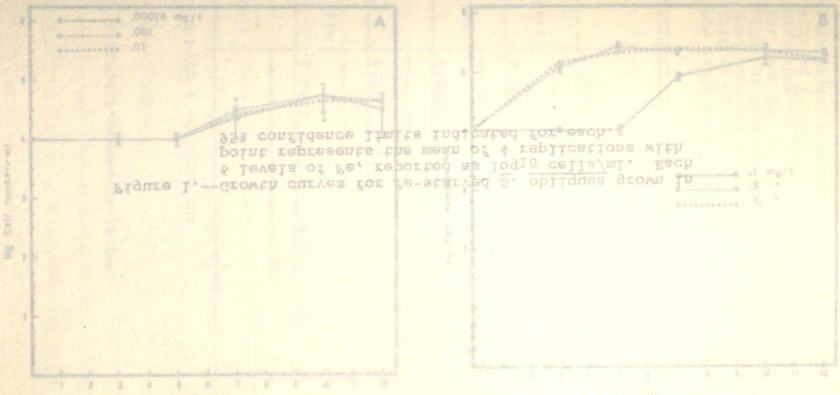

A
B
.0002 mg/l
.001
.01
Day
Mg Cell number/ml

Figure 1.--Growth curves for Fe-starved S. obliquus grown in 6 levels of Fe, reported as $\log_{10}$ cells/ml. Each point represents the mean of 4 replications with 95% confidence limits indicated for each.

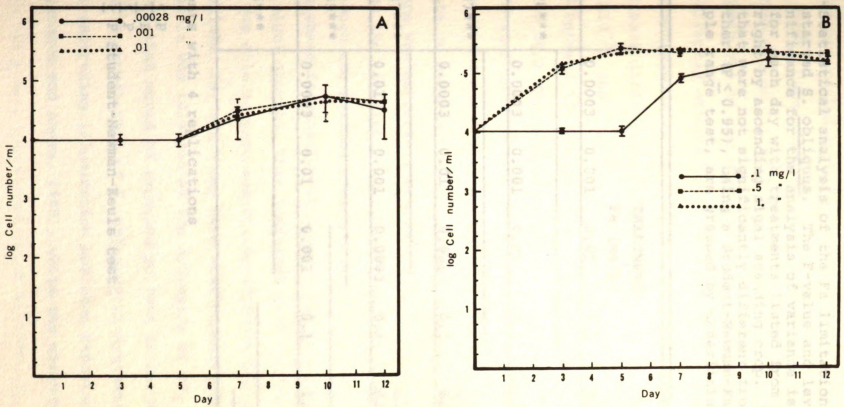

A
.00028 mg/l
.001 "
.01 "
log Cell number/ml
Day
B
.1 mg/l
.5 "
1. "
log Cell number/ml
Day

Figure 1

Table 1.--Statistical analysis of the Fe limitation of Fe-starved S. obliquus. The F-value and level of significance for the analysis of variance is listed for each day with treatments listed from left to right by ascending algal standing crop. Treatments that were not significantly different from one another ($P \leq 0.95$), using a Student-Newman-Keuls multiple range test, are grouped by underlining.

		TREATMENT Fe (mg/l)					
DAY	F	0.0003	0.001	0.01	0.1	0.5	1.0
3	84.98***	0.0003	0.001	0.01	0.1	1.0	0.5
5	70.37***	0.0003	0.01	0.001	0.1	0.5	1.0
7	54.47***	0.01	0.001	0.0003	0.1	0.5	1.0
10	12.39***	0.0003	0.01	0.001	0.1	1.0	0.5
12	40.06***						

6 treatments with 4 replications

*** $0.99 \leq P$
** $0.95 \leq P$
* $0.90 \leq P$
+ $0.95 \leq P$ Student-Newman-Keuls test

A concentration of 0.1 mg/l Fe caused a significantly greater relative growth rate after day 7, but there was still a lag phase. When 0.5 or 1.0 mg/l Fe were present in the culture medium, there was no lag phase and the relative growth rate was significantly enhanced over that found in the media with lower concentrations of Fe (Figure 1 and Table 1). There was no significant difference between the growth responses to $3X10^{-4}$, $1X10^{-3}$, and $1X10^{-2}$ mg/l Fe. No significant difference was found between the maximum standing crop in the presence of 0.5 and 1.0 mg/l Fe, indicating that the limiting level of Fe for the Fe-starved stock was 0.5 mg/l Fe or less. Increasing the Fe level above 0.5 mg/l caused no increase in relative growth rate or maximum standing crop. This was probably because there was sufficient Fe available, but another nutrient had become the limiting factor to further algal growth.

Although the maximum standing crop was less and there was a lag phase for the lowest 4 concentrations of Fe, there was some algal growth. This indicates that the Fe-starved S. obliquus was able to grow and divide after it had accumulated enough cellular Fe, which may have been removed from solution as soon as it was released by the hydroxide or PO_4 precipitates of Fe that would be expected to form in the medium. The relative growth rate of S. obliquus in synthetic growth medium at saturating illumination has been reported to be 2.2 (Hoogenhout and Amesz, 1965), while the maximum relative

growth rate for the cultures in these studies was 0.37, which occurred in the 0.5 mg/l Fe medium between day 0 and 3 (Appendix V, Table 21). This indicates that something other than Fe was limiting the algae in the exponential growth phase. The cultures may have been carbon limited, but this was not likely because air was bubbled through the medium (Appendix I) and the pH never rose above 9.5 (Appendix IV, Table 11). The cultures were grown under 375 ft candles continuous illumination which was possibly not the limiting factor.

For a given species, the relative growth rate is generally a constant for a particular set of environmental factors. These include temperature, light intensity, nutrient availability, etc. The relative growth rate also depends on the cell size and physiological state of the stock used. The dependency upon these conditions makes comparisons of relative growth rates under different culture conditions questionable and should not be done. The limiting level of Fe for growth by Fe-starved S. obliquus under these particular culture conditions was tentatively determined to be between 0.1 and 0.5 mg/l Fe.

Further experiments were conducted to more precisely determine the limiting level of Fe to the Fe-starved cells and study more closely the algal response to Fe nutrition near the limiting level. The responses were studied as before, but for a more restricted range of concentrations.

The growth response of S. obliquus to 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 mg/l Fe were plotted (Figure 2). There was no significant difference between the standing crops in the media containing 0.1, 0.2, 0.3 or 0.4 mg/l, at any sampling, although the mean standing crops were generally higher for the higher Fe concentrations (Table 2). After 7 days of growth, there was a significantly greater cell density in the medium containing 1.0 mg/l Fe than at all other Fe concentrations. The medium containing 0.4 mg/l Fe showed a significantly lower growth than all other Fe concentrations. The highest maximum relative growth rate was between days 0 and 7 in the 1.0 mg/l Fe medium, with the 0.5 mg/l Fe medium having the next highest relative growth rate for the same period (Appendix V, Table 22). All other concentrations of Fe had approximately the same relative growth rate for the same period.

The log growth phase continued until day 12 for all concentrations of Fe except 1.0 mg/l, which reached a maximum standing crop on day 7 and decreased until there was no significant difference between any of the final standing crops after 16 days of growth. This phenomenon may be caused by several factors. The final standing crop may be a function of the solubility of Fe precipitates, with the culture containing the highest free Fe concentration initially supporting the greatest growth until the Fe concentrations in all

Figure 1. Growth curves for two-fold serial dilutions of an
overnight culture of a heterotrophic strain
grown both heterotrophically and photosynthetically
at different levels of irradiance. Symbols as in
Figure 1. Each point is the mean of 4 observations.
Error bars are ± 1 standard deviation.

Figure 2.--Growth curves for Fe-starved S. obliquus grown at 6 different levels of Fe, reported as $\log_{10}$ cells/ml. Each point represents the mean of 4 replications with 95% confidence limits indicated for each mean.

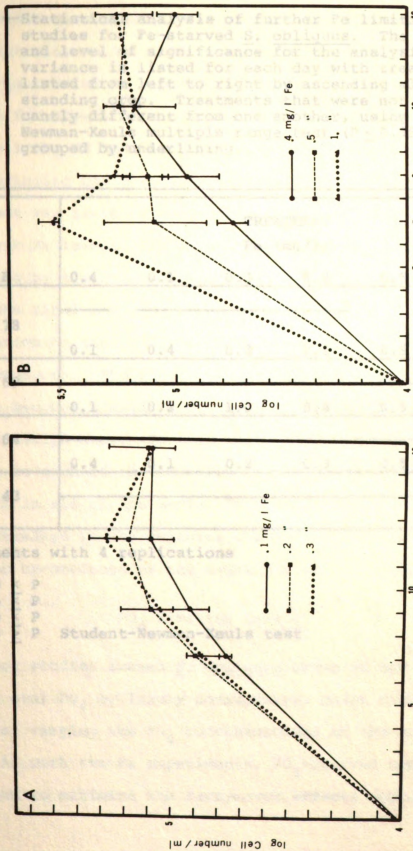


Figure 2

Table 2.--Statistical analysis of further Fe limiting level studies for Fe-starved S. obliquus. The F-value and level of significance for the analysis of variance is listed for each day with treatments listed from left to right by ascending algal standing crop. Treatments that were not significantly different from one another, using a Student-Newman-Keuls multiple range test ($P \leq 0.95$), are grouped by underlining.

		TREATMENT					
		Fe (mg/l)					
DAY	F	0.4	0.1	0.3	0.2	0.5	1.0
7	1.78				+		
		0.1	0.4	0.3	0.2	0.5	1.0
9	1.69						
		0.1	0.2	1.0	0.4	0.5	0.3
12	1.64						
		0.4	0.1	0.2	0.3	0.5	1.0*
16	1.43						

6 treatments with 4 replications

*** 0.99 $\leq$ P
** 0.95 $\leq$ P
* 0.90 $\leq$ P
+ 0.95 $\geq$ P Student-Newman-Keuls test

of the media were equalized by precipitation. The algae in the medium with an initially high free Fe concentration would be able to take up Fe rapidly and create an excess cellular Fe level for growth. This would cause an increased growth rate until the Fe had been partitioned between the algal cells, bringing the cellular concentration for growth to a level that was limiting to growth. The cells in the media with lower Fe levels grew at a lower rate which may have been controlled by the release rate of Fe from precipitates. The reason the final standing crops were the same may be due to Fe depletion or the build-up of wastes and algal extracellular by-products. The slower growing cultures may acquire Fe as it becomes available due to its dynamic equilibrium with Fe precipitates. To test this, additional Fe was added as $FeCl_3$ to each of the cultures. This caused a stimulation of growth in all of the media, indicating that the level of growth attained in the cultures was due to Fe limitation and not toxic by-products or the depletion of other nutrients.

PO_4 Limiting Levels

Pilot studies showed S. obliquus grown in AAP medium to carry over PO_4 by luxury consumption, which masked the effects of varying the PO_4 concentrations of the culture media. As with the Fe experiments, PO_4-starved stock algae were grown to minimize the carry-over effects (Appendix I).

Using the same methods that were used to study the algal growth responses to Fe, the growth responses of PO_4-starved S. obliquus to the following concentrations of PO_4 were studied: $2X10^{-4}$, $1X10^{-3}$, $5X10^{-3}$, $1X10^{-2}$, $1X10^{-1}$ and $5X10^{-1}$ mg/l. The PO_4 in the media from the impurities of the other nutrients used, was calculated to be $2x10^{-4}$ mg/l. This was the lowest PO_4 concentration attainable.

No growth occurred in the lowest 4 PO_4 concentrations so these were not plotted (Figure 3). Significantly greater growth was supported by the medium containing 0.5 mg/l PO_4 than that contained 0.1 mg/l (Figure 3). As well as supporting a greater final standing crop, the higher PO_4 concentration produced a higher maximum relative growth rate (Appendix V, Table 23).

A second PO_4-limitation experiment was performed to more precisely determine the limiting level of PO_4 for PO_4-starved S. obliquus under the experimental culture regime. The algal growth response was plotted for the following PO_4 concentrations: 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 mg/l PO_4.

The only PO_4 level producing a lag phase was 0.05 mg/l. The algae grown in the higher PO_4 concentrations showed no lag phases (Figure 4) and exhibited very similar maximum relative growth rates (Appendix V, Table 24). The final standing crops of the 3 lowest levels of PO_4 were significantly

Figure 3--Growth curves for E. coli strain B/r. Optimum growth temperature is 37°C.

Figure 3.--Growth curves for PO_4-starved S. obliquus grown at 2 levels of PO_4 in AAP culture medium, reported as $\log_{10}$ cells/ml. Each point represents the mean of 4 replications with 95% confidence limits reported for each mean. The minimum generation times (G) for each treatment are reported.

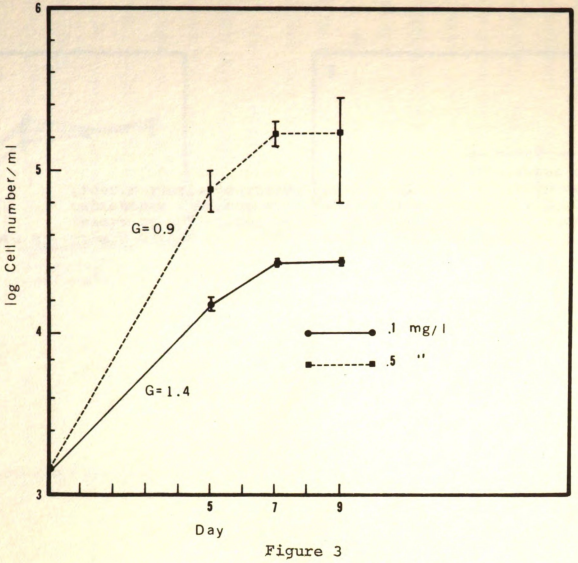


Figure 3

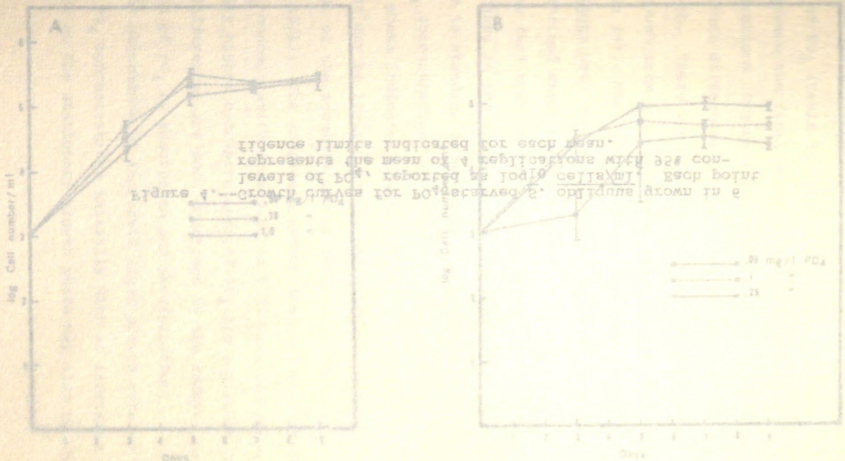


Figure 4.--Growth curves for bottlenose dolphin skin cells in a medium containing 2.5 mg/l PO_4 at 5 levels of PO_4, reported as total cell number. Each point represents the mean of 4 replicates with ±2SE confidence limits indicated for each mean.

Figure 4.--Growth curves for PO_4-starved S. obliquus grown in 6 levels of PO_4, reported as $\log_{10}$ cells/ml. Each point represents the mean of 4 replications with 95% confidence limits indicated for each mean.

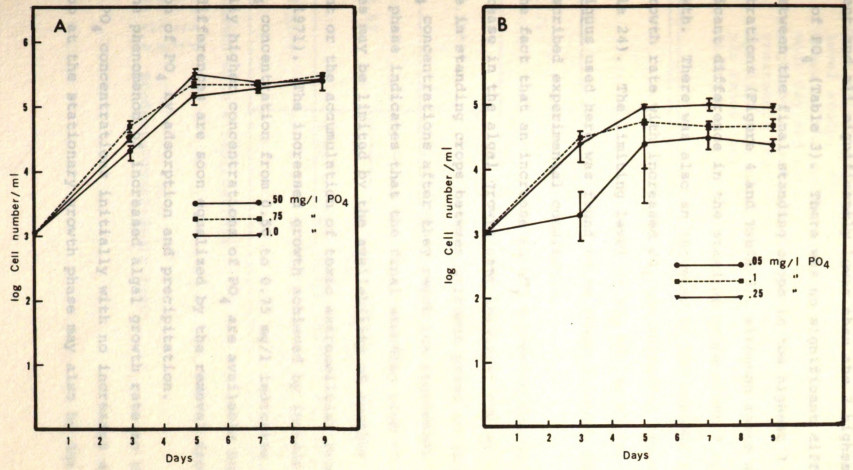

A
log Cell number/ml
Days
.50 mg/l PO4
.75 "
1.0 "
B
log Cell number/ml
Days
.05 mg/l PO4
.1 "
.25 "

Figure 4

different and all significantly lower than the 3 highest levels of PO_4 (Table 3). There was no significant difference between the final standing crops in the highest 3 PO_4 concentrations (Figure 4 and Table 3), although there was a significant difference in the standing crops after 3 days of growth. There was also an increase in the maximum relative growth rate with increased PO_4 concentration (Appendix V, Table 24). The limiting level of PO_4 to the PO_4-starved S. obliquus used here was found to be about 0.5 mg/l under the described experimental conditions.

The fact that an increase in PO_4 concentration causes an increase in the algal growth rate initially but no difference in standing crops between cultures grown in different PO_4 concentrations after they reach the stationary growth phase indicates that the final standing crop of these cultures may be limited by the availability of another nutrient or the accumulation of toxic extracellular products (Fogg, 1971). The increased growth achieved by increasing the PO_4 concentration from 0.50 to 0.75 mg/l indicates that initially higher concentrations of PO_4 are available but these differences are soon equalized by the removal from solution of PO_4 by adsorption and precipitation.

The phenomenon of increased algal growth rate to the higher PO_4 concentrations initially with no increased standing crop at the stationary growth phase may also be due to

Table 3.--Statistical analysis of the further PO_4 limiting level study. The F-value and level of significance on the analysis of variance is listed for each day and treatments are listed from left to right by ascending algal growth responses. The treatments that were not found to be significantly different, using a Student-Newman-Keuls multiple range test ($P \leq 0.95$), are grouped by underlining.

		TREATMENT PO_4 (mg/l)					
DAY	F	0.05	0.50	0.25	1.00	0.10	0.75
3	1.71						
		0.05	0.10	0.25	0.50	0.75	1.00
5	79.34***						
		0.05	0.10	0.25	0.50	0.75	1.00
7	3.62						
		0.05	0.10	0.25	0.50	0.75	1.00
9	20.91***						

6 treatments with 4 replications

*** 0.99 $\leq$ P
** 0.95 $\leq$ P
* 0.90 $\leq$ P
+ 0.95 $\leq$ P Student-Newman-Keuls test

luxury uptake consumption by the PO_4-starved S. obliquus cells. The actively growing cells at the beginning of the culture experiments may be limited by the rate with which they build up their cellular PO_4 levels, but by the end of the experiment after the deficient cells have satisfied their cellular PO_4 requirement for growth, there may be very little advantage to increasing the PO_4 level.

Many of these growth effects are artifacts caused by growing algae in batch culture. The small volume and continued justaposition of the algal cells and precipitates is different than the situation observed in most natural systems.

Fe-PO_4 Interactions

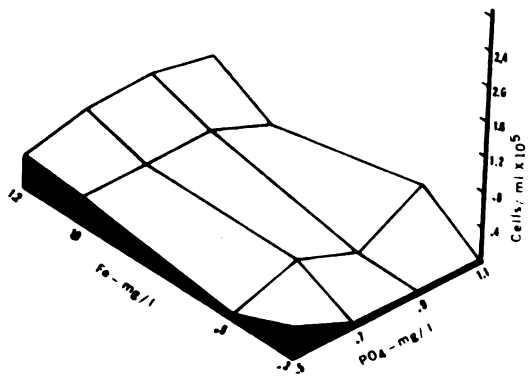
The effects of PO_4 and Fe in combination, on the growth of Fe-PO_4-starved stock S. obliquus were studied, using a 4X4 factorially designed experiment. The concentrations of PO_4 studied were 0.50, 0.70, 0.90 and 1.10 mg/l, while the Fe concentrations were 0.30, 0.50, 1.00 and 1.20 mg/l. The base medium was the normal AAP medium without EDTA, to which Fe and PO_4 were added to make the appropriate concentrations. Fe-PO_4-starved stock S. obliquus was added to each experimental flask to make an initial cell concentration of $1X10^3$ cells/mg. The algae were cultured under the standard experimental conditions (Appendix I), with samples taken after 4,

5, 6, and 7 days and the standing crop determined by cell counts.

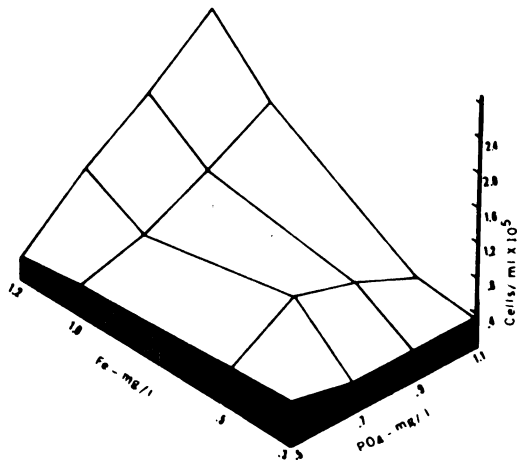
Neither PO_4 nor Fe added singly stimulated growth of Fe-PO_4-starved S. obliquus (Figure 5). There was a growth response only when Fe and PO_4 were added in combination. As the cultures grew, the responses became more and more variable with an increase in the growth response to PO_4 as well as an increased response to the lower levels of Fe. During the actively growing log phase (day 5), the greatest response was to Fe and PO_4 in combination at the highest levels of application. After 8 days, when the cultures had passed the point of maximum standing crop and senescence had set in, the response surface became more complex because the cell densities in the high Fe and PO_4 media had begun decreasing in intensity while some of the cultures with lower Fe and PO_4 levels continued to increase.

There was no decrease in the growth response at the high levels of either nutrient indicating that there was no decrease in the availability of nutrients due to mass action precipitation. Because both Fe and PO_4 were supplied at concentrations near the limiting levels of the stock S. obliquus there was an "inching up" effect of the growth responses. When more of one of the two nutrients being studied (Fe and PO_4) was added, the algae were limited by the other nutrient and an increase in that nutrient was able to cause a growth response.

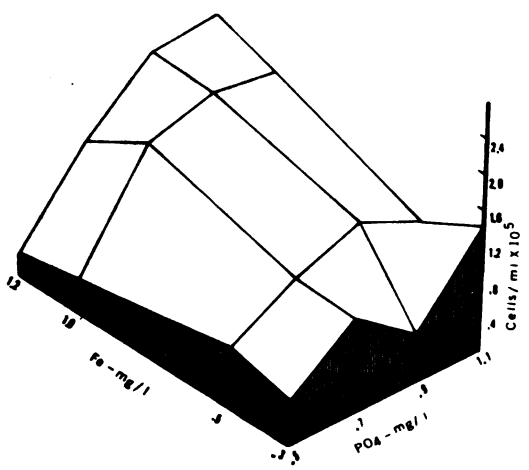
Figure 5.--Response surfaces of growth by Fe-PO_4-starved S. obliquus grown in 16 treatment combinations of Fe and PO_4. Expressed as cells/ml $X10^5$ reported as a function of Fe and PO_4 concentration after 4, 5, 6 and 8 days of growth.



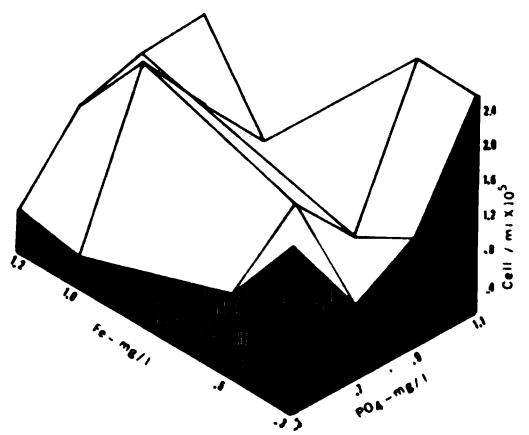
day 4



day 5



day 6



day 8

Figure 5

The media with 0.5 mg/l PO_4 supported the lowest final standing crop of the 4 PO_4 levels although all of the initial growth rates were similar (Figure 6 and Appendix V, Table 25). There was a lag phase only in the medium containing 0.5 mg/l Fe. The lower 2 levels of Fe showed the greater standing crop after 8 days. The higher Fe levels supported a smaller final standing crop of S. obliquus, indicating that there is an antagonism between Fe and PO_4. At the low level of PO_4 (0.5 mg/l PO_4), PO_4 is the limiting nutrient for growth because an increase in PO_4 concentration to 0.7 mg/l PO_4 causes an increase in the algal growth response. Increasing the level of Fe in the medium seems to cause a decrease in the available PO_4 and a concomitant decrease in the standing crop.

When 0.7 mg/l PO_4 was present in the culture medium, a different Fe response was noted (Figure 6b). At this level of PO_4 addition, there was sufficient PO_4 that the greatest final standing crop was supported by 1.0 mg/l Fe. Increasing levels of Fe supported higher final standing crops of algae until an Fe level of 1.2 mg/l was reached. This level of Fe caused a decrease in the final standing crop, indicating that the Fe was decreasing the availability of PO_4 at this point. As with the lower level of PO_4, there was an initial lag phase for cultures grown in the 0.3 mg/l Fe medium. Increasing the Fe concentration to 0.5 mg/l increased the initial growth rate to 0.37 (Appendix V, Table 25).

Figure 6. Growth curves for Fe-PO_4-starved S. obliquus in the Fe-PO_4 interaction studies. The growth response is reported as cells/ml X10^5 as a function of time.

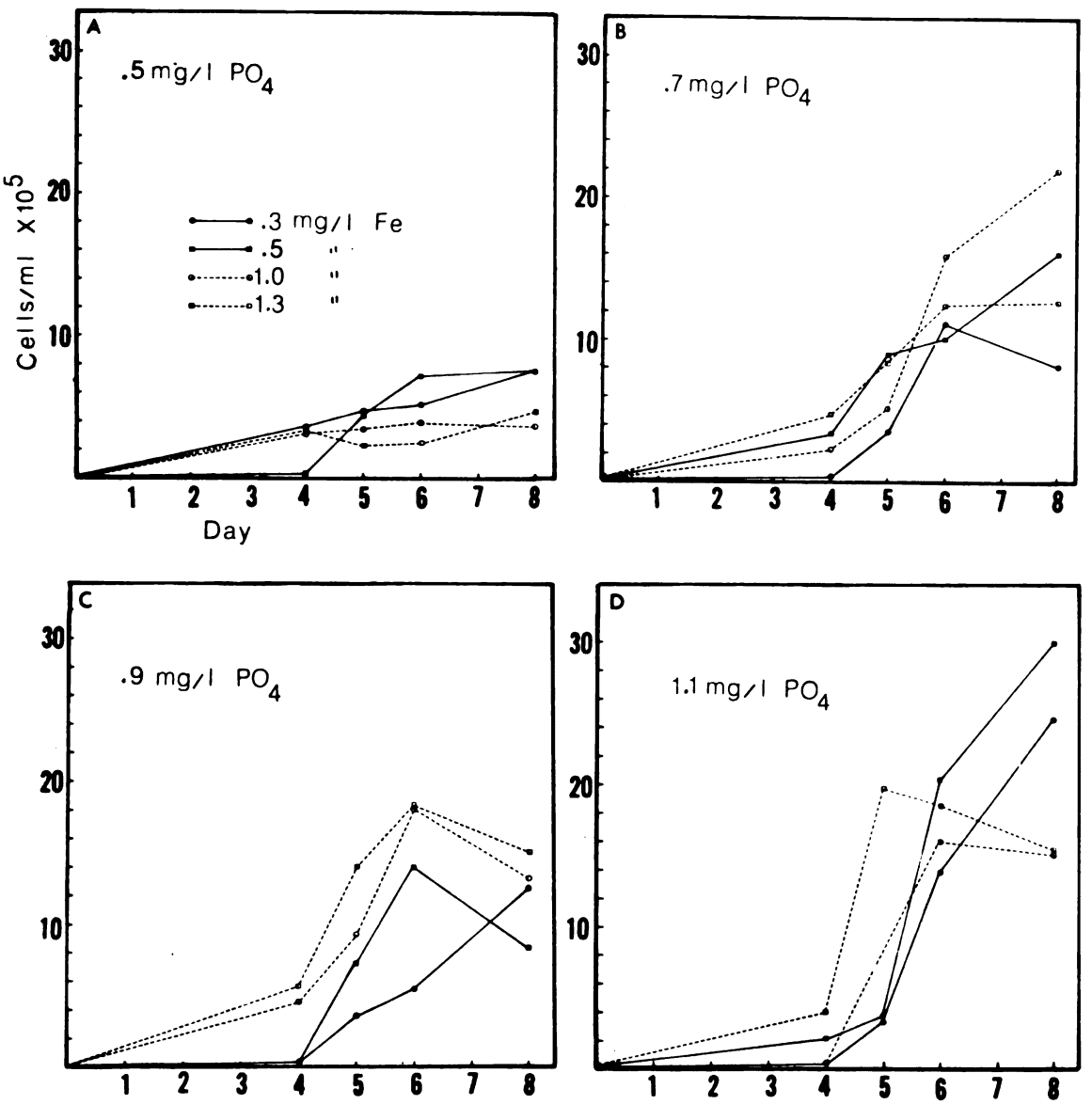

A
.5 mg/l PO_4
.3 mg/l Fe
.5 "
1.0 "
1.3 "
Cells/ml X10^5
Day
B
.7 mg/l PO_4
C
.9 mg/l PO_4
D
1.1 mg/l PO_4

Figure 6

Increasing the PO_4 level to 0.9 mg/l did not cause an increase in the final standing crop nor the maximum standing crop for any of the Fe levels, but there was an increase in the initial growth rate (Appendix V, Table 25). The antagonism of the PO_4 at this level was great enough to prevent growth in the cultures containing 0.3 and 0.5 mg/l Fe before day 4 (Figure 6c). The media with the higher 2 levels of Fe did support growth, with the greater growth occurring in the medium with 1.2 mg/l Fe. The maximum standing crops for the 3 highest levels of Fe were reached on day 6, while the cells in the medium containing only 0.3 mg/l Fe continued to grow at a lesser rate and were still dividing when the experiment was terminated on day 8. This indicated that the culture where Fe was limiting was growing at the rate of release of Fe from $FePO_4.2H_2O$ and not the maximum potential growth rate of S. obliquus. By day 8 the cultures in the 3 highest Fe media were in the death phase and had begun to decline.

At the highest PO_4 level (1.1 mg/l) there was still a lag phase for the lowest levels of Fe with the 2 highest Fe concentrations supporting the highest initial growth rate (Appendix V, Table 25). The maximum standing crop was reached on days 5 and 6 in the 2 highest Fe concentrations, after which the cell densities decreased. The algal cell density, in the media with the lower 2 Fe levels, continued to increase and was still increasing when the experiment was

terminated on day 8. At the highest PO_4 concentrations, the 2 lowest Fe levels supported the greatest final standing crop (Figure 6d), This was probably due to the fact that less Fe than PO_4 was required for growth and higher Fe levels reduced the PO_4 availability. The higher Fe levels supported a higher initial growth rate which was probably because at this point Fe was limiting algal growth. The higher final standing crop observed in the lower Fe levels was most likely due to PO_4 precipitation by the higher Fe concentration after PO_4 had become the limiting nutrient.

There was an interaction between Fe and PO_4 affecting the growth of S. obliquus. In general the effects can be explained by mass action chemical precipitation, making the Fe and PO_4 less available to the algae, although the algae seem to be able to grow after a lag phase when either Fe or PO_4 is in short supply, which indicates that algal growth may be mitigated by the kinetics of $FePO_4.2H_2O$ dissolution or the necessity to prepare the environment for growth by the excretion of extracellular by-products when Fe or PO_4 is in short supply due to chemical precipitation.

Ionic Fe in natural waters occurs in very small concentrations at the pH, oxygen concentration and redox potential normally found in the euphotic zone of temperate thermally stratified lakes (Hutchinson, 1957). Most of the suspended Fe exists as $Fe(OH)_3$ or $FePO_4.2H_2O$, sorbed to particulates,

as complexes or chelated by organic molecules (Hutchinson, 1957).

It is difficult to determine which forms of Fe and PO_4 will be determining the availability of these nutrients to algae in a particular system. In an oxygenated system, with a pH between 8.0 and 9.5, the solubility product of ferric hydroxide ($Fe(OH)_3$) determines the concentration of free Fe in solution because it is the most insoluble Fe-containing compound ($K_{sp}=10^{-39}$) and provides a sink for Fe^{+3}. The stabilization of the $Fe(OH)_3$ gel is a slow process so that over the short run, $FePO_4.2H_2O$ may exert some control over the system (Ellis, Personal Communication). The kinetics of the system are such that in short term bioassay systems both Fe and PO_4 may have an effect on the availability of the other. By mass action an increase in one nutrient would cause a precipitation of the other. The $\underline{p}$-H_2PO_4 of $FePO_4.2H_2O$ at pH 8.5 is approximately 26.5 (Lindsay and Moreno, 1960), which is a higher solubility than that of $Fe(OH)_3$ under similar conditions, but still low enough to make very little Fe or PO_4 available as free ions. Ferric phosphate solubility in soil and water systems increases with increasing pH while that of $Fe(OH)_3$ decreases with increasing pH (Lindsay $\underline{et\ al.}$, 1972). The important ionic species of Fe in natural waters include Fe^{+3}, Fe^{+2}, $Fe(OH)_2^{+}$, $Fe(OH)_2^{+2}$ and $Fe(OH)^{+}$, depending upon pH and Eh (Cooper, 1937).

In aerated water with a pH above 5.0, Fe^{+3} can be present in excess of 0.01 mg/l only as a suspension of $Fe(OH)_3$ (Hem and Crooper, 1959), Fe^{+3} forms a complex readily at pH values between 5.3 and 9.1 but PO_4 complexes were not able to prevent the precipitation of $Fe(OH)_3$ (Hem and Crooper, 1959),

It has been reported that algae are able to use PO_4 from very insoluble Fe-PO_4 compounds such as $FePO_4.2H_2O$ (Fitzgerald, 1972). Diatoms seem to be especially well adapted to using $Fe(OH)_3$ as a source of Fe (Harvey, 1937; Goldberg, 1952; Hayward, 1968). Fogg (1971) reports that PO_4 in the form of $FePO_4.2H_2O$ is largely unavailable to algae. The availability of nutrients to algal cells from insoluble chemical compounds may be species specific and is probably also dependent on environmental factors.

GROWTH EXPERIMENTS

Effect of Humic Acids on S. obliquus Growth

An experiment was conducted to determine the effects of various concentrations of humic acids on the growth of Scenedesmus obliquus. The algal growth response was tested for the following humic acid concentrations: 0.0, 2.0, 10.0, 15.0, and 25.0 mg/l in AAP medium without EDTA. This range of concentrations was chosen to bracket the concentrations frequently encountered in natural waters. Prakash and Rashid (1968) used humic acid concentrations of 6.0, 13.0, and 32.0 mg/l in their studies of the effects of humic acids on marine dinoflagellates. The experimental media were inoculated with Fe-starved stock S. obliquus to an initial concentration of $1X10^5$ cells/ml (Appendix I). The culture medium and methods used in this experiment were the standard experimental procedures (Appendix I).

Humic acids significantly increased the growth of S. obliquus grown in AAP culture medium (Figure 7a, 7b and Table 4). After 5 days, the lowest growth had occurred in the medium containing no humic acids while the humic acid containing media supported greater growth. A Student-Newman-Keuls test showed no significant differences between the growth responses of S. obliquus to 2.0, 5.0, or 10.0 mg/l

Figure 7.--Growth curves for Fe-starved S. obliquus cultures grown in 6 concentrations of humic acids. The growth responses are reported as cells/ml X10^5. Each point represents the mean of 4 replications with 95% confidence limits for the mean indicated.

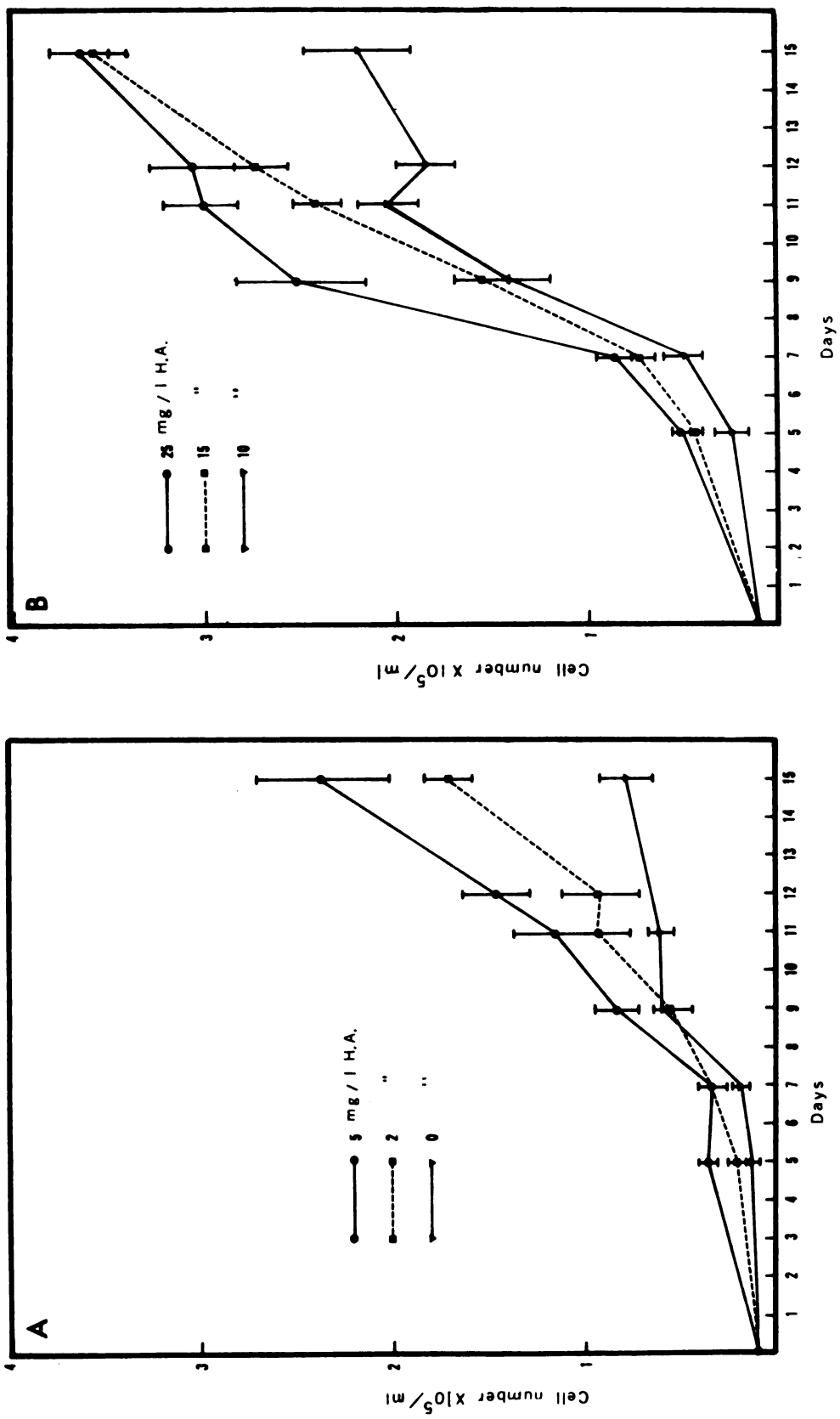


Figure 7

Table 4.--Statistical analysis of the algal growth responses of Fe-starved S. obliquus to 6 levels of humic acids. The F-value and level of significance for the analysis of variance is listed for each day with the treatments listed from left to right by ascending algal growth response. The responses that were not significant, using a Student-Newman-Keuls multiple range test ($P \leq 0.95$), were grouped by underlining.

		TREATMENT H.A. (mg/l)					
DAY	F	0.0	2.0	10.0	5.0	15.0	25.0
5	20.33***	+					
		0.0	5.0	2.0	10.0	15.0	25.0
7	13.75***						
		2.0	0.0	5.0	10.0	15.0	25.0
9	8.64***						
		0.0	2.0	5.0	10.0	15.0	25.0
11	10.20***						
		0.0	2.0	5.0	10.0	15.0	25.0
12	35.52***						
		0.0	2.0	5.0	10.0	15.0	25.0
15	18.85***						

6 treatments with 4 replications

*** $0.99 \leq P$
** $0.95 \leq P$
* $0.90 \leq P$
+ $0.95 \geq P$ Student-Newman-Keuls test

humic acids (Table 4). The responses to these levels of humic acids were significantly greater than when humic acids were absent, but significantly less than the response to 15.0 and 25.0 mg/l humic acids (Table 4). This trend continued throughout the experiment so that on day 15 the standing crop was not significantly different at the 2 highest concentrations of humic acid. The response to the 2 highest humic acid concentrations was significantly higher than the response to the next 3 highest concentrations which was in turn higher than the response to AAP medium in the absence of humic acids (Figure 7a, 7b and Table 4). In the presence of humic acids, S. obliquus exhibited a longer log growth phase than was observed in cultures where humic acids were absent. The algal cells in the medium where humic acids were absent reached a maximum standing crop after 5 days, while the algae in the humic acid containing media continued to grow.

Although generally greater standing crops of algal cells were found in the media containing humic acids before day 7, the growth rates for all of the media were approximately the same (Appendix V, Table 26). This seems to support the theory that humic acids are able to supply nutrients which become limiting in their absence. Since the stock S. obliquus cells used were Fe-starved, the nutrient that was

most likely to be in short supply was Fe. The ability of humic acids to sustain the log growth phase and support a greater final standing crop indicates that they may be stimulating growth by making Fe available for growth for a longer period of time. This may be misleading because earlier it was shown that S. obliquus was able to acquire Fe from Fe precipitates so that the final standing crops were the same at various Fe concentrations.

The humic acids were also able to shorten the lag time and decrease its severity at the beginning of the experiment. Bozniak (1969) found that humic substances were able to decrease the lag time of a species of Chlorella grown in batch culture. This decrease in lag time may be due to direct cellular stimulation by the humic acids or by making nutrients available sooner than in media containing no humic acids. Thus humic acids not only make growth in an Fe-limited system last longer, but enable it to begin sooner.

The fact that the final standing crops in the 2 highest humic acid concentrations were not significantly different indicates that a point of diminishing return is reached where the further addition of humic acids does not increase the growth response. Prakash and Rashid (1968) found that increasing concentrations of humic acids caused increased growth responses in marine phytoflagellates until a humic acid concentration of 35.0 mg/l was reached. This concentration caused a reduction in growth from that of the next lower

humic acid concentration. The Fe chelation theory explains this phenomenon by postulating that the high concentration of humic acids is able to compete with the algal cells for the available Fe and thus make Fe less available, decreasing the algal growth. The humic acids may also be directly stimulatory to algal cells at lower concentrations, but become toxic at the higher levels.

Because the growth studies were not done in axenic S. obliquus cultures (Appendix I) possible bacterial effects must be taken into account when explaining the stimulatory effects of humic acids on algal cells. The bacterium Azotobacter sp. has been found to be stimulated by a 25.0 mg/l humic acid solution (Burke, 1932b). It was thought that this may be due to a redox effect on the bacterial cell membranes, but may also be due to nutrient availability. The bacteria may be able to use parts of the humic acid molecules as a carbon source although humic acids seem to be very resistant to bacterial decomposition.

Although the mechanism is not well understood, humic acids do stimulate some species of bacteria and this may have an indirect effect on the growth of algal cells. It was found that Scenedesmus obliquus could not be grown in aqueous media in the absence of its normally associated bacteria (Appendix I). It was postulated that the bacteria may be

supplying a nutrient or service that the S. obliquus cells could not supply themselves. If this was true, a stimulation of the associated bacterial cells could also cause a stimulation of the algal cells. This explanation works well to explain the increased growth rates and decreased lag times, but does not explain the increased final standing crops in the media containing humic acids. If the supply of some bacterial product was determining the standing crop of S. obliquus, one would expect the final standing crops to eventually be the same.

Another possible explanation for the extended growth of S. obliquus is that humic acids may mitigate the possible toxic effects of metabolic by-products that build up in batch algal cultures. There are many metabolic by-products which are actively secreted and excreted from cells and others which simply leak from the cells (Zajic, 1970; Fogg, 1971). It has been found that the presence of some of these extracellular by-products are necessary for the logarithmic growth of some algal species (Fogg, 1971). If this is true and humic acids are able to decrease the lag phase, they must not be sequestering these extracellular products or are able to replace them. It is possible that there are different mechanisms acting at the beginning and end of the algal growth functions and there may be different metabolic products involved in causing the death or declining growth phase.

There may also be concentration effects enacted. That is, there may be a minimum concentration of extracellular by-products needed to support good growth and a maximum concentration above which growth is inhibited. None of these problems have been studied here and need further investigation.

Humic Acid-EDTA Interactions

Since the stimulatory effects of humic acids have been theorized to be due to the ability of the humic acids to form stable complexes and chelates with algal nutrients and thus make them available, an experiment was conducted to compare the stimulatory effects of humic acids to those of ethylene-diaminetetraacetic (EDTA). EDTA is a synthetic chelating agent which has been shown to stimulate the growth of Fe-starved algae by keeping Fe in solution and making it available for algal growth (Schelske et al., 1962). The possible interactions between EDTA and humic acids were also studied.

A 2^3 factorial experiment with 3 replications was used to determine the effects of humic acids and EDTA on Fe-starved and non-starved S. obliquus. The experimental conditions were those of the standard culture procedure (Appendix I). The 2 levels of EDTA were 0.0 and 0.3 mg/l while the experimental levels of humic acids were 5.0 and 15.0 mg/l.

The third factor studied was the nutrient condition of the stock S. obliquus. The stock algae were either normal actively growing cells from AAP medium or Fe-starved cells (Appendix I). The culture flasks were inoculated with enough stock cells to make an initial cell density of $1X10^3$ cells/ml.

EDTA in the presence of 5.0 mg/l humic acids caused an increased algal standing crop at each sampling (Figures 8 and 9). The increased standing crop was significant for day 4 and 7, but not for day 3 and 5 (Table 5). Humic acids also had a stimulatory effect on S. obliquus in the absence of EDTA.

Initially 15.0 mg/l humic acids in the absence of EDTA caused an increase in growth rate for both the non-starved and Fe-starved algae, but caused a significant decrease in growth rate of the cultures after day 5 (Figures 8, 9 and Table 5). By day 7 the response was reversed and there was a significant reduction of the standing crop of the culture inoculated with Fe-starved and non-starved stock (Figures 8, 9 and Table 5). In the absence of EDTA, 15.0 mg/l humic acids caused the algae to have a higher maximum relative growth rate than 5.0 mg/l humic acids, but the log growth phase was shorter when the higher humic acid concentration was present (Figure 9 and Appendix V, Table 27). Similar responses were obtained for cultures inoculated with starved and non-starved stock S. obliquus.

Figure 8.--Response surfaces of S. obliquus growth to humic acids and EDTA with the algal growth response in cells/ml reported for each treatment combination for Fe-starved and non-starved stock S. obliquus after 3, 4, 5 and 7 days of growth. Note the scale changes from cells/ml X 10^5 to cells/ml X 10^6 at day 7.

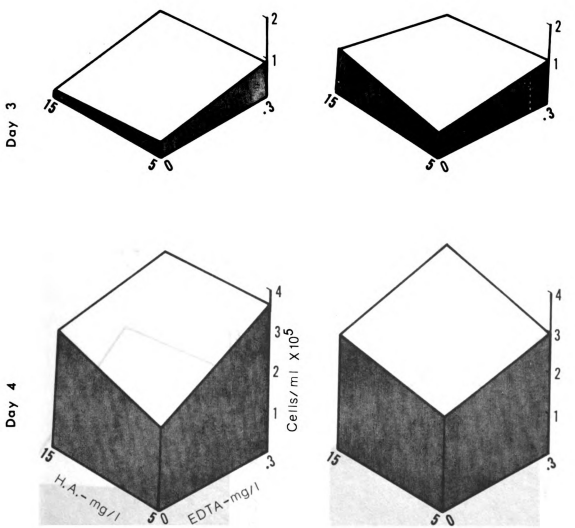

Day 3
Day 4
2
1
.3
15
5
0
4
3
2
1
Cells/ml X10^6
H.A.-mg/l
EDTA-mg/l
Iron starved stock
unstarved stock

Figure 8

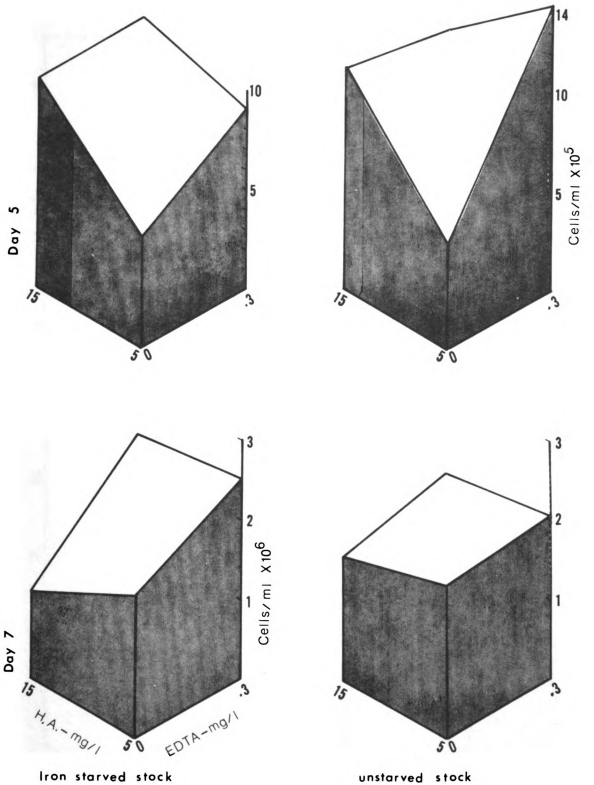


Figure 8 (cont'd)

Figure 9.--Growth response curves of Fe-starved and non-starved S. obliquus to humic acids and EDTA. Each point represents the mean of 3 replications with 95% confidence limits for each mean indicated.

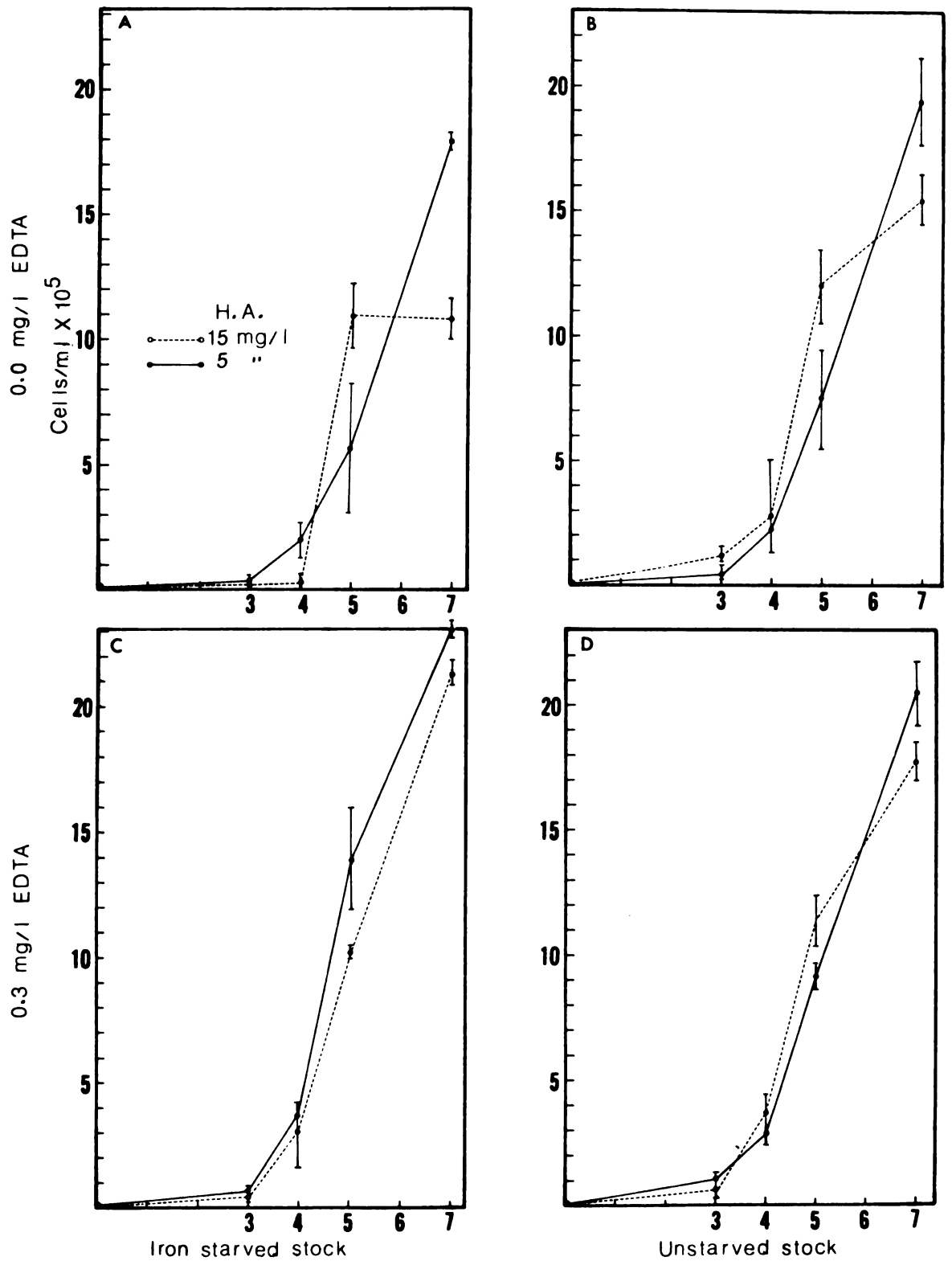

A
B
C
D
H.A.
15 mg/l
5 "
0.0 mg/l EDTA
0.3 mg/l EDTA
Cells/ml X 10^5
Iron starved stock
Unstarved stock

Figure 9

Table 5.--Statistical analysis of the EDTA-humic acid experiment. The effect means and levels of significance are tabled for each main effect and interaction for each day.

		TREATMENT EFFECTS						
DAY	(1)	S	E	SE	H	SH	EH	SEH
3	7.0×10^4	3.0×10^4 **	2.2×10^4 *	-2.1×10^4 *	-1.4×10^4 NS	6.2×10^3 NS	-2.4×10^4 *	-2.1×10^4 NS
4	3.0×10^5	3.3×10^4 NS	8.7×10^4 NS	2.4×10^4 NS	4.3×10^4 NS	-2.3×10^4 NS	2.6×10^4 NS	3.9×10^4 NS
5	2.0×10^6	-8.7×10^5 *****	1.9×10^5 ****	-2.0×10^5 ****	1.8×10^5 ***	9.3×10^5 *****	3.1×10^5 *****	1.5×10^5 **
7	1.9×10^6	4.1×10^5 *	5.7×10^5 NS	1.9×10^5 NS	-3.9×10^5 *	5.1×10^5 **	1.8×10^5 NS	3.3×10^5 NS

2^3 factorial design with 3 replications

***** $0.999 \leq P$
**** $0.995 \leq P$
*** $0.990 \leq P$
** $0.950 \leq P$
* $0.900 \leq P$
NS $P \leq 0.900$

	low	high	
Fe-Starved Algal Stock		Non-starved Algal Stock	(S)
0.0 mg/l EDTA		0.3 mg/l EDTA	(E)
5.0 mg/l H.A.		15.0 mg/l H.A.	(H)

When 0.3 mg/l EDTA was included in the medium, the presence of 15.0 mg/l humic acids caused a lower standing crop at every sampling in the culture inoculated with Fe-starved stock and the culture inoculated with the non-starved stock had a lower final standing crop than when 5.0 mg/l humic acids were present (Figure 9). There was a significant negative interaction between EDTA and the high level of humic acids after 3 days of growth (Table 5). When both of these chelating agents were present, there was an initial inhibition of growth (Table 5). The interaction was positive but not significant for the samples taken on day 4 and 7 while the positive interaction was highly significant for the sample taken on day 5 (Table 5).

The nutritional state of the algal inoculum had a significant effect on the growth response. Initially the non-starved algae grew better than the Fe-starved S. obliquus (Figures 8, 9 and Table 5). By the fifth day there was a significantly lower growth response by the non-starved algae because of the fact that the initial growth rate of this algae was greater than the starved cells and they had begun to enter the stationary growth phase (Table 5, Figure 9 and Appendix V, Table 27).

The only significant second order interactions between the presence of EDTA and the nutritional condition of the stock algal cells were in the samples taken on day 3 and 5.

EDTA caused a significantly lower growth response when non-starved stock algae were used than when Fe-starved S. obliquus stock was used (Table 5, Figure 9 and Appendix V, Table 27).

Humic acids on the other hand, significantly stimulated growth in the culture inoculated with non-starved stock after 5 and 7 days of culture (Table 5 and Figure 8).

The initial effect of the third order interaction was a decreased growth response of non-starved S. obliquus in the presence of EDTA and the higher concentration of humic acids. This initial response was not shown to be significant, but the increased standing crop on day 7 was significant (Table 5).

An additional set of experiments was conducted to more precisely determine the effect of humic acids and EDTA on the growth response of Fe-starved S. obliquus. A 6X2 factorially designed experiment with EDTA at 0.0 and 0.3 mg/l and humic acids at 0.0, 2.0, 5.0, 10.0, 15.0 and 25.0 mg/l was used to study the main effects and interactions of these chelating agents.

The results of the more detailed study of the effects of humic acids and EDTA on the growth responses of Fe-starved S. obliquus were similar to those of the previous experiment. Initially EDTA caused an increased growth rate in the absence of humic acids (Figure 10 and Appendix V, Table 28). After 2 days, EDTA alone caused a significantly

Figure 10.--Response surfaces of the standing crops of Fe-starved S. obliquus to humic acids and EDTA reported as cells/ml for each treatment after 2, 4, 5, 7, 9, 12 and 16 days of growth. Note that the scale of the response changes from cells/ml $X10^4$ to cells/ml $X10^5$ on day 5.

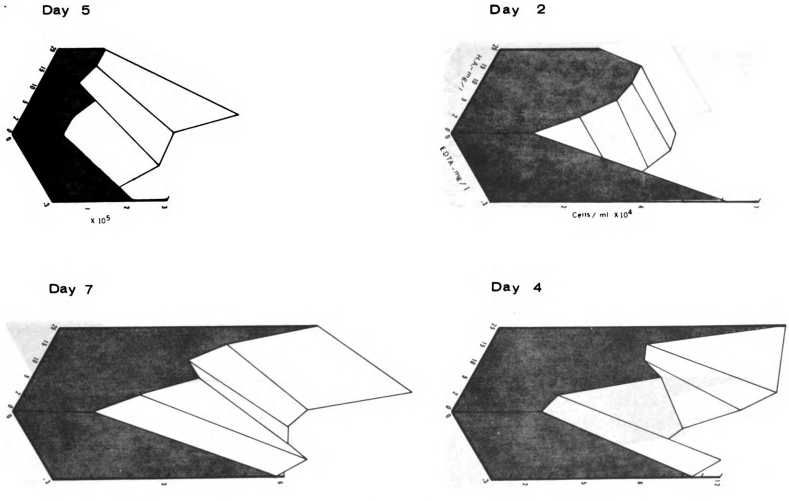
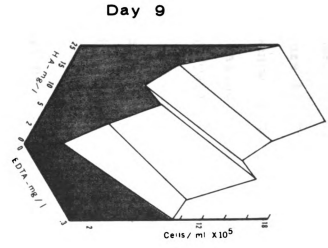
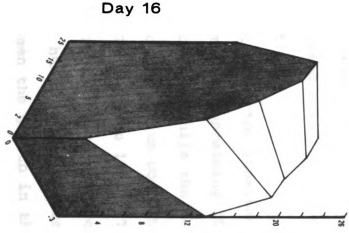


Figure 10





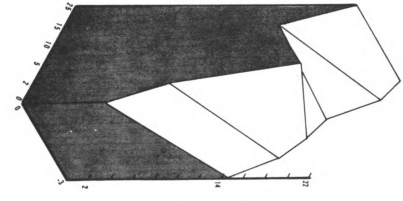


Figure 10 (cont'd)

greater growth rate than all combinations of EDTA and humic acids (Table 6). The lowest growth rate was observed in the medium which contained neither EDTA nor humic acids (Figure 10, Table 6 and Appendix V, Table 28). In the presence of humic acids, the growth response increased with increasing concentration of humic acids with and without EDTA (Table 6, Figures 10 and 11). This trend continued until a humic acid concentration of 25.0 mg/l was reached, which caused the same growth response as that for 2.0 mg/l humic acids (Figure 10).

Growth enhancement seemed to depend upon the presence of either humic acids or EDTA. While the addition of EDTA alone caused the highest single increased growth response, the next highest responses were caused by humic acids either with or without EDTA. When humic acids were present the presence of EDTA increased standing crops only slightly (Figure 10 and Table 6).

After 4 days of growth, the greatest growth response was observed for the medium containing 25.0 mg/l humic acids in the absence of EDTA while the second greatest growth response was to the medium containing 25.0 mg/l humic acids and 0.3 mg/l EDTA (Figures 10 and Table 6). The lowest growth response after 4 days was observed in the medium containing no EDTA or humic acids. There were no significant differences between the growth in the various media

Table 6.--The statistical analysis of a 6X2 factorial humic acid-EDTA experiment. The F-value and level of significance is listed for each main effect and interaction at each sampling. The treatment combinations are listed from left to right by ascending algal growth response, with the means that were not significantly different, using a Student-Newman-Keuls multiple range test ($P \leq 0.95$), grouped by underlining.

DAY	EFFECT	F
2	H	0.29
	E	5.08**
	HE	1.39

TREATMENT

	mg/l EDTA											
	0.0	0.3	0.0	0.3	0.0	0.3	0.3	0.0	0.0	0.0	0.3	0.3
mg/l H.A.	0.0	25.0	25.0	2.0	2.0	5.0	15.0	4.0	10.0	15.0	10.0	0.0

DAY	EFFECT	F
4	H	1.87
	E	1.26
	HE	1.42

	mg/l EDTA											
	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.3	0.3	0.3	0.3	0.0
mg/l H.A.	0.0	2.0	5.0	15.0	10.0	10.0	5.0	0.0	15.0	2.0	25.0	25.0

continued

Table 6--continued

DAY	EFFECT	F
5	H	1.05
	E	0.83
	HE	0.30

	TREATMENT											
	mg/l EDTA											
	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.3	0.3	0.3	0.0	0.3
	mg/l H.A.											
	0.0	10.0	15.0	2.0	10.0	5.0	2.0	0.0	15.0	5.0	25.0	25.0

DAY	EFFECT	F
7	H	3.48*
	E	15.80***
	HE	0.74

	mg/l EDTA											
	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.3	0.3	0.0	0.3
	mg/l H.A.											
	0.0	2.0	10.0	5.0	15.0	10.0	5.0	0.0	15.0	2.0	25.0	25.0

DAY	EFFECT	F
9	H	11.11***
	E	14.53***
	HE	0.65

	mg/l EDTA											
	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.3	0.3	0.3	0.0	0.3
	mg/l H.A.											
	0.0	10.0	0.0	2.0	2.0	15.0	5.0	10.0	15.0	5.0	25.0	25.0

continued

Table 6--continued

DAY	EFFECT	F										
12	H	9.58***										
	E	2.97*										
	HE	1.58										
			TREATMENT									
			mg/l EDTA									
			0.0	0.0	0.3	0.3	0.3	0.0	0.3	0.0	0.0	0.3	0.3	0.0
			mg/l H.A.									
			0.0	0.2	0.0	2.0	5.0	15.0	10.0	10.0	5.0	15.0	25.0	25.0
	EFFECT	F										
16	H	6.01***										
	E	0.45										
	HE	1.01										
			TREATMENT									
			mg/l EDTA									
			0.0	0.3	0.0	0.0	0.3	0.3	0.0	0.3	0.3	0.0	0.0	0.3
			mg/l H.A.									
			0.0	0.0	2.0	5.0	5.0	2.0	25.0	15.0	10.0	10.0	15.0	25.0

6X2 Factorial with 4 replications

*** 0.99 $\leq$ P
** 0.95 $\leq$ P
* 0.90 $\leq$ P
+ 0.95 $\geq$ P Student-Newman-Keuls test

H Humic acid
E EDTA

Figure 11.--Growth response curves of Fe-starved S. obliquus to humic acids and EDTA. Each point represents the mean of 4 replications with the 95% confidence interval for the mean reported.

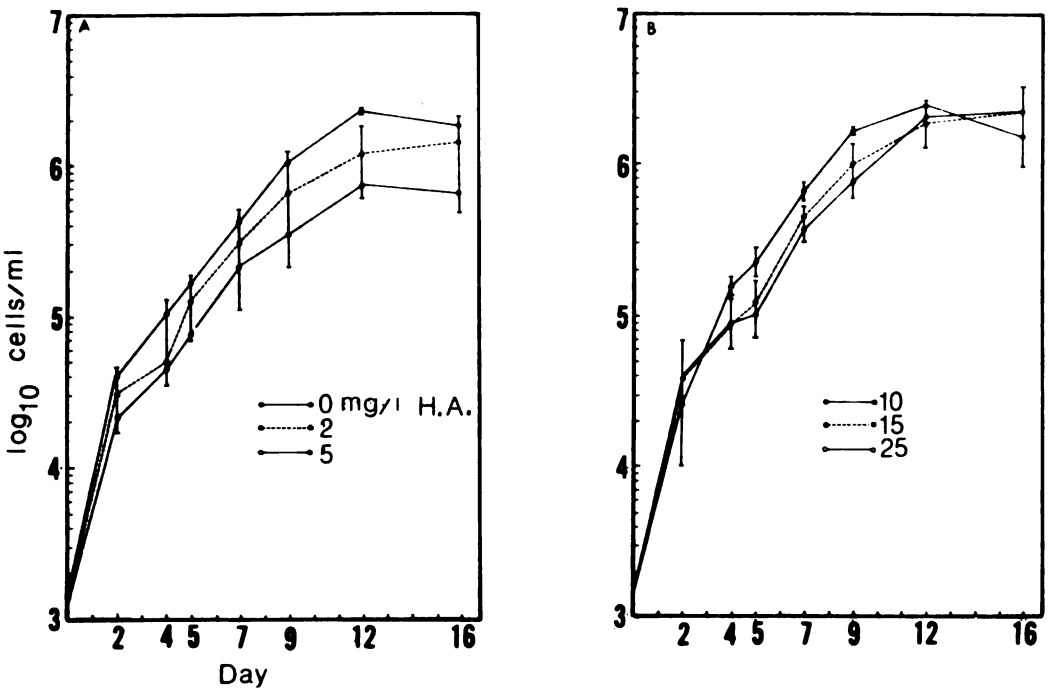

A
B
C
D
log10 cells/ml
Day
0 mg/l H.A.
2
5
10
15
25
0
2
5
10
15
25
2 4 5 7 9 12 16
3
4
5
6
7
0 mg/l EDTA
.3 mg/l EDTA

Figure 11

containing 5.0, 10.0, and 15.0 mg/l humic acids with and without EDTA, although the means were higher when EDTA was present (Table 7).

By the fifth day the relative growth rate was beginning to decrease (Figure 11 and Appendix V, Table 28). The media containing 0.3 mg/l EDTA in general supported better algal growth than the media containing no EDTA with the greatest growth response occurring in the medium which contained 25.0 mg/l humic acids as well as EDTA (Figure 10). In the absence of humic acids EDTA still caused an enhanced algal growth, but there was little difference between the responses to the various concentrations of humic acids without EDTA. There were 2 significantly different groups of algal growth responses. The higher growth response was obtained when EDTA was included in the culture medium with the higher humic acid concentrations supporting better growth than EDTA alone (Figure 10).

After 7 days the relationships between the growth responses to the various experimental treatments were essentially the same as they were on day 5 (Figure 10) and the growth rates were further decreased (Figure 11 and Appendix V, Table 28). The greatest growth response was still in the medium containing EDTA and 25.0 mg/l humic acids with the standing crops increasing with increasing concentrations of humic acids in the media. There was a higher response to

each level of humic acid when EDTA was present. Once again there were 2 statistically different groups of growth responses. As in the day 5 sample, the higher growth responses occurred in the media where EDTA was present, although when 25.0 mg/l humic acid was present in the medium in the absence of EDTA the growth response was almost as great as when both EDTA and 25.0 mg/l humic acid were present.

The standing crops for the various media displayed the same trend on day 9 and 12 as they did on day 7 (Figure 10, 11, Table 6 and Appendix V, Table 28).

The cultures were entering the stationary growth phase on day 12. By day 16 some cultures were in the stationary growth phase while others had entered the death phase (Figure 11). The more actively growing cultures began to decline first so that the response surface for the growth responses on day 16 showed a smaller standing crop for the media containing 25.0 mg/l humic acids with and without EDTA. Otherwise the response surface is proportionately the same as for earlier samples. There were two significantly different groups of responses. Because some of the cultures had entered the death phase of the growth curve there was a tendency for the differences between the growth responses to decrease, but the least growth still occurred in the media which contained neither EDTA nor humic acids (Table 6). The growth responses of S. obliquus to humic acids and EDTA were

very similar. Alone EDTA and humic acids caused almost identical growth responses and in combination there was a slightly higher response than when each was present singly. EDTA has been found to hold Fe in solution and make it available to algae in fresh water (Schelske et al., 1962). EDTA has also been found to prevent the precipitation of Fe from sea water and keep the Fe available to marine phytoplankton (Lewin, 1971). It has also been found that EDTA was able to dissolve $Fe(OH)_2.2H_2O$ when added to culture media (Davies, 1970).

EDTA has a stimulatory effect on algal growth by chelating Fe and other cationic nutrients and keeping them from precipitating. The stability constants of EDTA are such that they can make these metal ions available for algal growth. Since the growth responses of S. obliquus to EDTA and humic acids were very similar, it seems that humic acids may be acting in a similar manner to EDTA in stimulating algal growth. In the absence of a chelating agent, the reduced growth may also be due to the fact that the algal growth rate was the same as the rate of Fe release from $Fe(OH)_3$ and $FePO_4.2H_2O$.

The fact that humic acids seemed to enhance the algal growth even in the presence of EDTA suggests that the humic acids may have been acting to stimulate S. obliquus growth by some mechanism other than chelation alone. The addition

of humic acids at the highest level and EDTA both resulted in no over-chelation. Humic acids seemed to be acting in two ways: (1) Chelation such as that of EDTA and (2) some other stimulatory mechanism (see section of heterotrophic growth).

Effect of Humic Acids on the S. obliquus Growth Response to Fe and PO_4

Because humic acids may be stimulatory to algal growth by making Fe available to Fe-starved S. obliquus, the effects of humic acids on algal growth at 2 levels of Fe were studied. The earlier experiments indicated that Fe and PO_4 can have reciprocal effects on their availability to algal cells so the effects of humic acids on the Fe-PO_4 interaction were also investigated.

The 2^3 factorial experiments were performed to determine the effects of humic acids on the growth response of S. obliquus to Fe and PO_4. One experiment was done using Fe-starved stock algae, while the other was done using non-starved stock algae (Appendix I). The experimental levels of Fe were 0.5 and 1.2 mg/l, while the 2 levels of PO_4 studied were 0.5 and 0.9 mg/l. Humic acids were either absent or present at a concentration of 15.0 mg.l. The cultures inoculated with Fe-starved algae had an initial cell density of $3X10^3$ cells/ml and when the non-starved stock was used the initial cell density was $6X10^3$ cells/ml.

When non-starved stock algae was used, 1.2 mg/l Fe caused an increase in growth over 0.5 mg/l (Figure 12), which was significant at the $P \geq 0.95$ level (Table 7). After 2 days, the response to Fe was greater in the presence of PO_4 than to Fe alone (Figure 12).

The sample taken after 3 days showed the standing crop to have increased in all of the cultures (Figure 13), but at the low level of PO_4 application there was little difference between the 2 levels of Fe (Figure 12). At the higher level of PO_4 the response to the higher level of Fe was about the same as it was at the lower PO_4 level, but the standing crop of S. obliquus was lower in the low Fe medium when PO_4 was present at 0.9 mg/l.

After 4 days, the standing crop was the same for the high Fe medium at the low level of PO_4 application. The structure of the response surface was beginning to break down because the relative growth rates of the cultures were decreasing as the cultures moved into the stationary growth phase (Figures 12, 13 and Appendix V, Table 29).

By the fifth day of the experiment, the cultures were either in the stationary phase or the death phase. The initially more rapidly growing cultures were declining while the other cultures were in the stationary phase. This caused the standing crops of the various cultures to be more similar, with no difference between the 2 levels of Fe at an

Figure 12.--Response surfaces for the growth responses of non-starved S. obliquus to Fe and humic acids. The standing crop is reported as cells/ml after 2, 3, 4, 5 and 7 days of growth for each of the 2 PO_4 levels studied. Note the scale of standing crop changes from cells/ml $X10^5$ to cells/ml $X10^6$ on day 4.

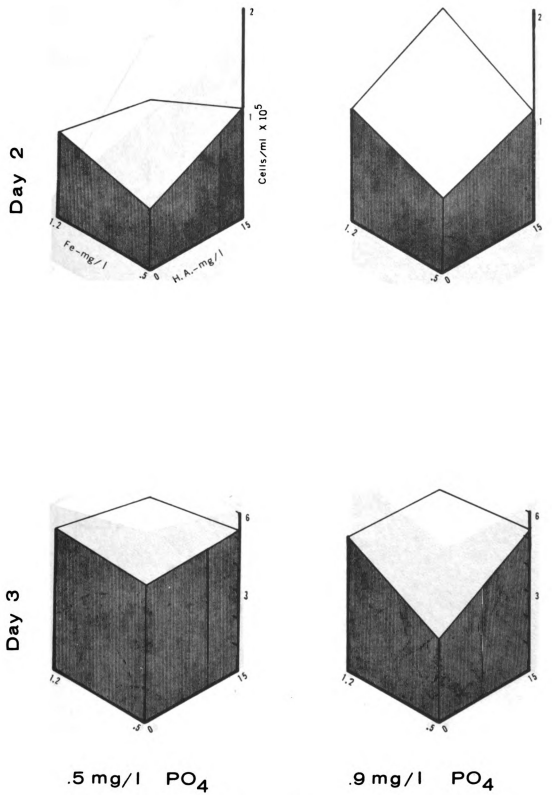

Day 2
Day 3
Cells/ml x 10^5
Fe-mg/l
H.A.-mg/l
1.2
.5
0
1.5
2
1
6
3
.5 mg/l PO_4
.9 mg/l PO_4

Figure 12

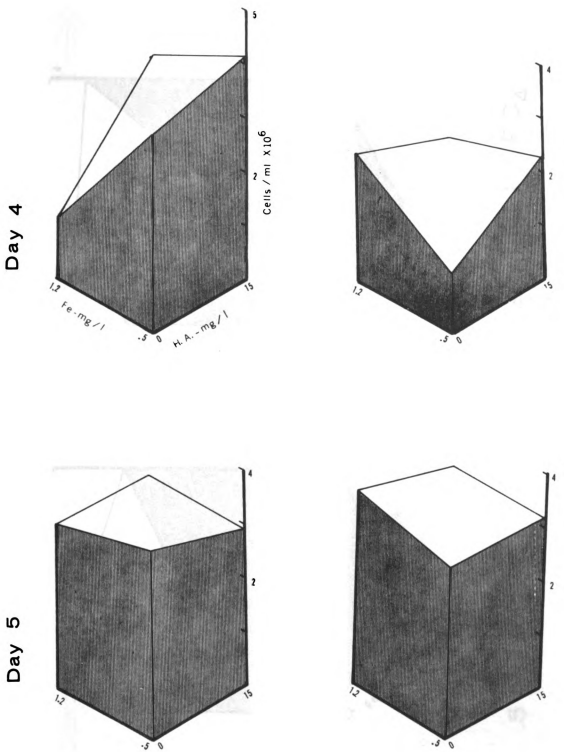


Figure 12 (cont'd)

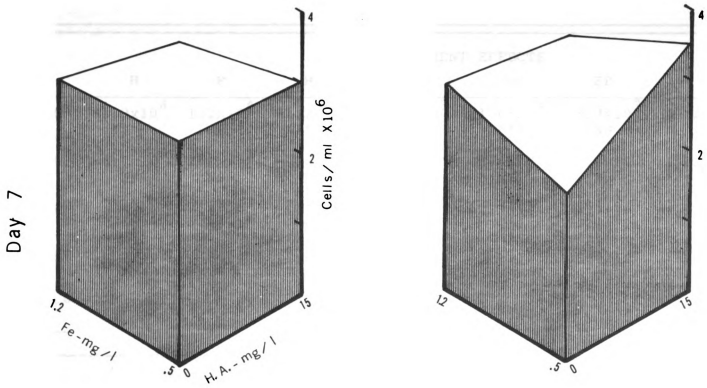


Figure 12 (cont'd)

Table 7.--The statistical analysis of the effects of humic acids and Fe on the growth of non-starved S. obliquus at 2 PO_4 levels. The effect means and significance levels are reported for each main effect and interaction at each sampling.

		TREATMENT EFFECTS						
DAY	(1)	H	F	HF	P	HP	FP	HFP
2	4.0×10^4	-1.9×10^4 ****	1.2×10^4 **	-1.2×10^4 **	-6.7×10^3 NS	1.6×10^4 ***	3.7×10^3 NS	-1.2×10^4 **
3	3.4×10^5	-4.7×10^5 NS	2.0×10^5 NS	-9.6×10^4 NS	-1.4×10^5 NS	2.4×10^4 NS	8.0×10^4 NS	1.3×10^5 NS
4	2.2×10^6	4.4×10^5 **	6.4×10^5 *****	-1.7×10^6 NS	-3.0×10^5 NS	-2.2×10^5 NS	4.3×10^5 NS	6.5×10^4 NS
5	2.9×10^6	7.3×10^5 ***	6.4×10^5 NS	1.0×10^6 NS	-4.2×10^5 NS	-3.1×10^5 NS	5.7×10^5 NS	1.7×10^5 NS
7	1.5×10^6	3.0×10^5 *	-4.8×10^4 NS	5.8×10^3 NS	-3.0×10^4 NS	-8.6×10^4 NS	8.4×10^4 NS	5.4×10^4 NS

2^3 factorial design with 3 replications

***** $0.999 \leq P$
**** $0.995 \leq P$
*** $0.990 \leq P$
** $0.950 \leq P$
* $0.900 \leq P$
NS $P \leq 0.900$

	low	high	
	0.0 mg/l H.A.	15.0 mg/l H.A.	(H)
	0.5 mg/l Fe	1.2 mg/l Fe	(F)
	0.5 mg/l PO_4	0.9 mg/l PO_4	(P)

Figure 13.--Growth curves for the growth responses of non-starved S. obliquus to Fe and humic acids at 2 levels of PO_4 application reported as $\log_{10}$ cells/ml. Each point represents the mean of 3 replications with 95% confidence intervals for each mean indicated.

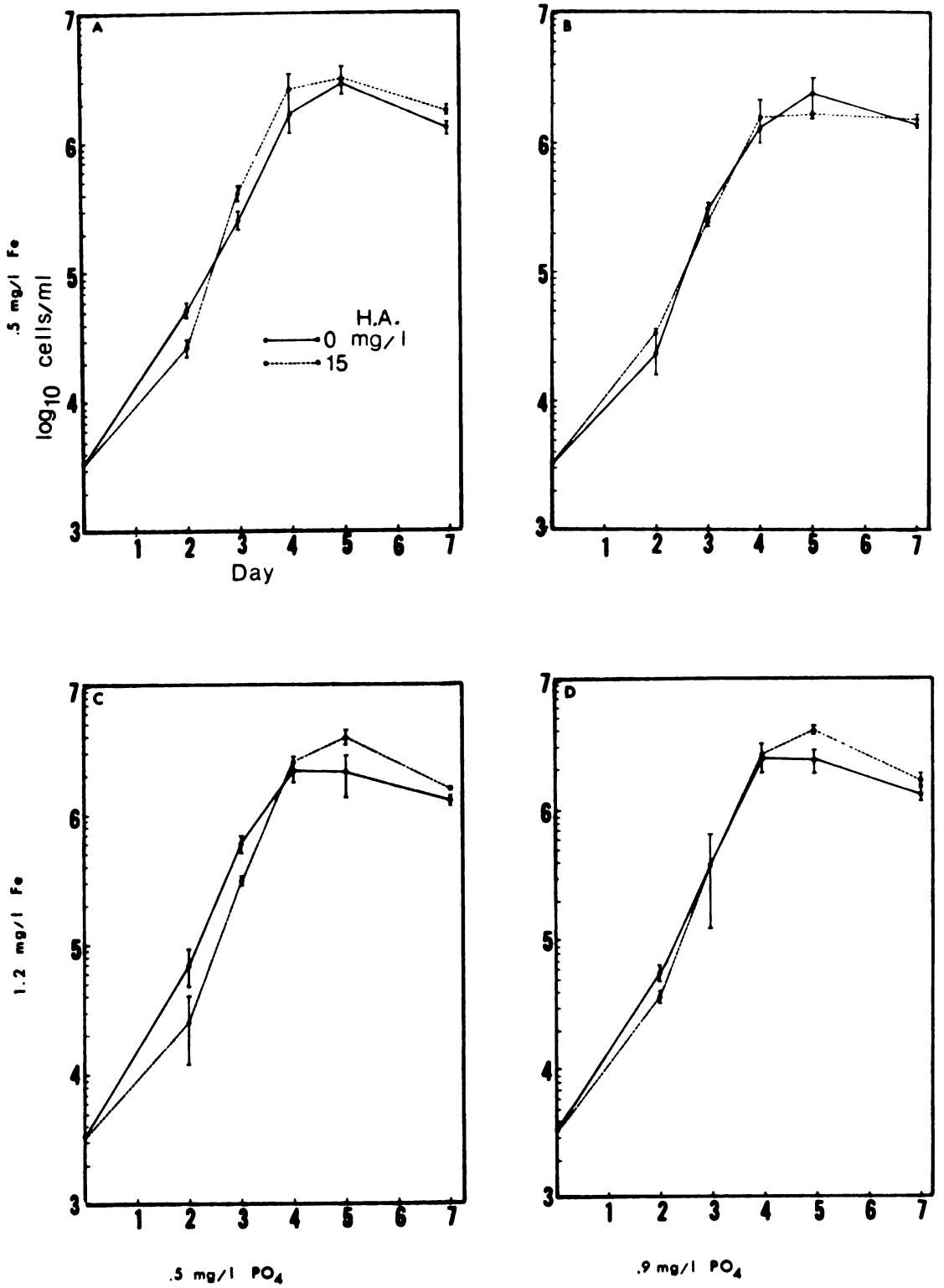

A
B
C
D
.5 mg/l Fe
1.2 mg/l Fe
log10 cells/ml
H.A.
0 mg/l
15
Day
.5 mg/l PO4
.9 mg/l PO4

Figure 13

application of 0.5 mg/l PO_4. The standing crop was greater in the higher Fe medium when 0.9 mg/l PO_4 was present.

Because of the large amount of experimental error, increasing the Fe concentration caused a significant stimulatory effect on the standing crop only on day 2 and 4 (Table 7). The main effect of PO_4 was always negative, but this main effect was not significant for any of the sampling. PO_4 did seem to have a positive effect on the growth response in some cases (Figure 13 and Appendix V, Table 29), but since the main effect was a measure of all PO_4 effects, the overall main effect was negative and not significant at each sampling.

After the first 2 days of growth, the main effect of the higher humic acid level (15.0 mg/l) was negative and highly significant (Table 7). The higher level of humic acids caused a decreased standing crop at both Fe levels when PO_4 was present at 0.5 mg/l and at the higher Fe concentration when PO_4 was present at 0.9 mg/l (Figure 13). The greatest standing crop after 2 days was in media containing humic acids in the presence of the higher Fe and PO_4 concentrations. When humic acids were present the growth response to the lower level of Fe was the same as the growth response to the higher Fe level in the absence of humic acids at both the higher and lower levels of PO_4 application (Figure 12).

After 3 days of growth, there were no significant main effects or interaction terms involving humic acids (Table 7). There were no differences in the responses to either level of Fe or humic acids at the lower PO_4 level, but at the higher level of PO_4 either humic acids or the higher Fe level caused an enhancement of growth. The presence of humic acids and 1.2 mg/l Fe did not cause a greater growth response than that observed for the higher level of Fe or humic acids alone.

Between day 2 and 3, the growth curves of the S. obliquus in humic acid containing media and non-humic acid media crossed (Figure 13). In the log growth phase, the growth rate of the algae in the media containing humic acids was greater (Appendix V, Table 29), allowing the algae in these media to surpass the standing crop of those in the media containing no humic acids. Although the humic acids caused an initially decreased growth rate in all the media, except where Fe was present at the lower concentration and PO_4 was present at the higher concentration, once the algae in these media surpassed the others the standing crop was always higher in the media containing humic acids (Figure 13). The sample taken on day 4 showed the greatest growth responses to be in the media containing humic acids. There were no differences between the responses to the levels of Fe at the high or low PO_4 level in the presence of humic acids. At the higher level of PO_4, the addition of humic

acids to the low Fe medium caused the same growth response as the higher level of Fe in the absence of humic acids.

There was a significant positive growth response to the higher level of humic acids as well as Fe (Table 7). The PO_4 main effect was negative but not significant and there were no significant interactions.

By day 5, the cultures had reached or passed their maximum standing crop (Figure 13). With some cultures in the death phase and some in the stationary phase the differences between the cultures were minimized and there was little difference between the media with and without humic acids present. At the lower PO_4 concentration, humic acids increased the growth response in the presence of 1.2 mg/l Fe but caused a decrease in the growth response when 0.5 mg/l Fe was present. At the higher PO_4 concentration there was a slight increase in the growth response to the lower level of Fe when humic acids were present, but humic acids had no effect on the growth response to 1.2 mg/l Fe.

The sample taken on day 7 of this experiment was well into the death or decline phase of all the cultures (Figure 13). The presence of humic acids in the culture media had no effect on the algal growth response at the lower PO_4 level, but at the higher PO_4 concentration the presence of humic acids caused an increase in the growth response. There was no significant main effect due to the presence of

high Fe or Fe-humic acid interactions. The humic acid main effect was significant, but none of the interactions involving humic acids were significant.

When Fe-starved stock S. obliquus was used to inoculate the experimental media a different set of responses was observed than those observed for non-starved stock. After 2 days of growth, the greatest algal growth response was to the medium with the higher level of Fe and no humic acids at both the higher and lower PO_4 levels. The greatest response to this combination occurred at the lower PO_4 level (Figure 14). At the lower PO_4 level the lowest growth response was to 15.0 mg/l humic acids and 0.5 mg/l Fe. There was little difference between the response to low Fe in the absence of humic acids and that to the higher Fe level in the presence of humic acids. The presence of humic acids in the culture medium caused a growth inhibition and decreased the difference between the higher and lower levels of Fe. When PO_4 was present at 0.9 mg/l the smallest growth response was observed in the medium where Fe was present at a concentration of 0.5 mg/l and humic acids were absent, while the greatest growth occurred in the medium containing no humic acids and the higher Fe level. Humic acids had a slight stimulatory effect at the lower Fe level, but decreased the growth response to the higher level of Fe. No significant main effects or interactions could be shown (Table 8).

Figure 14.--Response surfaces for the growth response of Fe-starved S. obliquus to Fe and humic acids at 2 levels of PO_4. Standing crops are reported as cells/ml after 2, 3, 4, 5 and 7 days of growth. Note the scale changes from cells/ml X 10^4 to cells/ml X10^5 on day 3.

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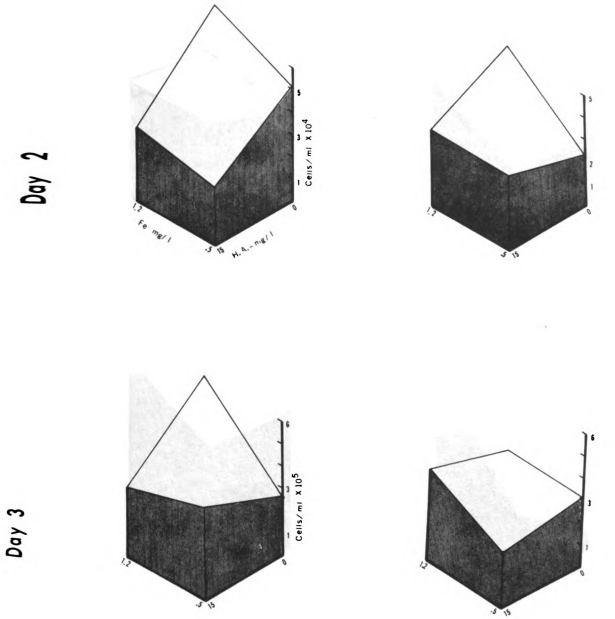


Figure 14

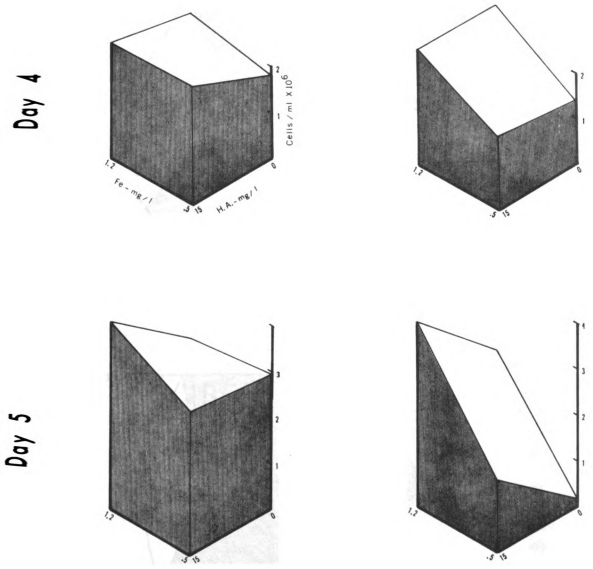


Figure 14 (cont'd)

Figure 14 (cont'd)

Table 8.--Statistical analysis of the effects of humic acids and Fe on the growth of *Fe*-starved *S. obliquus* at 2 PO_4 levels. The effect means and significance levels are reported for each main effect and interaction at each sampling.

DAY		TREATMENT EFFECTS (1)	H	F	HF	P	HP	FP	HFP
2	EFFECT MEAN	1.0×10^5	4.0×10^4	3.3×10^4	3.3×10^3	1.7×10^4	-3.0×10^3	1.3×10^4	1.8×10^3
	SIG. LEVEL	———	NS	NS	NS	NS	NS	NS	NS
3	EFFECT MEAN	4.8×10^5	3.5×10^4	2.8×10^3	-8.8×10^4	-4.3×10^4	7.0×10^4	6.6×10^4	-3.7×10^4
	SIG. LEVEL	———	NS	NS	NS	NS	NS	NS	NS
4	EFFECT MEAN	2.4×10^6	7.8×10^5	$+5.8 \times 10^5$	$+1.9 \times 10^5$	-1.1×10^6	$+6.3 \times 10^5$	9.3×10^5	-7.2×10^5
	SIG. LEVEL	———	*****	*****	**	*****	*****	*****	*****
5	EFFECT MEAN	3.2×10^6	-4.1×10^5	-7.6×10^4	1.1×10^5	2.2×10^5	4.3×10^4	8.4×10^4	1.8×10^5
	SIG. LEVEL	———	NS	NS	NS	NS	NS	NS	NS
7	EFFECT MEAN	3.3×10^6	-2.7×10^5	-9.7×10^5	-6.9×10^5	1.2×10^5	1.2×10^6	-1.6×10^5	-1.0×10^6
	SIG. LEVEL	———	***	NS	NS	NS	NS	NS	NS

2^3 factorial design with 3 replications. ***** $0.999 \leq P$; **** $0.995 \leq P$; *** $0.990 \leq P$; ** $0.950 \leq P$; * $0.900 \leq P$; NS $P < 0.90$.

After 3 days of growth, the greatest growth for the low PO_4 media occurred when humic acids were absent from the medium and Fe was present at 1.2 mg/l. There was very little difference between the other 3 treatments, although the humic acids did seem to enhance growth when present in the lower Fe medium. When the PO_4 concentration was 0.9 mg/l the greatest growth response was to the higher Fe concentration and humic acids in conjunction, but the differences between the various responses were not very great and could not be shown to be significant (Table 8)

After 4 days of growth, all of the main effects and interaction effects were highly significant. By day 4, the maximum standing crop in the low PO_4 media was found in the medium containing 1.2 mg/l Fe. There was no difference between the response to Fe in the humic acid and non-humic acid containing media. The response to the lower Fe level was nearly as great as the higher Fe level in the presence of humic acids, but in their absence this level of Fe supported the smallest standing crop observed at this sampling.

When PO_4 was present at the higher concentration, the greatest growth response was to the higher Fe level. This was true of both the medium where humic acids were present and the medium where they were absent. There was no significant effect of humic acids at the higher or lower Fe level.

By the fifth day of growth, all of the cultures were in either the stationary phase or death phase of growth (Figure 15). The standing crops were proportionately the same as they were after 4 days when 0.5 mg/l PO_4 was present in the culture media, but there was a difference in the relative standing crops for the media containing 0.9 mg/l PO_4. At the higher level of PO_4 the greatest standing crop occurred in the high Fe medium in the presence of 15.0 mg/l humic acids with the response to high Fe in the absence of humic acids being slightly less. The media containing only 0.5 mg/l Fe exhibited a much lower standing crop, with humic acids slightly enhancing the growth at this level of Fe application.

After 7 days of growth, the most rapidly growing cultures had entered the decline phase while the less rapidly growing cultures had reached their stationary phase (Figure 15 and Appendix V, Table 30). The presence of humic acids had a significantly negative effect on the standing crop (Table 8 and Figure 15).

The media containing humic acids initially supported a greater growth rate than the non-humic acid media (Figure 15). By the seventh day of the experiment, the standing crop was lower in all of the media containing humic acids except the high PO_4-low Fe medium.

Figure 15.--Growth curves for the growth responses of Fe-starved S. obliquus to Fe and humic acids at 2 levels of PO_4. Each point represents the mean of 3 replications reported as $\log_{10}$ cells/ml with 95% confidence intervals for each mean indicated.

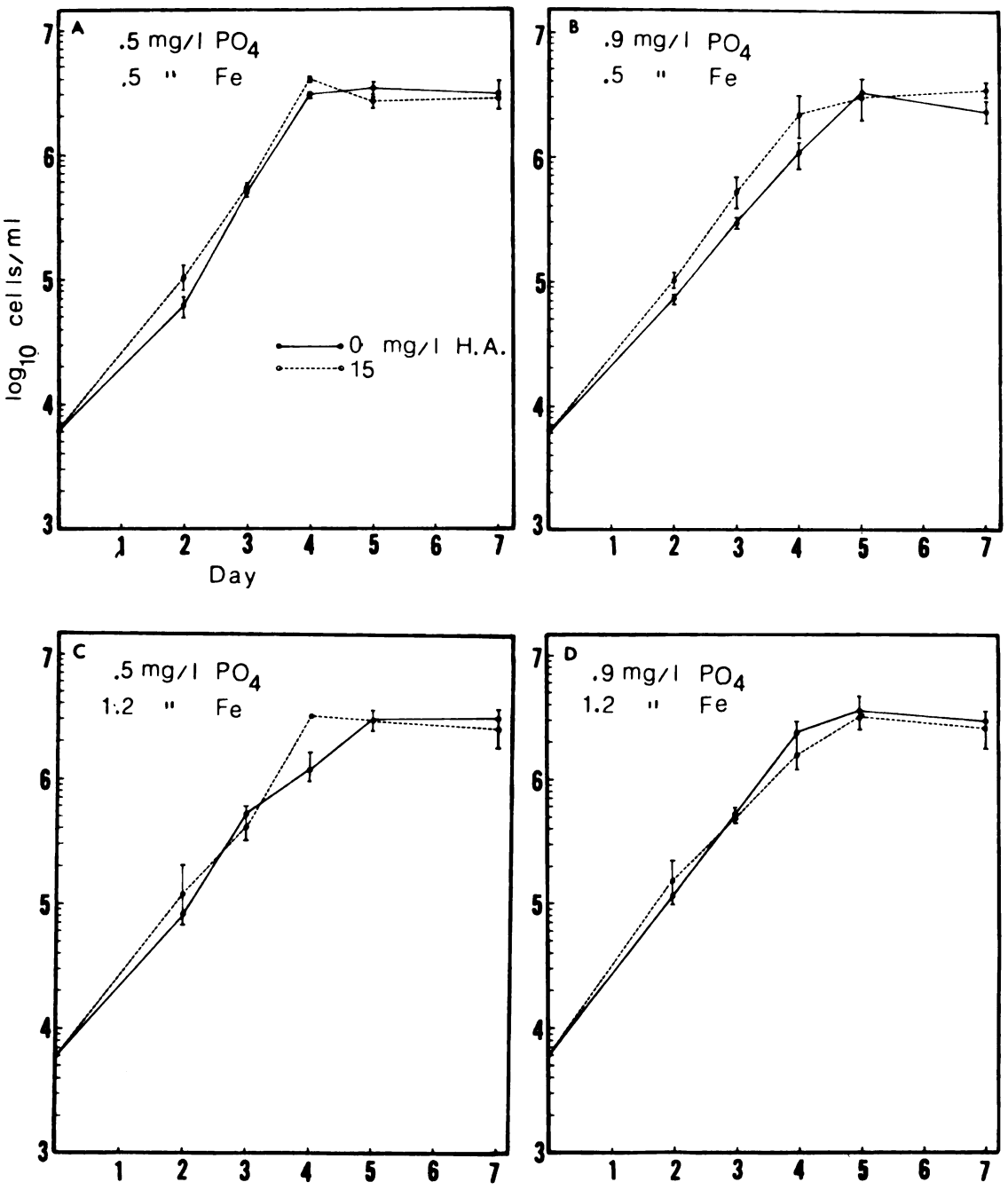

A
.5 mg/l PO4
.5 " Fe
0 mg/l H.A.
15
B
.9 mg/l PO4
.5 " Fe
C
.5 mg/l PO4
1.2 " Fe
D
.9 mg/l PO4
1.2 " Fe
log10 cells/ml
Day

Figure 15

The growth responses of the Fe-starved stock S. obliquus cells were much more variable than those of the non-starved stock. This made it very difficult to show any significant differences between the treatment main effects.

Fe and PO_4 have an antagonistic effect on the algal growth responses caused by each other. Similar responses were observed when Fe-starved and non-starved stock algal cells were used, although the responses of the Fe-starved stock were much more variable. The cultures grown at the lower PO_4 level responded to increased levels of Fe. This may be explained by the mass action precipitation of the available Fe by the presence of a high concentration of PO_4. The response to additional Fe was about the same as that to the addition of 15.0 mg/l humic acids in the presence of the low Fe concentration which seemed to indicate that the humic acids were somehow able to keep the PO_4 from precipitating the Fe, making the Fe available for algal growth. The initially decreased growth response in the presence of humic acids indicates that humic acids may initially decrease the availability of Fe but later make it more available.

The effect of the addition of Fe or humic acids to the culture media was not as great at the low level of PO_4 application when non-starved stock S. obliquus was used because this level of PO_4 was near the limiting level for

growth determined for S. obliquus grown under these cultural conditions. Thus the lack of differences between the various responses may have been due only to PO_4 limitation. When Fe-starved stock algal cells were used to inoculate the experimental media the responses to Fe were less at the higher PO_4 concentration. This was probably due to precipitation of Fe as $FePO_4.2H_2O$.

Humic acids did not decrease the negative effects of PO_4 on the availability of Fe. Growth responses were even decreased in the presence of humic acids and the higher PO_4 concentration in conjunction.

There were differences in the growth rates and standing crops for the early samples taken, but as the cultures aged the growth rates and standing crops became more alike. This was due in part to the decreasing growth of the more actively growing culture while the more slowly growing cultures continued to grow. The fact that the more slowly growing cultures continued to grow indicated that the algal cells were able to eventually get the Fe and PO_4 necessary to attain the same final standing crop as the cells grown in higher concentrations of these nutrients.

In a closed and confined system, such as a culture flask, the precipitates formed are kept in contact with the algal cells. Because of this the cells are able to obtain the needed nutrients by active solubilization of the precipitates

or by the uptake of the nutrients as they are solubilized by the dynamic equilibrium of the chemical species. In a natural system the precipitates formed are often sedimented out of the euphotic zone where algal cells are actively growing. This effectively removes the nutrients and makes them unavailable to algal cells for growth. If chelating agents are able to bind Fe and keep it from precipitating and thus sedimenting out of the euphotic zone, they could play a role in supplying the needed Fe to algal cells. Shapiro (1957) found this to be the case in Linsley Pond.

Humic acids as well as the numerous other large organic fractions that occur in natural waters are also subject to sedimentation (Otsuki and Wetzel, 1972). At low Fe levels the addition of humic acids caused a reduction in the initial growth response of Fe-starved S. obliquus, indicating that the humic acids were making Fe less available, but the humic acids caused an enhanced growth response in the presence of a higher Fe concentration. This effect may be due to a simple chelation and release phenomenon or may be more complex. The humic acids may be functioning in two ways; by chelating Fe and making it more or less available to algae or directly stimulating the algal cells by some other mechanism.

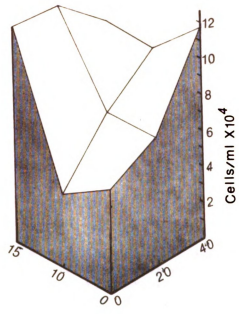
Effects of Ca on the S. obliquus Growth Response to Humic Acids

A 3X3 factorial experiment with 2 replications was used to study the effects of 3 levels of Ca on the algal growth responses to 3 levels of humic acids. The Ca concentrations studied were 0.0, 20.0 and 40.0 mg/l and the experimental humic acid concentrations were 0.0, 10,0, and 15.0 mg/l in AAP base medium without EDTA. Each culture flask was inoculated with enough Fe-starved S. obliquus to make an initial cell concentration of $7X10^3$ cells/ml.

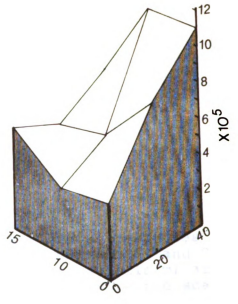
After 2 days there was an increased standing crop in the presence of 15.0 mg/l humic acids as well as in the presence of 40.0 mg/l Ca (Figure 16), but an analysis of variance showed no significant differences between any of the experimental treatments (Table 9).

By the sample taken on the third day there were some significant differences between the treatments (Figure 17 and Table 9). The greatest growth response was to 40.0 mg/l Ca with the same standing crop occurring at all 3 levels of humic acid at this level of Ca. In the absence of humic acids there was a growth response to 20.0 mg/l Ca that was intermediate between that to 0.0 and 40.0 mg/l Ca. The mean standing crop for the medium containing 15.0 mg/l humic acids in the absence of Ca was slightly greater than that for the media with lower concentrations of humic acids.

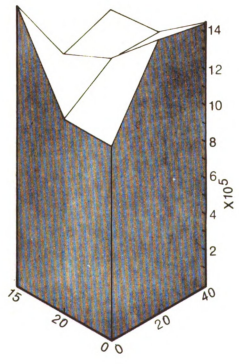
Figure 16.--Response surfaces for the growth responses of Fe-starved S. obliquus to humic acids and Ca with standing crops reported as cells/ml after 2, 3, 4 and 5 days of growth. Note the scale changes from cells/ml X 10^4 to cells/ml X10^5 on day 3 and to cells/ml X10^6 on day 5.



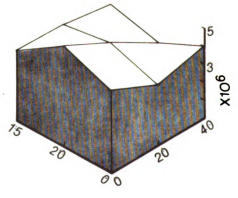
day 2



day 3



day 4



day 5

Figure 16

Table 9.--Statistical analysis of the algal growth responses of Fe-starved S. obliquus to Ca and humic acids. The F-value and level of significance for each main effect and interaction are listed for each sampling. The treatment combinations are listed from left to right by ascending standing crops. The means that were not found to be significantly different, using a Student-Newman-Keuls multiple range test ($P \leq 0.95$), are grouped by underlining.

DAY	EFFECT	F							
2	H	0.75							
	C	1.21							
	HC	2.60							
	EFFECT	F							
3	H	12.67***							
	C	39.97***							
	HC	9.97***							
						TREATMENT			
						mg/l Ca			
	0	20	0	20	0	40	20	40	40
						mg/l H.A.			
	10	15	0	10	15	15	0	10	0
	+								
	EFFECT	F							
4	H	1.23							
	C	0.39							
	HC	3.68**							
						mg/l Ca			
	0	0	20	40	40	20	40	20	0
						mg/l H.A.			
	0	10	15	15	10	10	0	0	15
	EFFECT	F							
5	H	1.03							
	C	6.31**							
	HC	2.59							
						mg/l Ca			
	40	20	40	20	0	40	20	0	0
						mg/l H.A.			
	10	0	15	15	15	0	10	0	10

3X3 factorial with 2 replications. *** $0.99 \leq P$; ** $0.95 \leq P$; * $0.90 \leq P$; + $0.95 \leq P$ Student-Newman-Keuls test; H humic acid; C Ca.

Figure 17.--Growth response curves of Fe-starved S. obliquus to humic acids and Ca. Each point represents the mean of 2 replications reported as $\log_{10}$ cells/ml with 95% confidence intervals indicated for each mean.

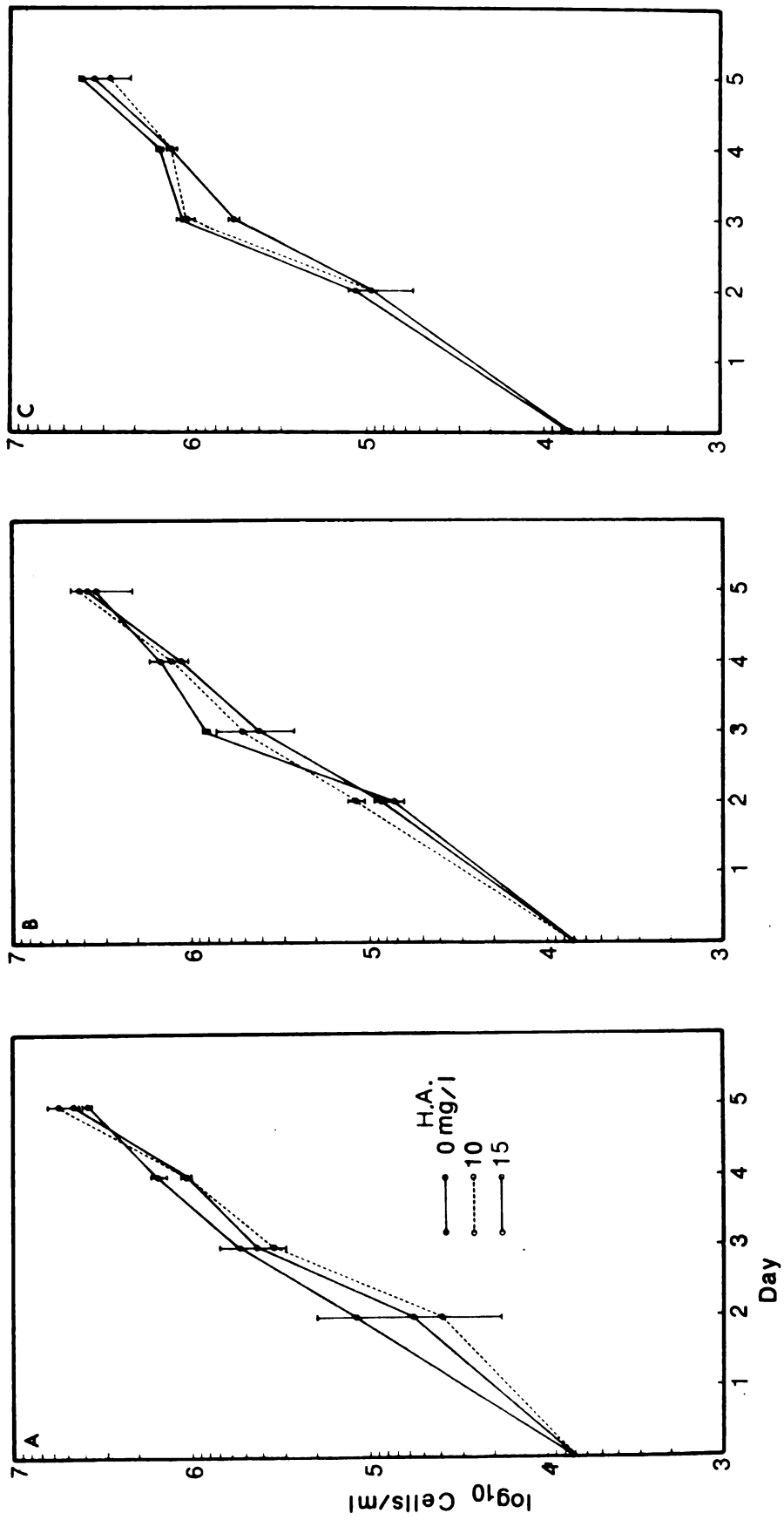


Figure 17

There were no statistically significant differences among most of the treatments due to the large amount of variability in some of the samples (Figure 17). There was a significantly lower standing crop in the medium containing no Ca in the presence of 10.0 mg/l humic acids and in the medium containing 20.0 mg/l Ca in the presence of 15.0 mg/l humic acids than all of the other treatment combinations.

A Student-Newman-Keuls multiple range test showed no significant differences among the growth responses to the various treatments after 4 days of growth, but there was a significant interaction between the humic acid and Ca effects (Table 9), although there seemed to be a growth enhancement by either Ca or humic acid addition (Figure 16).

After 5 days, the differences in standing crops on the response surfaces (Figure 16) were not as pronounced, but the standard deviations of the means were smaller (Figure 17) so that some significant differences were shown by the Student-Newman-Keuls multiple range test. The highest standing crops were in the media containing the lowest Ca concentrations, while the higher concentrations of Ca supported lower growth (Table 9).

Wilson and Collier (1955) reported that humic acid-bound Fe carried into the marine environment was released thus making it available to the phytoplankton. The effects of Ca on the growth of S. obliquus were not as expected.

The presence of Ca was expected to exchange humic-bound Fe thus making the Fe available for algal growth.

The Ca did have a stimulatory effect that was manifested in the absence of humic acids. In fact, the presence of humic acids in the media decreased the effect of Ca on algal growth. This was probably due to the fact that the humic acids were chelating the Ca and making it unavailable. Ca did not seem to be able to release Fe, but did seem to be chelated or otherwise have its stimulatory effect negated by humic acids.

It was thought that the stimulatory effect of Ca may have been due to the increased buffering capacity of the media in the presence of the Ca, but the pH values in the media containing Ca were not any lower than those where it was present (Appendix IV, Table 20).

The effect was a direct stimulation of the algal cells which was effective only for the first few days of culture. By the fifth day of growth, there was no difference between the various Ca concentrations, indicating that the stimulatory effect of the Ca was probably not due to Ca limitation in the culture media. This direct effect of Ca on S. obliquus growth was not studied here.

UPTAKE EXPERIMENTS

$^{32}PO_4$ Uptake in the Presence of Humic Acids

Bremner (1954) reported that humic acids were able to sequester the phosphorus that is available to plants while Manojlovic (1965) found that humic acids were able to sequester H_3PO_4 and reduce adsorption onto soil particles from super phosphate solutions. This ability of humic acids to sequester PO_4 may cause a reduction in the availability of PO_4 to algal cells. If humic acids are present in aquatic systems as colloids they may hold PO_4 by surface binding phenomena. Since it has already been shown that Fe and PO_4 can affect the availability of each other, it is important to understand the effects of humic acids on both PO_4 and Fe availability. Hutchinson (1957) reports an unknown substance which he calls the phosphorus sparing factor which allows algae to grow with a lower PO_4 level. This was discovered when cultures made with lake water and distilled water were compared. Since it has already been shown that soil extracts added to culture media stimulate growth it was thought that the humic acids may be acting as a phosphorus sparing factor. This effect was thought to be due to humic acids sequestering PO_4, thus preventing its precipitation,

but still keeping it available for uptake by algal cells.

An experiment was designed to study the effects of humic acids on the uptake of PO_4 by S. obliquus from AAP culture medium. The effects of various concentrations of humic acids (molecular weight 30,000 or greater) on the uptake of PO_4 from 200 ml of non-EDTA AAP base medium were studied radiologically, using carrier free PO_4 in 300 ml Erlenmeyer flasks.

The stable PO_4 concentration of the AAP culture medium was 0.186 mg/l P (added as K_2HPO_4) and the $^{32}PO_4$ concentration was 4.28×10^{-10} mg/l P (850 CPM/ml). After the tracer was added to the medium the system was allowed to equilibrate for 4 h. The loss of $^{32}PO_4$ due to adsorption to the flask was less than 1%.

After the equilibration period enough PO_4-starved stock S. obliquus (Appendix I) was added to make a final cell density of 2×10^5 cells/ml in the experimental media.

Twenty ml samples of the algal suspension were removed at known time intervals from 0.25 to 4.0 h with a volumetric pipette and filtered through a 5 μ cellulose-ester membrane filter at a vacuum of 5 psi or less to separate the algal cells from the culture medium. Microscopic examination indicated that filtration at this vacuum level did not rupture the S. obliquus cells. The filters and associated algal cells were washed with a 50 ml portion of 0.01 N HCl

followed by a 50 ml portion of distilled water to remove $^{32}PO_4$ from the outside of the cells and from the membrane filter. Tests indicated that this procedure removed between 98.5% and 99.5% of the activity adsorbed on the cellulose-ester filters and did not rupture the algal cells.

The filters were air dried and the activity counted, using a gas flow geiger-muller detector. The detectable background radiation was less than 2 counts per minute (CPM) and the activities high enough that a 10.0 min count gave at least 90% confidence to the counting statistics. All samples were counted for 10.0 min or 10,000 total counts, whichever came first. All samples were corrected for background and also for decay to the beginning of the experiment if there was a time lapse of more than 2.0 h between countings. In samples where only relative differences were meaningful all comparisons of uptake were made in CPM. If there were differences of specific activities between treatments the comparisons were made in mg/l. The uptake of $^{32}PO_4$ by PO_4-starved S. obliquus cells was determined in the presence of 0.0, 10.0, 15.0, 20.0 and 25.0 mg/l humic acids.

The presence of 20.0 mg/l humic acids reduced the rate of $^{32}PO_4$ uptake and the cells in this medium had the lowest final cellular $^{32}PO_4$ level (Figure 18). When 25.0 mg/l humic acids were present, the final cellular $^{32}PO_4$ concentration was slightly reduced but the initial uptake rate

Figure 18.--Uptake curves for the uptake of $^{32}PO_4$ from AAP media containing 5 levels of humic acids by PO_4-starved S. obliquus. The specific activities are the same so all of the uptakes are reported as CPM.

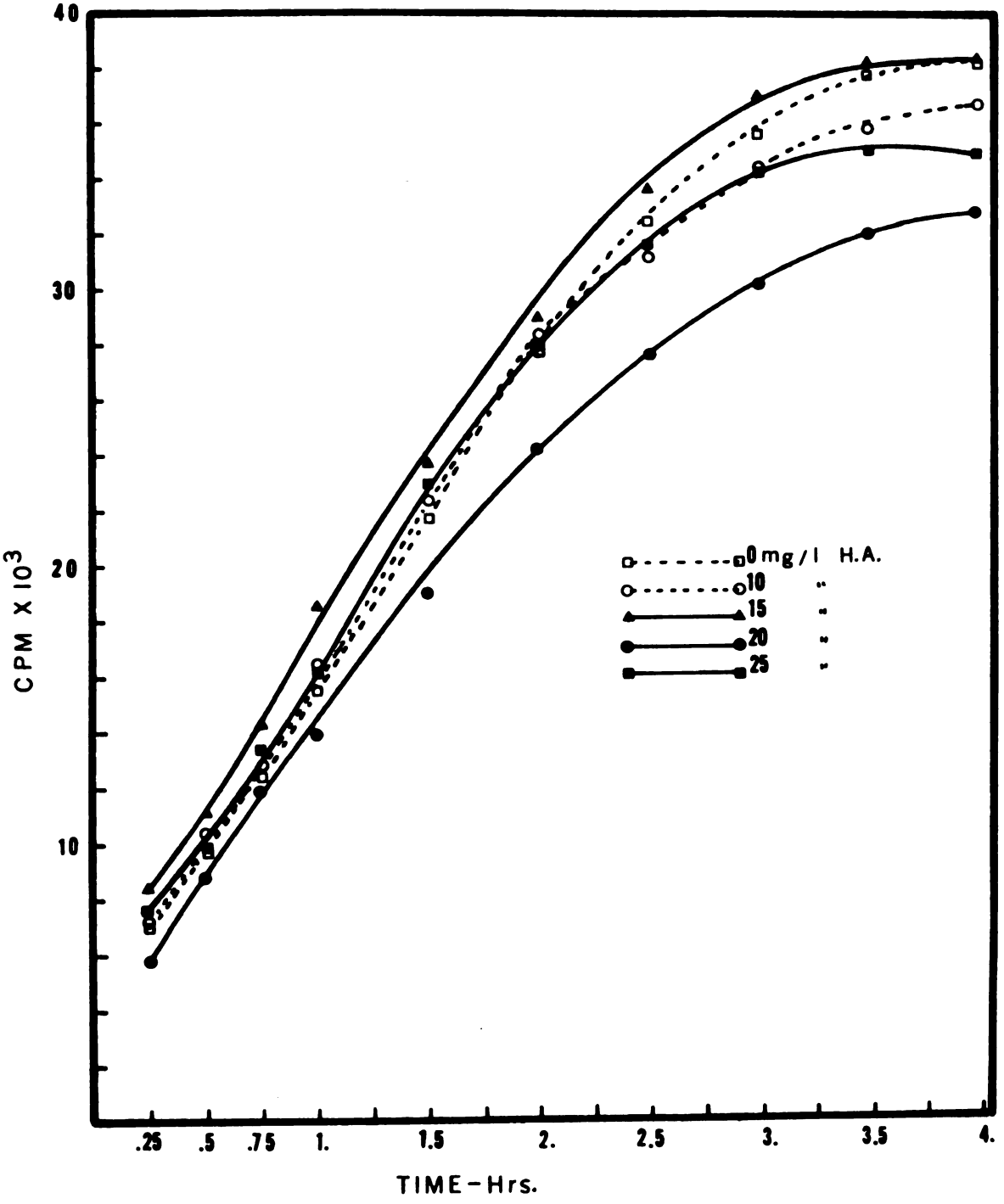

40
30
20
10
CPM X 10^3
0 mg/l H.A.
10 "
15 "
20 "
25 "
.25
.5
.75
1.
1.5
2.
2.5
3.
3.5
4.
TIME-Hrs.

Figure 18

was the same as when humic acids were absent or present at 10.0 mg/l. The highest final cellular $^{32}PO_4$ level was achieved in the medium containing no humic acids while the next highest final cellular level was in the medium containing 10.0 mg/l humic acids. This shows a trend of increasing $^{32}PO_4$ uptake with decreased humic acid concentration with the exception of the medium containing 15.0 mg/l humic acids which supported a final cellular $^{32}PO_4$ concentration as high as that of the algae in the medium containing no humic acids.

This $^{32}PO_4$ uptake enhancement may be either an artifact or a true humic acid effect. If this is a true effect, it indicates that there is an optimum humic acid concentration. Concentrations greater or less than 15.0 mg/l cause a decrease in the $^{32}PO_4$ uptake.

The presence of humic acids in the nutrient medium had no effect on the amount of $^{32}PO_4$ which adsorbed to the cellulose-ester filters and also had no effect on the amount of $^{32}PO_4$ activity lost to the glass culture flasks by adsorption.

The uptake results and the fact that the humic acids did not affect the adsorption of PO_4 suggests that the sequestering of PO_4 by purified humic acids of molecular weight 30,000 or greater is slight and of little importance in directly determining the availability of PO_4 to algae.

^{59}Fe Uptake in the Presence of Humic Acids

The mechanism of Fe uptake in algae is not well-known, but one possibility that has been suggested is that the cellular metabolic Fe is simply in equilibrium with free Fe released from Fe precipitates such as $Fe(OH)_3$ which is adherant to the cell membrane (see section on membrane effects). The uptake of Fe by higher plants may involve the movement of Fe across the cell membrane by sideramines (Prelog, 1964). The same mechanism is probably not operating in microorganisms, but there may be a similar mechanism. As early as 1953, Waris suggested that some algae may have chelating agents on their surfaces to make insoluble Fe compounds available for uptake.

Lewin (1971) found that some species of marine phytoplankton were unable to utilize unchelated Fe from sea water.

If humic acids are chelating Fe or if Fe is surface-bound to humic acids it is important to know if Fe held by the humic acids is available to algal cells. If humic acids can bind Fe and keep it in solution or suspended in the epilimnion of lakes but bind the Fe in such a way that it is unavailable to phytoplankton, the theory that humic acids stimulate algal growth by supplying Fe is not valid.

The effect of humic acids on the uptake of Fe from AAP media was studied using ^{59}Fe as a tracer. Fe uptake from

culture media made with an AAP base medium was studied in the presence of 0.0, 5.0, 10.0 and 25.0 mg/l humic acids. In studying the effects of humic acids on Fe uptake the EDTA called for by the AAP medium was omitted. The experimental media contained $3X10^{-2}$ mg/l stable Fe and $5.56X10^{-10}$ mg/l ^{59}Fe (123 CPM/ml) added as carrier-free $FeCl_3$. As in the PO_4 uptake study the media were allowed to react for 4 h after the tracer was added. After 4 h, less than 1.0% of the Fe was lost by adsorption to the glass culture flasks. This was true whether humic acids were present or absent.

The cell density in the experimental flask was made up to $5X10^5$ cells/ml with Fe-starved stock S. obliquus and 20 ml samples of the algal cell suspension were taken over time as in the PO_4 uptake study.

Another Fe uptake study was conducted to better define the Fe uptake during the first 30 min of the uptake and further clarify the length of time needed for the algal cells to reach saturation. In this experiment the Fe concentration was $3X10^{-2}$ mg/l and the ^{59}Fe concentration was $5.75X10^{-10}$ mg/l (250 CPM/ml) and the media were inoculated with Fe-starved stock S. obliquus to make a cell density of $1X10^6$ cells/ml.

The samples were filtered and washed as in the PO_4 uptake study, but instead of drying the filters, each filter was placed in a 9 dram plastic counting vial and dissolved

in 10.0 ml of methanol, which kept the counting geometry of the samples constant. The ^{59}Fe gamma radiation was detected with a well type, NaI crystal scintillation detector and corrected for background, which was a constant 5 counts per minute (CPM). As with the ^{32}P, the activity was counted for 10 min or 10,000 counts immediately after sampling with no correction made for decay.

When humic acids were present in the culture medium, there was a decrease in the Fe uptake by the S. obliquus cells (Figure 19). In the absence of humic acids the S. obliquus cells had an activity of more than 1,300 CPM after 30 min, but in the presence of 5.0 mg/l humic acids the final activity of the algal cells was only 130 CPM and when the humic acid concentration was 10.0 mg/l the uptake was even less. A concentration of 25.0 mg/l humic acids allowed the least uptake of Fe although this uptake was not much less than that observed in the medium which contained 10.0 mg/l humic acids.

Not only was there a decrease in the final activity of the cells but there was also a decrease in the rate of uptake in the media containing the higher concentrations of humic acids. Uptake of ^{59}Fe was complete in about 1 h when humic acids were present in the culture medium but uptake was complete after ½ h in the medium where humic acids were absent.

Figure 19.--Uptake of ^{59}Fe by Fe-starved S. obliquus from AAP media containing 4 concentrations of humic acids. The specific activities for the media were the same so the uptake was reported as CPM for the algal cells in a 20 ml sample.

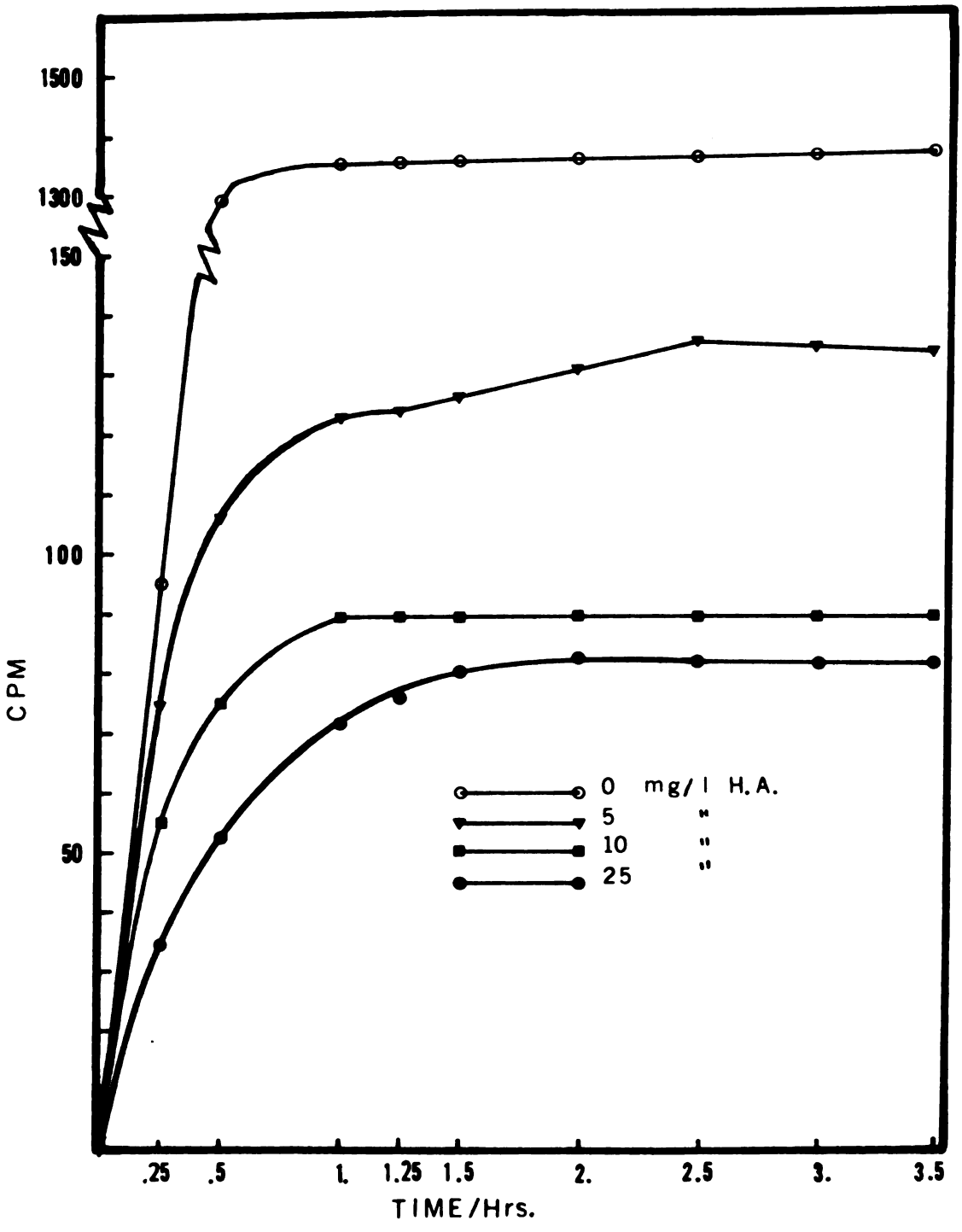

1500
1300
150
100
50
CPM
0 mg/l H.A.
5 "
10 "
25 "
.25
.5
1.
1.25
1.5
2.
2.5
3.
3.5
TIME/Hrs.

Figure 19

Similar results were obtained when the experiment was repeated with samples being taken at shorter intervals. Although the ^{59}Fe activity in the culture was slightly higher and cell density was greater, the final cellular ^{59}Fe activity was less, but the greatest uptake still occurred in the medium where humic acids were not present (Figure 20). There was little difference in the uptake of Fe from the media containing humic acids, but the higher the humic acid concentration, the lower the cellular Fe level. The uptake of Fe was complete after 5 min in the presence of humic acids and virtually complete after 10 min when humic acids were not present in the culture medium, although there was a slight increase in the cellular Fe activity with time.

The addition of 10.0 mg/l humic acid to a solution of ^{59}Fe and stable Fe caused a 97% decrease in the amount of Fe adsorbed onto cellulose-ester filters when they were washed with 50 ml of 0.5 N HCl and 50 ml of distilled water. After allowing culture media containing ^{59}Fe to stand for 36 h, the activity in the medium containing no humic acids decreased from 250 CPM/ml to 190 CPM/ml, while in the media containing 5.0, 10.0 and 25.0 mg/l humic acids the activity decreased to 226, 231 and 245 CPM/ml respectively. The humic acids were able to bind or otherwise make Fe unavailable to undergo adsorption reactions with the surfaces in which it came in contact.

Figure 20.--Uptake of ^{59}Fe by Fe-starved S. obliquus from AAP media containing 4 concentrations of humic acids. The specific activities of all the media were the same so the uptake is reported as CPM in the algal cells in a 20 ml sample of the cell suspension.

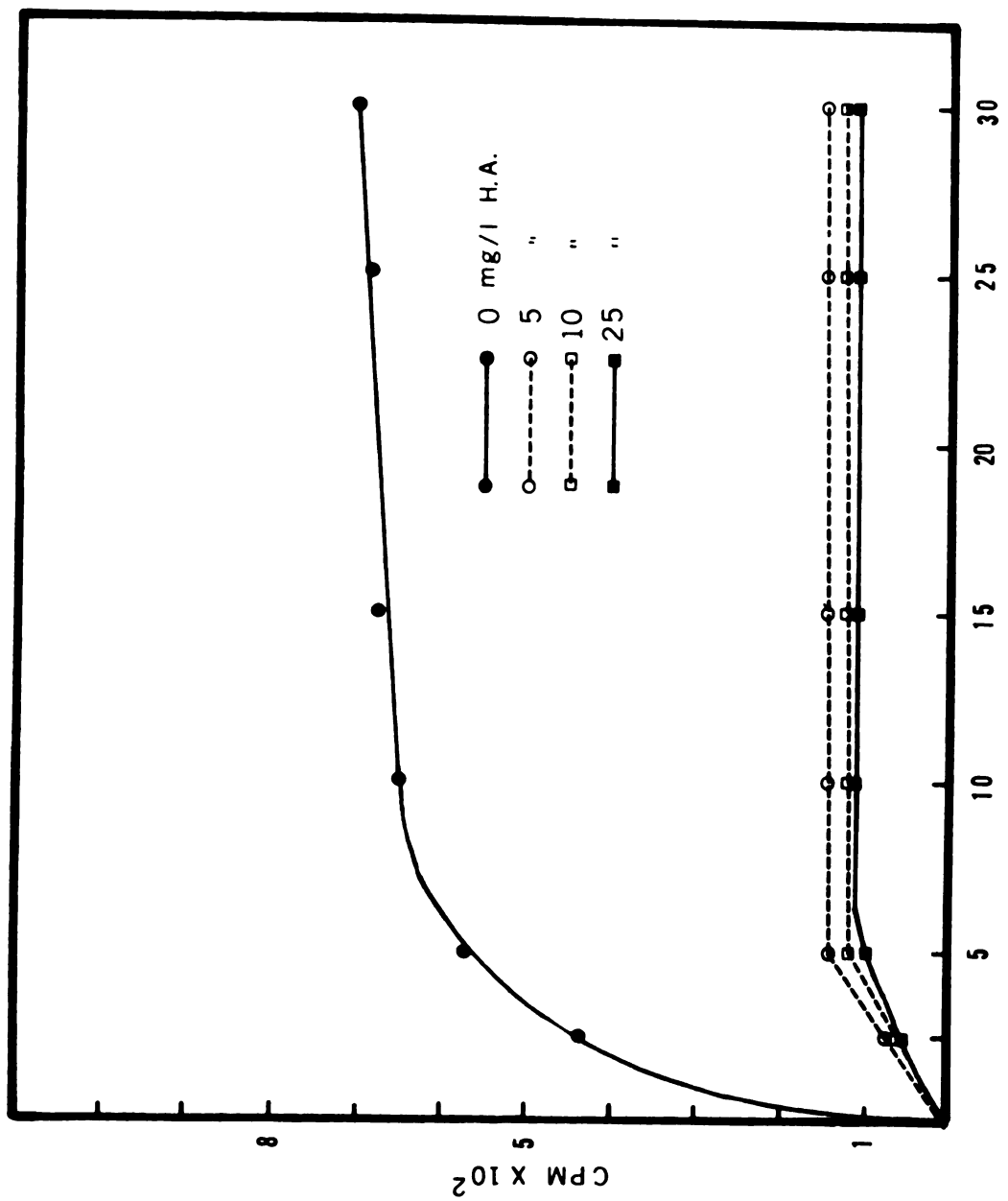


Figure 20

In another experiment the loss of ^{59}Fe activity due to adsorption was 50% less in culture medium containing 25.0 mg/l humic acids than in culture medium containing no humic acids.

The greater adsorption of ^{59}Fe in the absence of humic acids was a source of error in the uptake experiments. Despite the fact that there was a decrease in the total Fe concentration because of adsorption in the absence of humic acids, the greatest algal uptake of Fe still took place in the medium where humic acids were not present. There may also have been error introduced by increased adsorption of Fe onto filters in the absence of humic acids.

Removal of Humic Acid-Bound Fe by S. obliquus

An experiment was conducted to determine the ability of S. obliquus to remove Fe that was complexed or otherwise bound to humic acids. Because humic acids may exist as colloids instead of soluble molecules the bound Fe may exist in a surface bound state as well as a complex or chelate.

Humic acid-bound Fe was formed by allowing a mixture of stable Fe and ^{59}Fe to react with a solution of humic acids for 24 h and dialyzed against distilled water to remove the unbound Fe. One hundred ml of the humic acid-Fe solution were placed in a dialysis bag and suspended in 3,000 ml of distilled water and dialyzed for 7 days, resulting in

a 200 mg/l humic acid solution with a specific activity of $6.98X10^4$ CPM/mg Fe (Appendix II).

Ten ml of the humic acid-bound Fe solution were added to 180.0 ml of Fe-free AAP medium without EDTA and 10.0 ml of Fe-starved S. obliquus making an algal suspension with a cell density of $1X10^4$ cell/ml and an ^{59}Fe activity of 22.5 CPM/ml. A control flask to estimate adsorption losses was created by adding 10.0 ml of humic acid-bound Fe solution to 180 ml of Fe-free AAP medium with 10.0 ml of distilled water in place of the stock algae solution.

Twenty ml samples from each flask were taken after 0.5, 2.5 and 24.0 h, filtered through 5 μ cellulose-ester membrane filters and washed as in the other Fe uptake studies. The dissolved membrane filters with the S. obliquus as well as the filters through which the control medium was passed, were counted and the counts corrected for background. The activity of each sample was also corrected for decay to the beginning of the experiment so that the cellular Fe activity could be compared over time.

There was no significant difference between the activity of the filtered algae and the control filters, indicating that S. obliquus was unable to remove the Fe bound to humic acids of molecular weight 30,000 or greater. Even after 24 h, the algal cells showed no greater activity than that adsorbed on the membrane filters from the control medium.

The results of the Fe-uptake studies and decreases in Fe losses to glass and filter papers due to adsorption in the presence of humic acids indicate that Fe is bound tightly by humic acids and thus less available for uptake by the S. obliquus cells. It has long been known that humic acids may compete with terrestrial plant roots for Fe in soils (Alben et al., 1960, 1961a, 1961b). The unavailability of Fe and the resulting chlorosis has long been recognized as a problem of soils with a high humic acid content. Marine phytoflagellates have been shown to be unable to use chelated Fe (Goldberg, 1952; Provasoli, 1963; Johnson, 1964). Goldberg (1962) also demonstrated that the marine diatom Asterionella japonica was unable to utilize Fe from Fe-humate complexes, while work by Gran (1933) showed that the growth of another marine diatom, Sceletonema costatum was stimulated by soil extract containing 0.2 μ M Fe/l. Gran (1933) did not demonstrate that the stimulation of the growth response was due to the availability of Fe, but theorized that the stimulation was due to the additional Fe made available to the diatom by the soil extract. Prakash and Rashid (1968) also demonstrated that Fe-humic acid complexes stimulated the growth of marine phytoflagellates but thought that the stimulation was not due to increased availability of Fe.

Natural and synthetic chelating agents have been shown to bind metals and reduce their toxicity to algae and

Prakash and Rashid (1968) suggest that the chelation of trace metals may reduce their toxicity to marine dinoflagellates. Waris (1953) found that chelation decreased the toxicity of heavy metals to freshwater algae, while the toxicity of copper to phytoplankton has been reduced by the addition of EDTA to the culture media (Goldman, 1972). Wetzel (1967) found that chelaters enhanced the phosphorus effect by binding metal ions that might precipitate the phosphorus. When 500 mg/l EDTA was added to culture media the optimum Fe concentration for a particular algal species was increased from 0.05 mg/l to 5.0 mg/l because of the chelation of the Fe (Eyster, 1967).

Humic acids, like EDTA seem to bind Fe tightly, making it less available to the S. obliquus cells. It was also found that the Fe bound to humic acids was completely unavailable to S. obliquus and could not be removed during a 24 h period. The fact that the addition of humic acids to AAP media decreased Fe uptake and that S. obliquus was unable to remove the Fe from humic acids indicated that the stimulation of the growth responses of S. obliquus by humic acids was not due to the increased availability of Fe.

It has been suggested by some workers that high concentrations of humic acids may over-chelate metal ions and make them less available for uptake by algal cells (Provasoli et al., 1957; Siegel, 1971; Prakash et al., 1973). The uptake

studies and growth studies reported here were done over the same range of humic acid concentrations. In the growth studies the growth stimulation was increased by increasing humic acid concentrations while the same concentrations of humic acids caused a decrease in Fe availability in the uptake experiments. This does not seem to be consistent with the idea that the humic acids were stimulating growth in an Fe limited culture system by supplying Fe. This indicates that the stimulating effects of humic acids may be due to some mechanism other than chelation alone. The low rate of PO_4 sequestering by humic acids indicates that the effect was not due to increased Fe due to decreased PO_4-Fe interactions.

Effects of Humic Acids and PO_4 on ^{59}Fe Uptake

A 2X4 factorial experiment was used to study the effects of humic acids and PO_4 on the uptake of Fe by Fe-starved S. obliquus. The 2 PO_4 levels studied were 0.0 and 1.0 mg/l, while the experimental concentrations of humic acids were 0.0, 5.0, 10.0 and 25.0 mg/l. The stable Fe concentration was that of the AAP medium ($3X10^{-2}$ mg/l) while the ^{59}Fe tracer concentration was $1.7X10^{-8}$ mg/l (365 CPM/ml).

The cell density was made up to $1X10^4$ cells/ml with Fe-starved S. obliquus and 20.0 ml samples were taken after 0.5, 1.5, 7.0 and 23.0 h. The samples were taken and treated as above with the ^{59}Fe uptake measured as CPM.

After 0.5 h the greatest ^{59}Fe uptake had occurred in the medium containing no humic acids (Figure 21). The presence of 5.0 mg/l humic acids caused a decrease in the cellular ^{59}Fe activity with the higher humic acid concentrations causing only slightly lower cellular concentrations of ^{59}Fe. The presence of PO_4 caused a reduction in the uptake of ^{59}Fe, especially in the absence of humic acids.

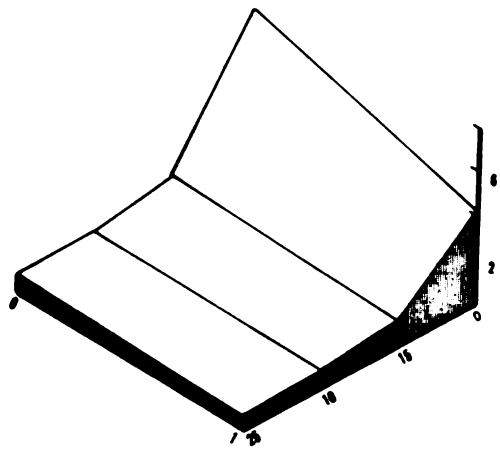
The sample taken after 1.5 h showed the same trends and there had been an increase in the cellular ^{59}Fe in the medium containing no humic acids, but little increase in the media containing humic acids.

After 7 h there was an increase in the cellular ^{59}Fe level in the medium where humic acids were absent, but very little increase in the humic acid containing media. The cellular ^{59}Fe concentration was slightly decreased by 1.0 mg/l PO_4 in the absence of humic acids but caused no effect on the cellular ^{59}Fe activity in the medium where humic acids were absent.

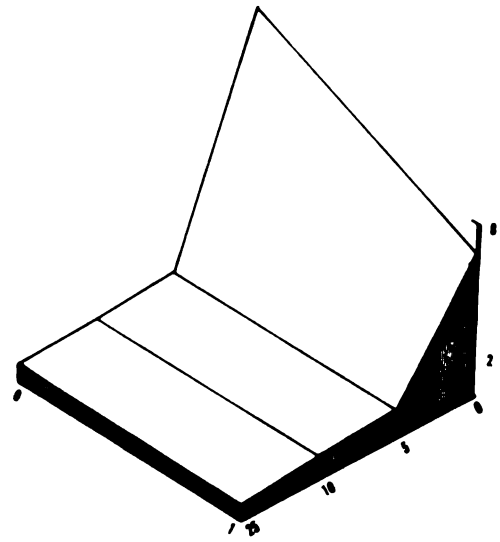
The 23 h sample exhibited the same trends as the earlier samples except that the cellular ^{59}Fe levels were higher. In the absence of humic acids the cellular ^{59}Fe activity had increased to 2,000 CPM while the activity of the cells in the culture medium containing 25.0 mg/l humic acids was less than 20.0 CPM.

The presence of PO_4 caused a lower uptake of ^{59}Fe in the absence of humic acids, as it did in the earlier samples but

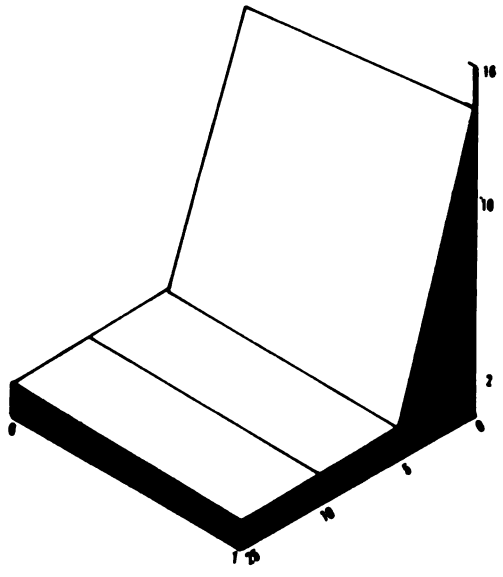
Figure 21.--Response surfaces for the uptake of ^{59}Fe from AAP media by Fe-starved S. obliquus in the presence of 2 levels of PO_4 and 4 levels of humic acids. Samples were taken after 0.5, 1.5, 7.0 and 23.0 h with the response reported as CPM contained in algae in a 20 ml sample.



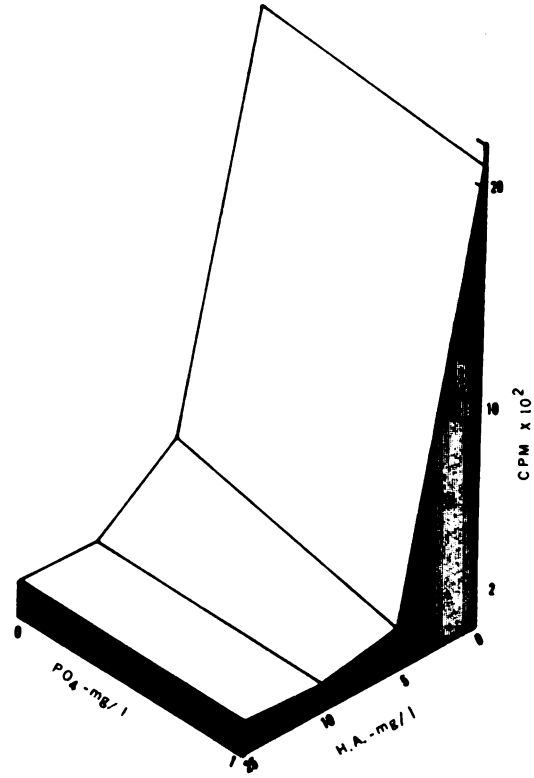
.5 hr



1.5 hr



7 hr



23 hr

Figure 21

there was also a PO_4 induced decrease in the cellular ^{59}Fe activity for the algae in culture media containing 5.0 and 10.0 mg/l humic acids. There was no difference in the uptake of ^{59}Fe between the PO_4 levels in the presence of 25.0 mg/l humic acids.

The presence of humic acids decreased the uptake of ^{59}Fe by S. obliquus as in earlier uptake studies. The slightly increased Fe uptake from the medium containing the lower PO_4 concentration was probably due to less precipitation of Fe as $FePO_4.2H_2O$. This effect was most pronounced after 23 h which was probably due to the time involved in forming the precipitate. When humic acids were present in the culture media the PO_4 effect was much reduced, indicating that the binding of Fe by the humic acids was the controlling factor determining the availability of Fe to S. obliquus.

Effects of Fe Concentration on Fe Uptake in The Presence of Humic Acids

The effects of increased Fe concentration on Fe uptake by Fe-starved S. obliquus were studied in a 4X2 factorially designed experiment. If the decrease in Fe uptake by algae caused by humic acids is strictly a chelation or binding phenomenon, the addition of Fe to the culture medium should cause an increase in the Fe uptake if the binding sites of the humic acids are saturated. Two levels of Fe, 0.03 and

and 1.03 mg/l, were studied in conjunction with 4 humic acid concentrations: 0.0, 5.0, 10.0, and 25.0 mg/l. The ^{59}Fe tracer concentration was $1.7X10^{-8}$ mg/l (365 CPM/ml) in each culture flask. Because the specific activity was not constant the Fe uptake could not be compared using counts per minute, but by knowing the specific activity of each solution the uptake of Fe as mg Fe/20 ml sample of cell suspension could be calculated. All of the algal uptake responses were reported as $mgX10^{-4}$ Fe. The cell density was made up to $1X10^{4}$ cells/ml with Fe-starved stock S. obliquus and samples were taken at 0.5 and 7.0 h after the algal cells were added. Each sample was filtered through a 5 μ cellulose-ester filter, washed and counted as in previous uptake studies.

As was reported earlier, the presence of humic acids caused a decreased uptake of Fe by S. obliquus (Figure 22). The greatest Fe uptake was from the medium which contained no humic acids. There was little difference between the uptake of Fe from the 3 media containing humic acids. When the Fe concentration of the culture medium was 0.03 mg/l, there was very little Fe uptake after 0.5 h or 7.0 h, but when 1.03 mg/l Fe was present in the cutlure medium the uptake was much greater (Figure 22). This greater uptake was most evident in the absence of humic acids, but there was also an increased Fe uptake in the culture media containing humic acids.

Figure 22.--Response surfaces for the uptake of Fe from AAP media by Fe-starved S. obliquus in the presence of 2 levels of Fe and 4 levels of humic acids. Samples were taken after 0.5 and 7.0 h with the Fe uptake reported as mgX10^{-4} Fe per 20 ml sample.

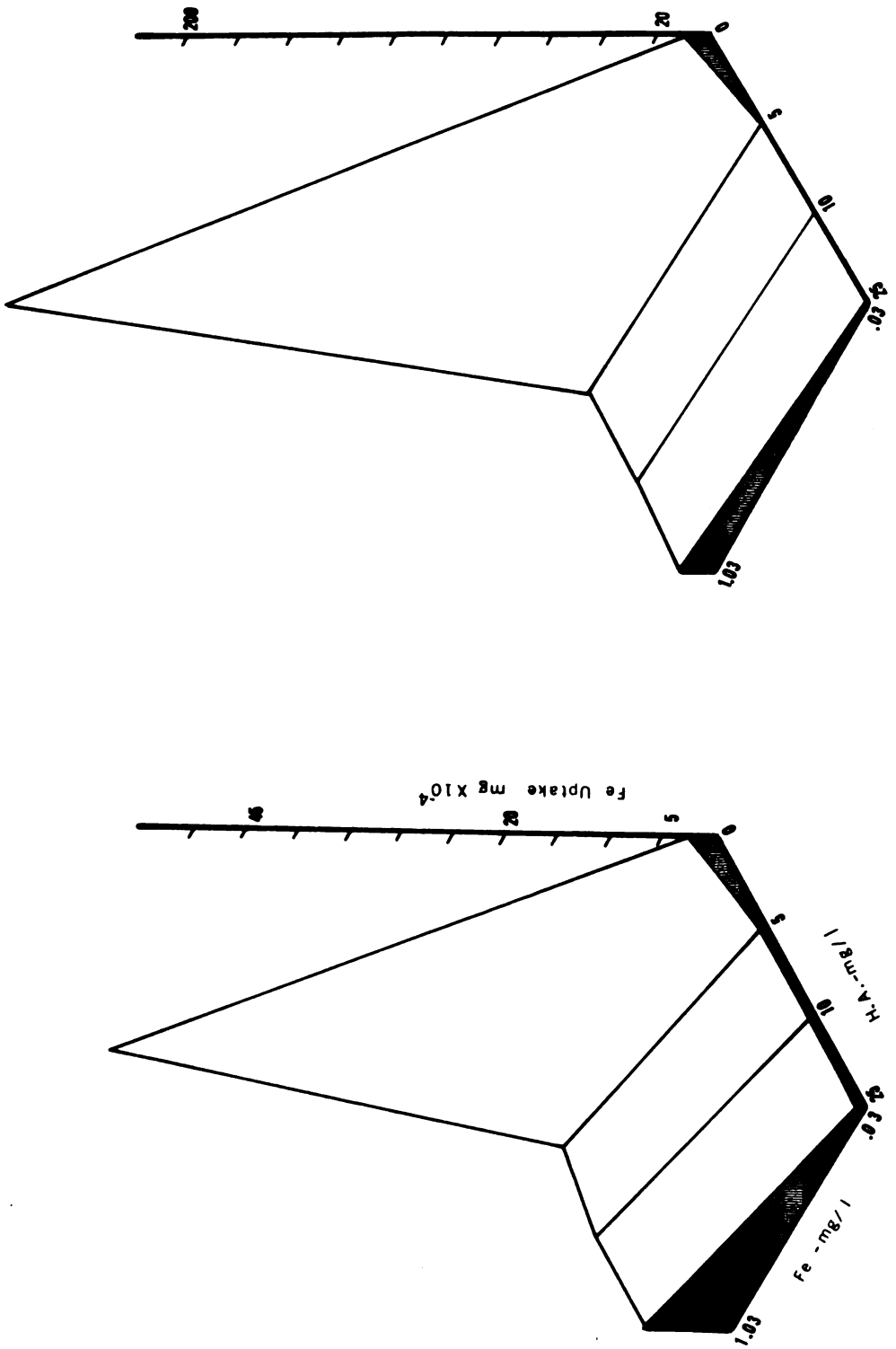


Figure 22

The Fe uptake in the absence of humic acids was dependent on the Fe concentration in the medium. In the presence of humic acids, the higher Fe concentration made more Fe available for uptake, but even the higher Fe concentration was not enough to completely mask the uptake inhibition of the humic acids. The Fe binding capacity of the purified humic acids was calculated to be approximately $1.5X10^{-2}$ mg Fe/mg humic acids. At this binding capacity, the 25.0 mg/l humic acid solution used here would be expected to bind 0.05 mg of Fe. This is much less than the 0.260 mg Fe which was added to the high Fe medium, but more than that added to the low Fe medium. It would be expected that there would be almost no uptake from the low Fe medium containing 25.0 mg/l humic acids, but one would expect a greater uptake from the high Fe medium than was observed in the presence of 25.0 mg/l humic acids. This exceptionally low uptake was not due to precipitation or adsorption because the medium containing no humic acids supported a much higher Fe uptake. The humic acids effectively blocked uptake to a greater level than would be predicted from their Fe binding capacity indicating some other mechanism was operating to reduce Fe uptake.

Effects of pH on ^{59}Fe Uptake in The Presence of Humic Acids

Because of the importance of pH on the availability of inorganic Fe and possible effects on the Fe binding capacity

of humic acids, an experiment was conducted to determine the effects of 3 pH regimes on the uptake of Fe from AAP culture medium in the presence of humic acids. A 3X3 factorial experiment was used with pH levels of 4.0, 7.0 and 10.0 and humic acid concentrations of 0.0, 10.0 and 25.0 mg/l. The pH was adjusted by the addition of 5.0 N HCl or 5.0 N NaOH. The uptake was studied as in the previous uptake studies, with 200 ml of non-EDTA AAP medium in 300 ml Erlenmeyer flasks. The stable Fe concentration was 0.03 mg/l, while the ^{59}Fe tracer concentration was $1.03X10^{-8}$ mg/l (225 CPM/ml). The algal density was made up to $1X10^{4}$ cells/mg, using Fe-starved stock S. obliquus.

A sample taken after 4 h showed the same response to humic acids that was observed in all of the previous Fe uptake studies: The greatest ^{59}Fe uptake occurred in the medium containing no humic acids, with a pronounced decrease in the uptake of ^{59}Fe in the presence of 10.0 mg/l humic acids (Figure 23). When the humic acid concentration was 25.0 mg/l, the ^{59}Fe uptake was not much less than that observed when 10.0 mg/l humic acids were present in the medium.

In the absence of humic acids, increasing the pH caused an increase in the ^{59}Fe uptake by S. obliquus. When the humic acid concentration was 10.0 mg/l, the greatest ^{59}Fe uptake was from the pH 7.0 medium. At a humic acid concentration of 25.0 mg/l the lowest ^{59}Fe uptake was from the

Figure 23.--Response surface for the uptake of ^{59}Fe from AAP media by Fe-starved S. obliquus in the presence of 3 concentrations of humic acids and 3 pH levels. The sample was taken after 4 h and uptake reported as CPM ^{59}Fe in 20 ml.

pH 7.0 culture medium, while the uptake from the pH 4.0 and 10.0 media was only slightly greater.

This experiment was designed to study the effects of pH on the uptake of ^{59}Fe from a humic acid containing medium because of the possible effects of the hydrogen ion concentration on exchanging bound Fe from humic acids. The physiological effects of the pH on the algal cells could not be separated from the possible effects of the hydrogen ion concentration on the exchangeability of the Fe from the humic acids, so it was difficult to determine if the pH had an effect on the availability of humic acid-bound Fe.

It was expected that the lower pH would make more Fe available for uptake. The fact that the uptake was greatest in the medium where the pH was highest in the absence of humic acids indicated that the physiology of S. obliquus was best suited to ^{59}Fe uptake at the higher pH. This may also have been due to the increased solubility of $FePO_4.2H_2O$ with increased pH (see Fe and PO_4 Dynamics section). Only when humic acids were present at the highest concentration was the uptake of ^{59}Fe enhanced by the lower pH values. Increasing the hydrogen ion concentration does not seem to increase the availability of humic acid-bound Fe to S. obliquus, as would be expected if the ^{59}Fe uptake inhibition was due to chelation and adsorption phenomena only.

Effects of Ca on ^{59}Fe Uptake in The Presence of Humic Acids

Because Fe and Ca both exist as cations they may compete for binding sites on humic acids. Ca may be important in displacing Fe from humic acids and thus making the Fe available for uptake by Algae. Ca also acts as a coagulating agent on humic acids in natural waters (Ruttner, 1972).

The effects of Ca on the uptake of ^{59}Fe from AAP medium by Fe-starved S. obliquus in the presence of humic acids were evaluated, using a 3X3 factorial experiment. The 3 Ca concentrations used were 0.0, 20.0 and 40.0 mg/l, while the humic acid concentrations studied were 0.0, 10.0 and 25.0 mg/l. The Ca was supplied by $CaHCO_3$ and the humic acids used were the purified stock solution used in all of the experiments (Appendix II). Stable Fe was present at a concentration of 0.5 mg/l while the ^{59}Fe concentration was $1.63X10^{-8}$ mg/l (349 CPM/ml). The algal cell density was made up to $1X10^4$ cells/ml with Fe-starved stock S. obliquus and sampled after 0.5 and 4.0 h.

After 0.5 h the greatest ^{59}Fe uptake was by the cells in the medium containing 15.0 mg/l Ca in the absence of humic acids (Figure 24). When humic acids were present in the medium the ^{59}Fe uptake was much less than in the absence of the humic acids. When humic acids were absent from the medium Ca caused a greater ^{59}Fe uptake, but when humic acids

Figure 24.--Response surface for the uptake of ^{59}Fe from AAP media by Fe-starved S. obliquus at 3 levels of humic acids and 3 levels of Ca. The sample was taken after 4 h and the uptake reported as CPM ^{59}Fe in a 20 ml sample.

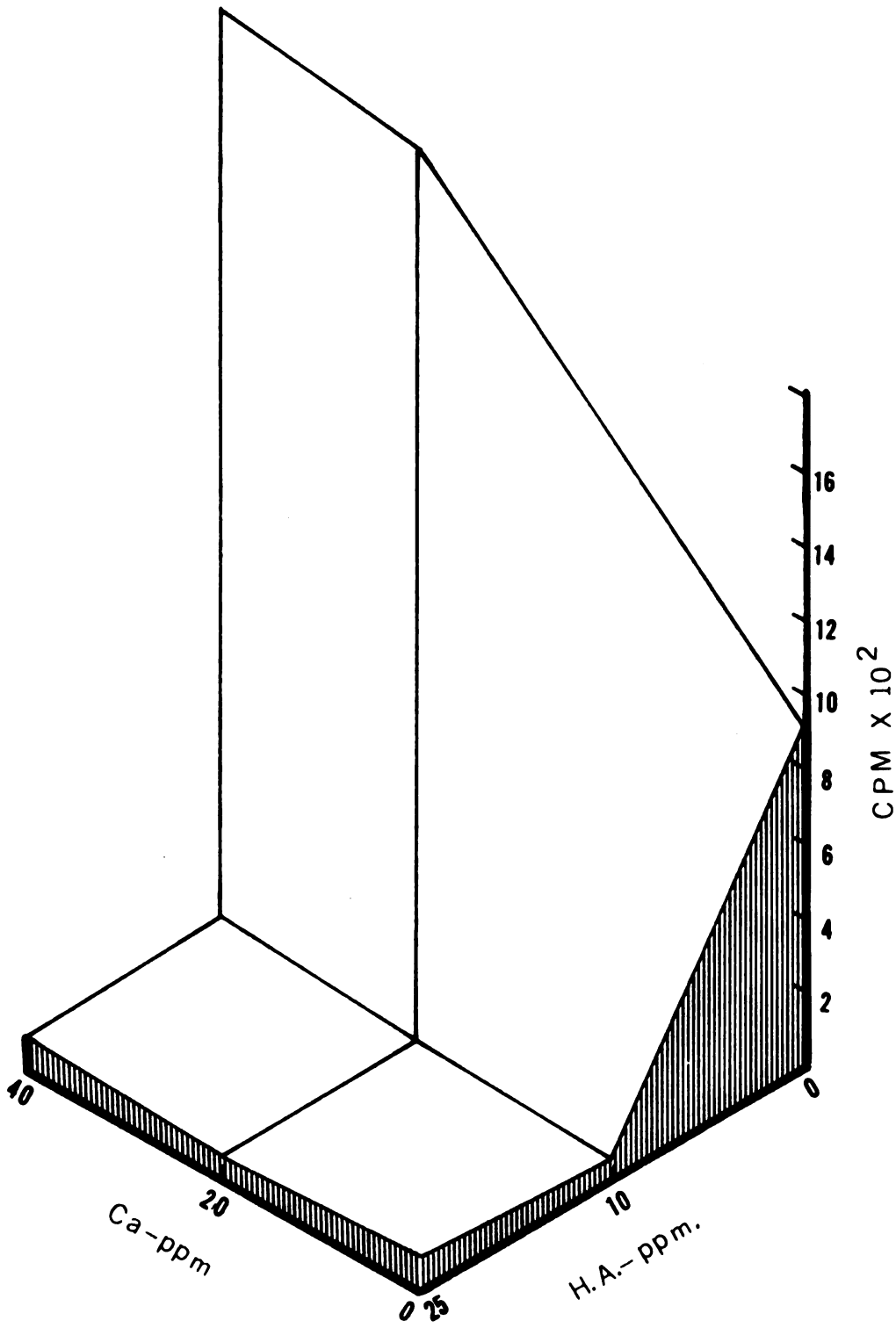


Figure 24

were present there was no increased uptake due to the presence of Ca.

After 4 h the ^{59}Fe activity in the cells had increased in the media containing no humic acids, but not in the media where humic acids were present. The relative intensities of the cellular ^{59}Fe activity remained the same in the 3 Ca concentrations in the absence of humic acids as well as in the media where the cellular ^{59}Fe activity did not increase.

The increased uptake of ^{59}Fe in the absence of humic acids, caused by the higher Ca concentrations may have been caused by a physiological stimulation which increased ^{59}Fe uptake when it was not bound to humic acids (see section on the effects of Ca on S. obliquus growth in the presence of humic acids). This possibility was not studied. Another possibility was that the Ca ions were able to bind negatively charged sites which may have been competing for ^{59}Fe. The excess PO_4 in the AAP culture medium may have been precipitated by the addition of Ca, thus decreasing the amount of ^{59}Fe tied up with PO_4.

The fact that Ca caused no increase in the uptake of ^{59}Fe from AAP medium in the presence of humic acids indicated that the Ca was unable to make ^{59}Fe available for uptake by displacing humic acid-bound ^{59}Fe. In the presence of only 10.0 mg/l humic acids, 40.0 mg/l Ca did cause a slight increase in the ^{59}Fe uptake which may have been due to the increased availability of humic acid-bound ^{59}Fe.

This may not be a true effect of Ca on the availability of Fe from humic acids, but the increased ^{59}Fe uptake may have been due to a residual effect that was seen of Ca in the absence of humic acids. The presence of 10.0 mg/l humic acids may have bound some of the Ca and caused a decrease in the effect of Ca on ^{59}Fe uptake.

The fact that the addition of Ca does not greatly increase the uptake of ^{59}Fe from AAP medium has significance for marine as well as freshwater systems. It has been postulated that humic acids may be responsible for the red tide organism reaching bloom conditions off the coasts of the United States (Wilson and Collier, 1955; Martin, 1971). Martin (1971) has suggested that this effect may be due to the ability of humic acids to chelate Fe and supply it to the algae in the Fe-limited marine surface waters. Although the effects on the uptake of Fe bound to humic acids need to be studied further, it seems clear that Fe bound to humic acids washed into a freshwater system high in Ca would still be held in a form which is unavailable to S. obliquus.

In freshwater systems, Ca may even act to remove humic acids from the epilimnions of lakes with their load of bound Fe. Ruttner (1972) indicated that Ca coagulates humic acids in natural waters, causing them to settle out, while Otsuki and Wetzel (1973) found that humic acids were removed from solution by adsorption to $CaCO_3$ crystals. In a natural

system this too would have an important decreasing effect on the cycling of Fe.

Displacement of Fe From Humic Acids by Ca

An experiment was designed to test the simple displacement of humic acid-bound Fe by Ca. Ten ml of a solution of humic acid-bound Fe was placed in each of several dialysis bags and suspended in 200 ml of AAP culture medium, in the absence of algal cells. The humic acid-Fe solution was that used in the experiment to determine the availability of humic acid-bound Fe to S. obliquus and had a specific activity of $6.98X10^4$ CPM/mg Fe (Appendix II). One medium was the normal AAP medium without EDTA, while the other had 100 mg/l Ca added as $CaHCO_3$.

The experimental systems were allowed to stand for 5 days, after which time the solutions outside of the dialysis bags were acidified with 5 ml of concentrated HCl to remove Fe adsorbed to the glass sides of the flasks. Several 20 ml samples were drawn from each flask and counted for gamma radiation in plastic counting vials. The samples were counted for 10,000 counts and corrected for background.

The solution containing 100 mg/l Ca had an activity of 26.2 CPM/20 ml sample, while the normal AAP medium had an activity of only 1.95 CPM/20 ml. Although the previous studies did not indicate that Ca was able to make Fe available from humic acids for algal growth or uptake, it was

found that Ca was able to increase the unbound Fe in solution. This may have been from direct displacement of Fe from the humic acids or may have been due to the binding of negative sites that could otherwise bind or precipitate Fe. Less than 5% of the humic acid-bound ^{59}Fe was released by Ca. This small amount may not have been sufficient to cause a growth response or detectably increase the uptake of Fe from humic acid containing culture media. Even when this amount of Fe was released by the addition of Ca it did not increase the uptake of Fe by S. obliquus indicating that humic acids may be able to block Fe uptake by some mechanism in addition to simply binding up Fe.

ALTERNATIVE STIMULATORY MECHANISMS

Heterotrophic Use of Humic Acids by *S. obliquus*

Algae, for the most part, obtain their carbon from carbon dioxide or some carbon dioxide containing compound such as bicarbonate. Some algae can use only inorganic carbon sources while others are able to use reduced carbon sources. Some algae such as *Prototheca zopfii* are colorless and are obligate heterotrophs (Baker, 1935). Other species of algae have been found to be facultative heterotrophs. That is, they can form inorganic carbon into reduced carbon compounds by photosynthesis, but are also able to use exogenous reduced carbon sources (Zajic and Chiu, 1970). The genus *Scenedesmus* has been found to have actively heterotrophic species, capable of using a wide variety of carbon sources. Taylor (1950) found that *S. obliquus* was able to use glucose, cellulose and acetate as carbon sources, while Algeus (1949) found this species to be able to use glycocol and alanine. Dvorakova-Hladka (1960) reported *S. obliquus* also used cellobiose as a carbon source for heterotrophic growth.

Because the stimulatory effects of humic acids on *S. obliquus* may be due to the use of humic acids or their

degradation products as a carbon source for heterotrophic growth, a 2^2 factorial experiment was designed to study the possible heterotrophic growth of S. obliquus in the presence of humic acids of molecular weight 30,000 or greater. The factors studied were humic acids and light. Humic acids were either absent or present at a concentration of 20.0 mg/l. The cultures were either grown in the dark or under 375 ft candles of continuous fluorescent illumination from "grow lux" bulbs.

The cultures were grown in 20 ml "Pyrex" screw top vials, with the dark treatment vials covered with aluminum foil and black tape. Each vial was filled with 15.0 ml of AAP medium containing the appropriate concentration of humic acids and the cell density made up to an initial density of $1X10^5$ cells/ml with non-starved stock S. obliquus. After addition of the stock algae, the vials were capped and placed under the lights. The vials were shaken 3 times a day for 21 days, after which time the cell densities were determined by microscopic cell counts. The cultures of S. obliquus were not axenic so were associated with bacterial cells of the type described in Appendix I.

There was significantly greater growth in the cultures grown in the light than those grown in the dark (Figure 25). When humic acids were present in the media there was a greater standing crop in both the cultures grown in the light and dark.

Figure 25.--Histogram representing the heterogrophic growth standing crop of S. obliquus after 21 days growth. Each bar represents the mean of 4 replications with 95% confidence limits indicated.

(A) AAP medium, grown in dark.

(B) AAP medium + 20.0 mg/l humic acids, grown in the dark.

(C) AAP medium, grown under 375 ft candles of continuous fluorescent illumination.

(D) AAP medium + 20.0 mg/l humic acids, grown under 375 ft candles of continuous illumination.

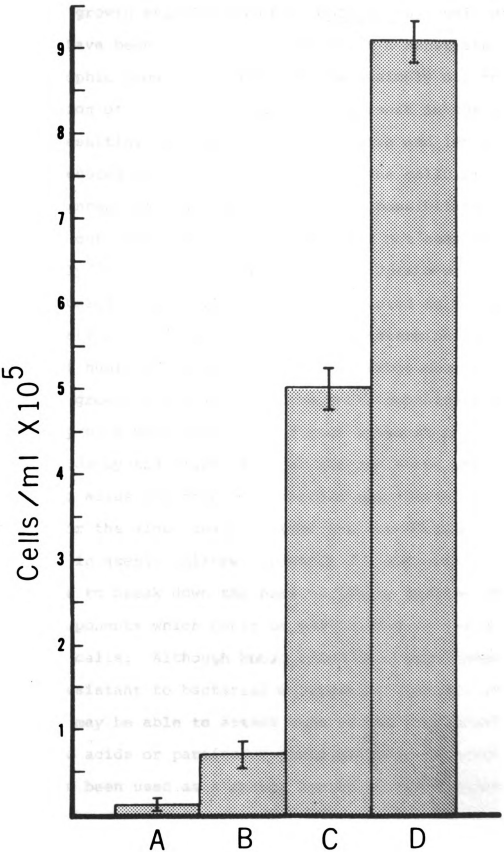


Figure 25

The growth stimulation of S. obliquus by humic acids may not have been due to the addition of a substrate for heterotrophic growth, but may have been simply due to a stimulation of division of the cells present in the inoculum, with a resulting decrease in cellular size and level of stored reduced carbon supplies. Since the cellular size and starch content were not monitored, this possibility cannot be ruled out, although the cell size did not seem to decrease noticeably from the stock cells added. Uptake studies using radio-labeled humic acids could be conducted to monitor the incorporation of ^{14}C to more precisely determine the possibility of humic acids being used as a carbon source.

The growth stimulation in the media containing humic acids may have been due to the direct heterotrophic use of humic acids by the algal cells or the breakdown products of the humic acids may have been another possible source of carbon for the algal cells. Since the growth studies were not done in axenic culture (Appendix I), bacteria may have been able to break down the humic acids to smaller structural components which could be assimilated by the S. obliquus cells. Although humic acids have been found to be highly resistant to bacterial degradation (Barber, 1968), bacteria may be able to attack some of the functional groups. The humic acids or particular parts of their structure may also have been used as a carbon source by the bacteria with

the reduced carbon compounds produced by the bacteria in turn being used by the S. obliquus (see section on stimulation of S. obliquus by humic acids). No measure of bacterial degradation was made in these studies, but the possibility of this additional source of carbon is attractive because the cultures were grown in sealed vials containing only 15.0 mg/l $NaHCO_3$ as an inorganic carbon source. The bacteria may also have been able to supply CO_2 directly to the algal cells. Because of the size of the molecules involved, Pratt (1955) concluded that the stimulation of algal growth by humic acids was probably not due to their direct use as an algal nutrient.

In the light-grown cultures the growth response to humic acids was almost twice that to AAP medium alone, which was further support for the heterotrophic growth theory. Many workers have found that the introduction of light greatly increases heterotrophic growth (Dvorakova-Hladka, 1966; Karlander and Krauss, 1966). Light may be used to supply ATP through photophosphorylation. Scenedesmus sp. grown in glucose had its growth increased threefold by illuminating the culture (Myers, 1947). The use of organic carbon sources only in the presence of light may not be true heterotrophic growth, but may explain, in part, the stimulatory effect of humic acids on S. obliquus.

Simply because humic acids are able to stimulate increased algal growth one could not expect the use of humic

acids as a carbon source to enhance growth alone to explain the growth stimulation in an Fe-deficient system. If the system was Fe-deficient the increased supply of carbon in any form would not increase the algal growth. Since one of the major functions of Fe in algal growth is the synthesis and maintenance of chlorophyll, the heterotrophic use of humic acids or their breakdown products may reduce the need for photosynthetic activity and thus the Fe requirement of the algal cells would also be reduced. Humic acids could then be thought of as Fe sparing factors instead of supplying additional Fe for growth (see section on PO_4 and Fe dynamics).

Surface Membrane Effects of Humic Acids

Prakash and Rashid (1968) suggested that the stimulatory effect of humic acids on marine phytoplankton may be a membrane phenomenon. They found that the growth rates, yield and uptake of radio-labeled CO_2 was not entirely attributable to metal chelation. Burke (1932b) thought that the stimulatory effects of humic acids on bacteria may be due to the redox potential effects on cell membranes. Chaminade (1956) found that humic acids stimulated growth in violet epidermal cells by allowing mineral transport across the cytoplasmic membrane. It was thought by Saunders (1957) that humic acids may exert a stimulatory effect on the cell membranes of phytoplankton, allowing an influx of bound metal nutrients,

while Waris (1953) suggested that humic substances may exert an effect on the cytoplasmic membrane directly. The low molecular weight humic acid fractions and fulvic acids may be able to penetrate the cell membranes of phytoplankton (Prakash et al., 1973), but the high molecular weight humic acids (molecular weight 30,000 or greater) under study here probably could not. Prát et al. (1959 and 1961) found that humic acids are not able to penetrate plant cell membranes, but the humus substances that were able to penetrate cell membranes were found to be inhibitory to all processes (Prát, 1968). It has been realized that many plant cells have chelating agents associated with their cell membranes which may have an effect on the uptake of inorganic metal nutrients. Humic acids may act to facilitate this chelation aided inorganic nutrient uptake (see section on Fe uptake in the presence of humic acids).

To test the possibility that the stimulatory effect of humic acids on S. obliquus growth was a membrane effect, a dialysis experiment was designed so that the humic acids could be added to the culture medium, but kept from direct contact with the cell membranes of the algal cells, while allowing free exchange of smaller molecules. The experimental design was a completely randomized block with 4 treatments and 4 replications.

The cultures were grown in 300 ml Erlenmeyer flasks with 200 ml of EDTA-free AAP medium. Treatment "A" was a

control treatment of 200 ml AAP medium only. Treatment "C" was also a control to determine the effects of the dialysis membrane on the growth of S. obliquus. Treatment "C" had 100 ml of AAP medium inside a dialysis bag and 100 ml of AAP medium outside of the dialysis bag. Treatments "B" amd "D" were humic acid treatments. The medium used in treatment "B" had 10.0 mg/l humic acids in the 100 ml outside the dialysis bag as well as in the 100 ml of AAP inside the bag. Treatment "D" contained a concentration of 20.0 mg/l humic acids in the 100 ml of AAP medium inside the dialysis bag, but no humic acids outside of the bag. Fe-starved stock S. obliquus was added to the 100 ml of culture medium outside of the dialysis bag in each flask to make a cell density of $1X10^4$ cells/ml (based on 200 ml of medium). The experimental flasks were then placed randomly in a culture rack and bubbled with air for 5 days (Appendix I).

The media containing humic acids supported greater growth than those without (Figure 26). There was no difference between the control treatments indicating no effect due to the dialysis membrane alone. There was also no difference between the media where humic acids were present, demonstrating that the growth stimulation caused by the humic acids of molecular weight 30,000 or greater was not a membrane phenomenon.

Keeping the humic acids separated from the S. obliquus cells kept them from direct contact but did not affect the

Figure 26.--Histogram of the growth response of S. obliquus separated from humic acids by dialysis membranes. Each bar represents the mean of 4 replications with 95% confidence limits indicated.

(A) 200 ml of AAP medium with no dialysis bag.

(B) 100 ml of AAP medium containing 10.0 mg/l humic acids inside as well as outside of the dialysis bag.

(C) 100 ml of AAP medium without humic acids inside and outside of the dialysis bag.

(D) 100 ml of AAP medium outside of the dialysis bag with 100 ml of AAP medium containing 20.0 mg/l inside of the dialysis bag.

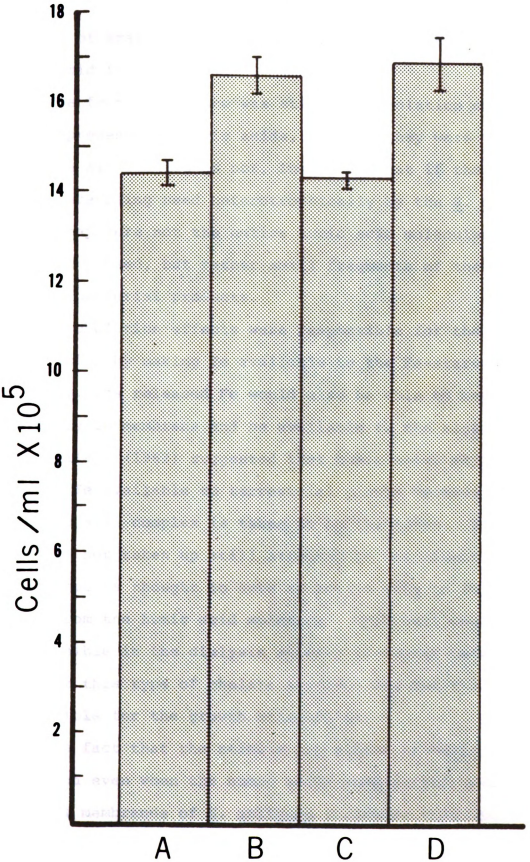


Figure 26

passage of small inorganic molecules such as Fe or small humic acid fragments.

The fact that there was still a stimulation of growth in the presence of humic acids, whether they were in contact with the algal cells or not, indicated that if the humic acids were being used heterotrophically by the S. obliquus cells they were not the entire humic acid molecules that were being used, but rather small fragments of the molecules or some bacterial products.

If chelation effects were responsible for the growth stimulation by making Fe available to the Fe-starved S. obliquus, the released Fe would also be able to move across the dialysis membrane and be available to the algal cells.

Wallace (1962) suggested that humic acids may be able to make Fe available to terrestrial plants because the entire Fe-humic acid complex is taken up by the roots. Even if the metal is not taken up still attached to the ligand, the plant root is thought to have an active role in removing the metal from the humic acid molecule. This cell contact was not possible in the dialysis studies presented here indicating that this type of chelate transfer was not the mechanism responsible for the growth stimulation.

The fact that the stimulatory effect of humic acids was found even when the humic acids were denied contact with the cell membranes of S. obliquus indicates that the

stimulatory effect on algal growth is not a membrane effect alone, although this is not conclusive evidence that surface membrane effects do not take place.

BOG WATER EFFECTS

Bioassay of Natural Bog Water

In an effort to determine the effects of naturally occurring, dissolved, unconcentrated organic acids on the growth of S. obliquus, a bioassay was conducted to compare a medium made with bog water to one made with distilled water.

Bog water was collected from Bear Lake Bog south of the Michigan State University campus, Ingham County, Michigan (Lansing Township, SE¼, NW¼, Sec. 35, T4N, R2W). Five liter samples were collected in polyethylene carboys on 10 October 1973. At the time of collection there was a dense bloom of Chlorella sp. and Staurastrum sp. in the surface waters of the bog. Samples were returned to the laboratory within 1 h of collection and filtered through washed, 0.45 μ membrane filters. Chemical determinations were made on the filtered water immediately and the remaining water stored in polyethylene sample bottles at 4.0 C until it was used for the algal assays.

The total alkalinity was found to be 10.0 mg/l as $CaCO_3$, using the H_2SO_4 titrametric method, while the hardness was found to be 36.0 mg/l as $CaCO_3$ by the EDTA complexation method. The total dissolved PO_4, as determined by the

stannous chloride-ammonium molybdate method on a concentrated sample, was found to be 0.015 mg/l PO_4 in the original water sample. Most of the PO_4 present in the bog water was tied up in the algal biomass and not in the water. The Fe concentration was determined by the bathyphenanthroline method (Lee and Stumm, 1960). No Fe could be detected in an undigested sample when it was concentrated by a factor of 10. After digesting with HNO_3, the total Fe concentration was found to be 10.0 mg/l. This indicates that most of the Fe in solution or suspension was held in a form unavailable to algal cells. The amount of Fe removed from these associations may not be indicative of the total bound Fe because some Fe may not have been removed by the HNO_3 digestion.

A simple experiment using 2 treatments with 4 replications was used to test the effect of bog water on the growth responses of S. obliquus. The regular AAP medium was made up using distilled water as the control medium with filtered bog water used to make the experimental AAP medium. Each experimental flask was inoculated with Fe-starved S. obliquus stock (Appendix I) to make an initial cell density of $9X10^3$ cells/ml. Samples were taken after 2, 3, 4, and 5 days and the cell density determined by cell counts.

After 2 days, there was greater growth in the medium made with bog water, but from day 3 to the end of the

experiment the greater standing crop was in the distilled water medium (Figure 27). These results were dissimilar to those obtained by the addition of purified humic acids of molecular weight 30,000 or greater in AAP medium. In fact, the algal growth response to bog water was exactly opposite to that obtained with purified humic acids.

Although it is impossible to determine the active ingredients of the bog water causing the observed results it is evident that the results obtained by using bog water in the culture medium were different from those observed when purified humic acids were used. This indicates that the humic acids of molecular weight 30,000 or greater are not responsible for the effect caused by filtered bog water. The results of this experiment are inconclusive and may be confounded by other limiting nutrients as well as a multitude of organic compounds.

Effects of Bog Water on The Growth Response of *S. obliquus* to Humic Acids, Fe and PO_4

Further studies were conducted with bog water to determine its effect on the algal growth responses to the addition of humic acids, Fe and PO_4 to AAP culture medium. The PO_4 concentrations were 0.0 and 0.9 mg/l, while the humic acid concentrations were 0.0 and 10.0 mg/l. Fe was either absent or present at a concentration of 10.0 mg/l. Each of the experimental treatments was made up in filtered bog water.

Figure 27.--Growth response curves of Fe-starved S. obliquus to AAP media made with distilled water and filtered bog water reported as $\log_{10}$ cells/ml. Each point represents the mean of 4 replications with 95% confidence limits reported.

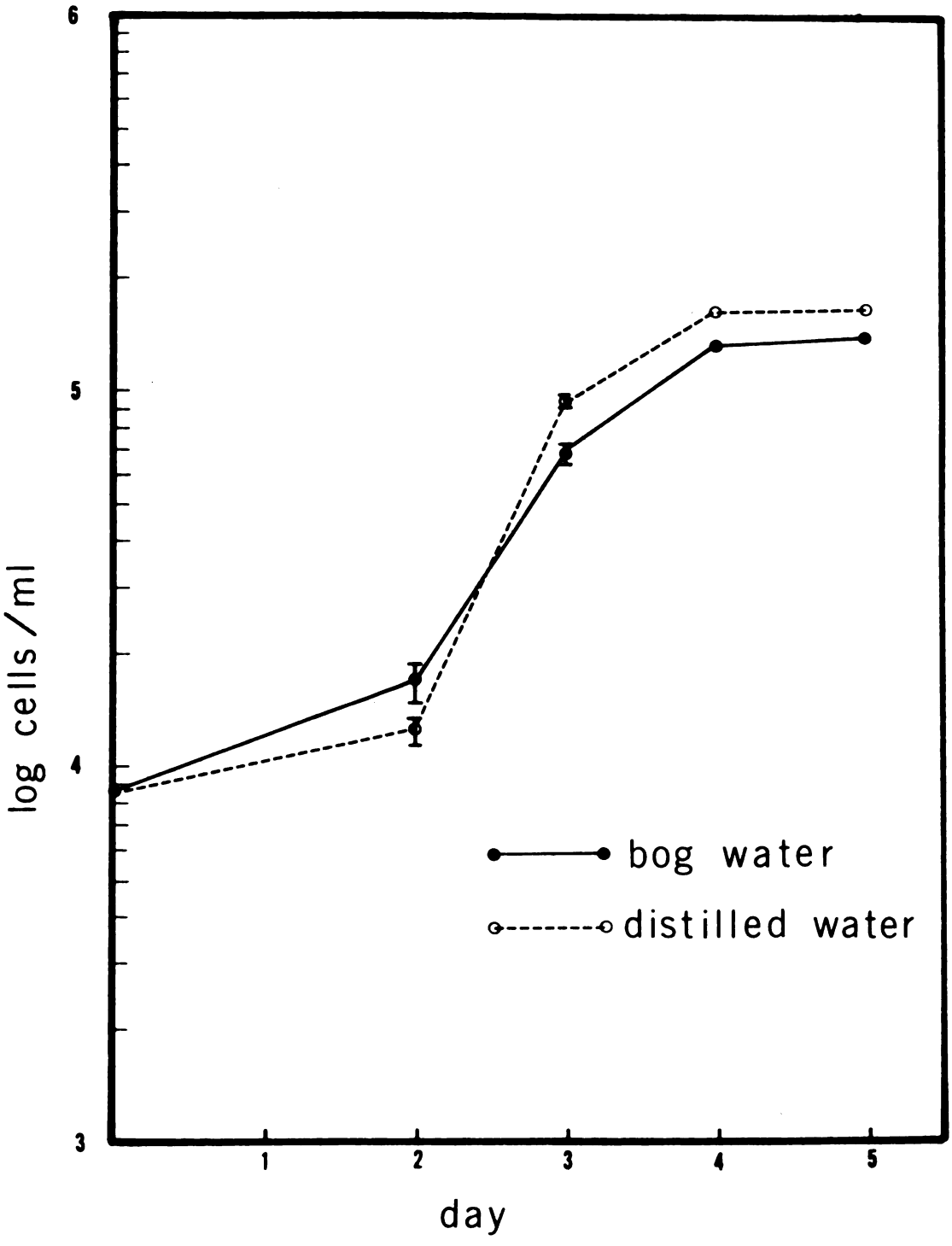


Figure 27

Each 300 ml Erlenmeyer culture flask was filled with 200 ml of culture medium, autoclaved and inoculated with enough Fe-starved stock S. obliquus to make a concentration of $7X10^3$ cells/ml. The culture flasks were placed in a culture rack under 375 ft candles of continuous fluorescent illumination and bubbled with 20 ml/min air. Samples were taken after 3, 4, 5 and 7 days.

There was no growth in the media without PO_4 so these cells were dropped from the analysis. After 3 days of incubation, the greatest growth response was to the medium containing 1.0 mg/l Fe and 10.0 mg/l humic acids, while the least growth occurred in the medium which contained no Fe or humic acids (Figure 28). When humic acids were present in the absence of Fe, there was an increased standing crop that was intermediate between that to Fe alone and to Fe and humic acids in combination.

After 4 days, the differences in standing crops between the 4 media had decreased although the standing crop in the medium containing no Fe or humic acids still supported the least growth. After 5 days, the growth responses of the 3 media were not very different, although there was a slightly greater growth response to the medium containing both 1.0 mg/l Fe and 10.0 mg/l humic acids.

By the seventh day, the greatest standing crop was in the medium containing 10.0 mg/l humic acids in the absence

Figure 28.--Response surfaces for the response of Fe-starved S. obliquus to Fe and humic acids added to AAP medium made with filtered bog water. Samples were taken after 3, 4, 5 and 7 days. Note the scale of the algal standing crop changes from cells/ml $X10^5$ to cells/ml $X10^6$ on day 4.

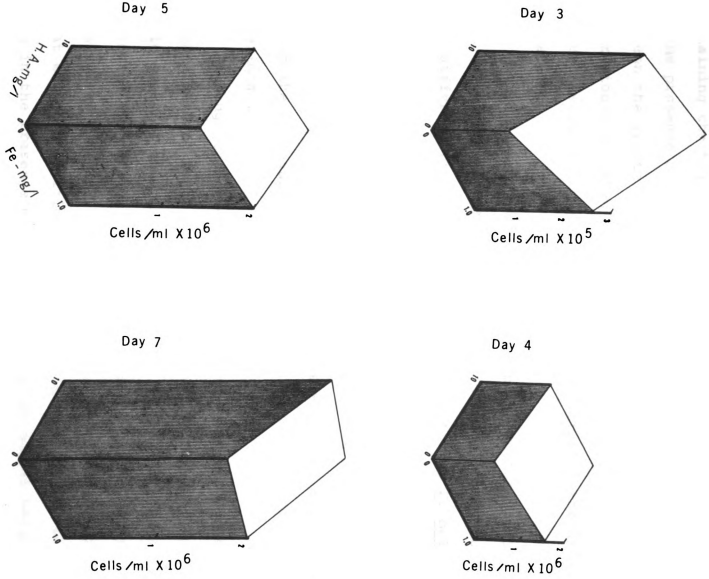

Day 5
Fe-mg/l
H.A.-mg/l
Cells/ml X 10^6
Day 3
Cells/ml X 10^5
Day 7
Cells/ml X 10^6
Day 4
Cells/ml X 10^6

Figure 28

of Fe, while the lowest growth response was to the medium containing the high Fe level in the absence of humic acids. In the presence of humic acids there was no difference between the growth responses to the higher or lower Fe concentrations, but when humic acids were absent from the culture medium there was a lower standing crop in the high Fe medium.

When bog water was used to make up non-EDTA AAP media with various levels of Fe, PO_4 and humic acids, the results were different from those observed for Fe-starved S. obliquus in similar media prepared with distilled water. The lack of a stimulatory response to the addition of Fe after day 3 indicated that the availability of Fe from the bog water medium was such that the algal cells were able to acquire all of the Fe required for growth at the beginning of the experiment.

Although there were dissolved colored organics present in the filtered bog water used, the addition of purified humic acids of molecular weight 30,000 or greater caused a growth stimulation indicating that the dissolved organics causing the stimulation were not present in the bog water in great enough quantities to allow the maximum stimulation. Even in the presence of the many smaller molecular weight fractions, the large purified humic acids still caused a growth stimulation. This increased growth response does not

seem to be the effect of humic acids providing Fe because the addition of Fe to the AAP medium made with filtered bog water caused very little growth stimulation.

SUMMARY

The initial studies of the PO_4 and Fe nutrition of Scenedesmus obliquus indicated that each of these nutrients could have a negative effect on the availability of the other. When Fe was added as $FeCl_3$ and P was added as PO_4, the 2 nutrients were precipitated as strengite ($FePO_4 \cdot 2H_2O$). This interaction was found to cause a decrease in the initial growth rates of cultures, but not in the final standing crops of the cultures indicating that the algal cells were able to use the Fe and PO_4 in the precipitated form or take up the 2 nutrients as soon as they became available due to the solubilization of $FePO_4 \cdot 2H_2O$.

Humic acids stimulated the growth of S. obliquus, with the stimulation being humic acid concentration dependent. Increased humic acid concentrations caused increased growth responses up to 25.0 mg/l, above which there was no increased growth. The presence of this high concentration of humic acids often caused a reduction in the growth response to levels below those observed in the media containing lower concentrations of humic acids. This was especially true in the presence of a low Fe concentration or high PO_4 concentration. The presence of humic acids increased the maximum relative growth rate, maximum standing crop and final

standing crop as well as decreasing the lag phase over that of AAP.

The stimulation of the growth responses of S. obliquus caused by humic acids was similar to the responses to EDTA, but the humic acid induced stimulation seemed to be not only by chelation. Humic acids did seem to act as chelating agents, but also stimulated algal growth by other mechanism. The addition of humic acids to Fe-limited culture systems caused the same growth response as the addition of $FeCl_3$. These results seem to indicate that the humic acids were acting as chelating agents in much the same way as the synthetic chelating agent EDTA is known to work. The responses of the Fe-starved S. obliquus cells indicated that the humic acids were stimulating increased growth, in part, by making additional Fe available to the algal cells.

When Ca was added to the culture media, the algal growth was stimulated in the absence of humic acids. There was a lesser stimulatory action by the Ca when humic acids were present and there was no stimulatory effect due to Ca in the presence of a humic acid concentration of 10.0 mg/l or greater. The Ca, when added to an Fe-limited system containing humic acids, was unable to increase algal growth by displacing humic acid-bound Fe, thus making the Fe available for algal growth. In fact, the presence of humic acids even decreased the stimulatory effects on the early growth of

S. obliquus cultures. This effect was probably due to the chelation effects of humic acids on Ca.

Humic acids caused a slight decrease in the uptake of $^{32}PO_4$ from AAP media by S. obliquus, but this effect was slight and may have been due to adsorption reactions between the PO_4 and humic acids.

The presence of humic acids decreased the uptake of ^{59}Fe from AAP media greatly. The presence of 5.0 mg/l humic acids decreased the uptake of ^{59}Fe from AAP culture medium by more than an order of magnitude. Increasing the humic acid concentration in the medium further reduced the uptake of ^{59}Fe, but to a lesser extent. The cellular capacity of S. obliquus was found to be saturated by Fe in less than 10 min by all humic acid concentrations. Humic acid-bound Fe was found to be tightly bound and unavailable to S. obliquus indicating that the stimulatory effect of humic acids on S. obliquus was not due to supplying Fe by chelation processes alone, even though the growth responses were similar for humic acids, Fe and EDTA. The growth studies seemed to support the theory that humic acids are able to bind Fe and make it available to algal cells by preventing precipitation, but the uptake studies reported here do not support this theory.

The presence of 1.0 mg/l PO_4 in the media caused a decrease in the ^{59}Fe uptake over that observed when PO_4 was

absent from the media. This effect was more pronounced when humic acids were absent from the media and was thought to be due to the precipitation of Fe by the PO_4.

Increasing the Fe concentration from 0.03 mg/l to 1.03 mg/l caused an increase in the Fe uptake by Fe-starved S. obliquus in the absence of humic acids and to a lesser extent in the presence of various concentrations of humic acids. The addition of more Fe than could be bound by the humic acids did not overcome the inhibitory effect of the humic acids on Fe uptake which indicates that the humic acids may be able to block Fe uptake by processes other than the simple Fe binding.

Ca and pH had very little effect on the uptake of Fe by Fe-starved S. obliquus from culture media containing humic acids, although the addition of Ca did increase the uptake of Fe in the absence of humic acids. Ca was found to be able to release humic acid-bound Fe in the absence of S. obliquus, but only about 5% of the bound Fe was displaced in 4 days. This small amount of release was not enough to cause a detectable increase in the growth responses or uptake of Fe and it was concluded that Ca had only a slight effect on the availability of Fe from humic acids for algal uptake and growth.

Humic acids were found to support heterotrophic growth of S. obliquus with this growth being enhanced by light

indicating that the humic acids were used by the algal cells in a photoheterotrophic process.

Culturing S. obliquus in media separated from humic acids by dialysis membranes demonstrated that the stimulatory effects of humic acids were not due to surface membrane effects nor the direct uptake of intact humic acid molecules as had been suggested. There was a growth stimulation of S. obliquus cells whether the humic acids came in contact with the algal cells or not.

It was also discovered that algal culture media made from filtered bog water supported algal growth responses differing from those to purified humic acids of molecular weight 30,000 or greater, but the addition of purified humic acids to the bog water media caused a further growth stimulation.

It was concluded that humic acids of molecular weight 30,000 or greater had a stimulatory effect on the growth of Scenedesmus obliquus. Although the growth responses of Fe-starved S. obliquus cells were similar for humic acids and EDTA, the humic acids did not seem to be able to supply Fe for uptake to Scenedesmus obliquus.

Several alternative mechanisms of stimulation such as heterotrophic growth of S. obliquus using humic acids or their degradation products as reduced carbon sources and indirect stimulation of algal growth by direct stimulation

of bacteria by humic acids may be responsible for the stimulatory effect observed. If humic acids were used as a reduced carbon source and the photosynthetic requirements of the cells were reduced, the Fe requirement of the cells for growth would be less. The humic acids may be acting as an Fe sparing factor instead of supplying Fe to the algal cells directly.

The studies presented here pose more questions than they answer, leaving many avenues of research into the stimulatory mechanisms of humic acids on the green alga Scenedesmus obliquus.

APPENDICES

APPENDIX I

Algal Culture Methods

APPENDIX I

Algal Culture Methods

Presently, algal bioassays are done in static (batch), continuous (chemostat) and *in situ* culture systems. *In situ* culture systems were not used in this study because of the difficulty in defining and controlling experimental parameters. *In situ* cultures are also less convenient when many treatment cells are used, as in the factorial experiments used here to study interactions. Each of these systems of bioassay has advantages which make it attractive. An advantage of chemostat systems over batch systems is that nutrient levels as well as other chemical and physical parameters can be held constant by continuous addition and flushing of the system. Because of this nutrient stability chemostats are recommended for studying uptake and growth kinetics (Torein *et al.*, 1971). Although batch cultures are thought to be inferior to the continuous culture systems by some, they do have certain advantages. Because of their low cost and ease of construction, batch culture bioassays are well suited to factorially designed experiments where a large number of experimental units are needed. The complexity and cost of the chemostat systems make their use almost prohibitive for these types of experiments.

Continuous culture systems maintain a constant nutrient regime which does not approximate the euphotic zone of most fresh water lakes. Because of the changes in nutrient concentration with time, batch cultures allow one to study all of the phases of algal growth: (1) lag phase; (2) exponential growth phase; (3) declining growth phase; (4) stationary growth phase; (5) and death phase (Fogg, 1971). The effect of nutrient availability does not only affect the exponential growth phase but has effects on all of the other growth phases. To get a good understanding of the effects of nutrient availability, all of these growth phases must be studied.

Because of their flexibility, batch culture bioassays were used for all of the growth experiments presented here. The limitations of batch culture were taken into account for each experiment.

Experimental Algae

Scenedesmus obliquus (Türp) Kütz was chosen as the test organism for these studies because species of Scenedesmus are easily cultured and ubiquitus, occurring in almost every freshwater environment (Bold, 1967). Scenedesmus is a common genus to small ponds and lakes with highly colored water (Loub et al., 1954). The genus Scenedesmus is important, ecologically, because species of the genus may make

up as much as 90% of some phytoplankton communities (Round, 1970; Loub et al., 1954). The fact that many species of Scenedesmus are not susceptable to destruction by zooplankton grazing, since individuals pass through the gut undigested or exist as tetrads of cells too large to ingest, make them even more important in phytoplankton blooms. Individuals of S. obliquus tend to be persistent and largely unavailable as an energy source to higher trophic levels. Finally, much is known about the physiology of the genus Scenedesmus which makes the results of a nutritional study more meaningful.

Stock cultures of Scenedesmus obliquus (Türp) Kütz (strain 1952 isolated by Krauss) were obtained from the Indiana University Culture Collection (Starr, 1971).

Stock cultures were maintained in 300 ml Erlenmeyer flasks containing 200 ml of AAP culture medium (Anon., 1971; Toerien et al., 1971). Each flask was stoppered with a two-hole #6 rubber stopper to allow for ventilating the stock with air (see section on culture racks). Stocks were maintained without a bubbling air supply except for the two days prior to their use in an experiment. This kept the growth rate depressed so that cultures needed to be transferred much less often. Bubbling air through the stocks for two days before they were used for experimentation increased the growth rate of the cells and insured that the cells were

in an actively growing phase when they were used. Stocks were kept under 375 ft candles continuous illumination from balanced spectrum "grow lux" fluorescent bulbs while a blower kept the temperature under the lights at 23 C.

Stock cultures were maintained in a viable form by transferring the cells to new culture medium and halving the cell density at 14 day intervals. This prevented senescence because of cell aging and nutrient depletion, and also prevented the build-up of extracellular by-products and wastes. After halving the cell suspension, the cells were centrifuged out of solution and the old medium decanted. They were then resuspended in 200 ml of fresh medium.

The stock cells used in each experiment were from the same part of the growth curve. By keeping the stocks on a regular schedule of medium transfer the stocks were kept in the same physiological state for all of the experiments. It is important to keep the stock algal cells in the same physiological state because if the physiological state of the stock cells is variable, the responses of the cells will be variable. For example, if stock cells are taken from the senescent phase they may not be viable or may show a lag phase.

AAP culture medium was created as a low nutrient medium to somewhat starve algal cells so that they would show a response to various nutrient regimes. However, stock

S. obliquus grown in the AAP medium, was found to carry over enough Fe to mask growth responses of algal cells grown in experimental media with varying Fe concentrations. Fogg (1965) found that Fe was most often the limiting nutrient in artificial algal culture media. Murry (1971) found that the Fe concentration in the AAP medium was limiting to Selenastrum capricornutum and Toerin et al. (1971), found that by increasing the nutrient concentrations of the AAP medium above the standard concentrations Selenastrum capricornutum showed an increased growth response.

Because of the problem of Fe carry-over, stock cultures of Fe-starved algae were grown. This was done by spinning down the normal cells in 100 ml of the actively growing stock (about 10^5 cells/ml) and washing them with 50 mg/l $NaHCO_3$. The cells were then resuspended in 200 ml of AAP medium minus Fe and bubbled with air. The cells were transferred in this way every day for seven days preceding their use for experimentation.

S. obliquus grown in AAP medium also showed a carry-over of PO_4 and PO_4-starved cells were obtained in a manner similar to the Fe-starved cells. When Fe and PO_4 effects were to be studied in combination, stock cells starved for Fe and PO_4 were grown by deleting both Fe and PO_4 from the AAP medium. The limiting levels of Fe and PO_4 and dynamics of algal growth responses to Fe and PO_4 were determined for

the Fe and PO_4-starved stock algae before any experiments were done.

Culture Media

The AAP medium (Table 10) was used as the base medium for stock and experimental media with stock solutions of each nutrient made up in glass containers to 1,000 times the final concentration in the nutrient medium, autoclaved, and stored in the dark. New stock solutions were made up every 4 weeks and only nutrient medium stocks from the same lot were used in each battery of experiments.

Experimental media were made up by using 100 ml of a double strength base medium to which the appropriate amount of experimental solution, such as humic acids, Fe or PO_4 was added. This was made up to 200 ml with distilled, double deionized water. The resulting medium had the desired concentration of experimental compound or nutrient without confounding the experiment with between flask variation introduced by mixing the base medium separately for each flask. The base medium was not always whole AAP medium, with varying concentrations of Fe and PO_4 and EDTA depending upon the experiments. In a pilot study to determine variability, no significant differences could be shown between flasks with media mixed in this way. The experimental media were always made up fresh for each experiment and autoclaved in the experimental flasks.

Table 10.--Synthetic algal assay procedure (AAP) medium developed by the National Eutrophication Research Program, Environmental Protection Agency (Anon., 1971).

Macronutrients			
Compound	Concentration mg/l	Element	Concentration mg/l
$NaNO_3$	25.500	N	4.200
K_2HPO_4	1.044	P	0.186
$MgCl_2$	5.700	Mg	2.904
$MgSO_4.7H_2O$	14.700	S	1.911
$CaCl_2.2H_2O$	4.410	Ca	2.143
$NaHCO_3$	15.000	Ca	1.202
		Na	11.001
		K	0.469

Micronutrients			
Compound	Concentration µg/l	Element	Concentration µg/l
H_3BO_3	185.520	B	32.460
$MnCl_2$	264.264	Mn	115.374
$ZnCl_2$	32.709	Zn	15.691
$CoCl_2$	0.780	Co	0.354
$CuCl_2$	0.009	Cu	0.004
$Na_2MoO_4.2H_2O$	7.260	Mo	2.878
$FeCl_3$	96.000	Fe	33.051
$Na_2EDTA.2H_2O$	300.000	Na	36.900

Physical Conditions for Culture

Experiments were conducted in 300 ml Erlenmeyer flasks fitted with two-hole rubber stoppers. Each flask had a Pasteur pipette inserted through the rubber stopper and bent to reach the bottom of the flask when these were placed at a 45° angle in wooden culture racks (Figure 29 A, B, C). Each flask also had a glass exit vent with a polyethylene plug to prevent foreign matter from falling into the flask as well as to minimize evaporation.

Pilot culture studies found actively growing cultures of S. obliquus to be carbon limited. When not bubbled with air the pH often exceeded 11.0. At this pH carbon becomes limiting to most algae (Goldman, 1972). Increased pH may also cause changes in the solubility and valence state of nutrients and there may be pH induced physiological effects on algae. For example, fluctuations in CO_2 concentration affects nitrate utilization by Scenedesmus (Brown, 1969). The more actively growing cultures reach carbon limiting conditions sooner than do the less actively growing cultures, causing a dampening effect on the differences between experimental treatments. For the algal cells to reach their maximum potential response to a particular treatment, in bioassays, their growth must not be limited by carbon availability.

Figure 29.--Photographs of the algal culture apparatus.

(A) Algal culture rack with 300 ml Erlenmeyer flasks.

(B) Algal culture rack; note aquarium gang valves, tygon delivery system and bent glass delivery tubes.

(C) Algal culture rack in place under fluorescent lights.

(D) In-line 0.45 μ membrane filter.

(E) Algal sampling.

(F) System for axenic culture.

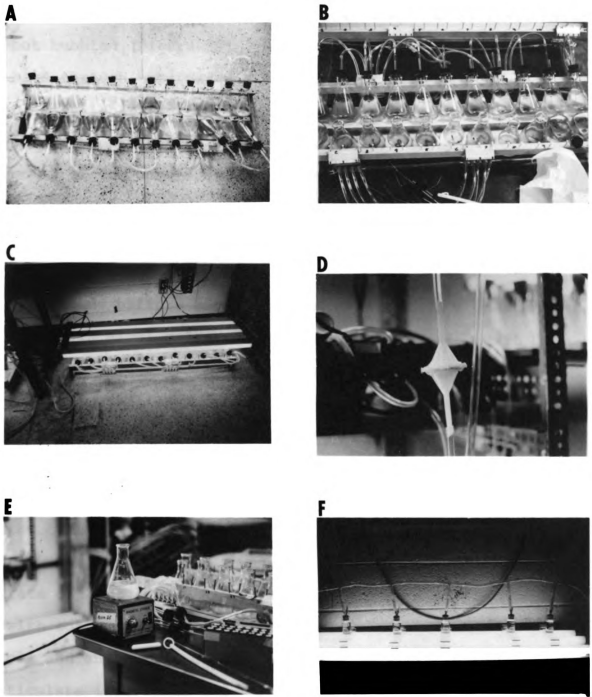


Figure 29

When air was bubbled through the AAP culture medium, much better growth was realized than when the cultures were not bubbled (Figure 30). When 5% CO_2-enriched air was bubbled through unbuffered AAP medium, S. obliquus was completely inhibited but when the CO_2 bubbling ceased, the culture rapidly recovered to the same level as that achieved by cells in the unbubbled medium (Figure 30). Bubbling CO_2-enriched air through the culture media caused the pH to decrease to 4.5 which was probably responsible for the growth inhibition.

Amine buffers are often used to maintain a constant pH regime in algal cultures but these have been found to be inhibitory to S. obliquus and cause morphological changes (Giesy, unpublished data). For this reason amine buffers were not used to maintain a constant pH. Because of the difficulty of controlling the pH when CO_2-enriched air is bubbled through culture media, air only was bubbled through the culture media in this study.

Air was supplied to each flask by piston pumps at a rate of 20 cc/min. After passing through a column packed with cotton and activated charcoal to remove residual pump oil and an in-line of 0.45 μ membrane filter to remove particulate impurities and bacteria, the air was metered to each flask with stainless steel aquarium gang valves mounted on the culture rack (Figure 28b). The flasks were incubated

Figure 30.--Algal growth response (cells per ml) as a function of time (days) to AAP media bubbled with air, 5% CO_2-enriched air and unbubbled medium. Each point represents the arithmetic mean of three replications, with 95% confidence limits reported for each mean.

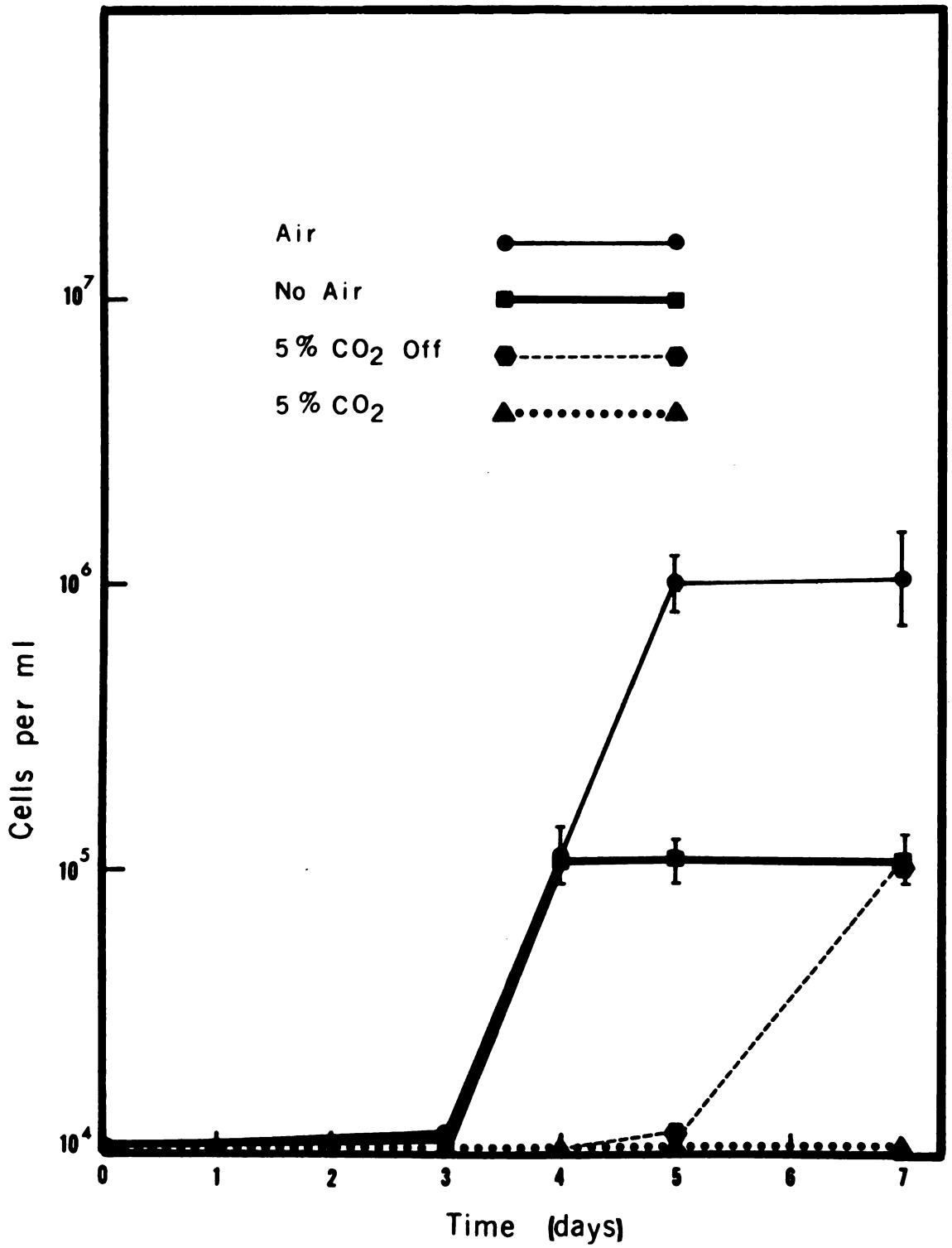

Air
No Air
5 % CO_2 Off
5 % CO_2
10^7
10^6
10^5
10^4
Cells per ml
0
1
2
3
4
5
6
7
Time (days)

Figure 30

at 23 C under 375 ft candles of continuous illumination from ballanced spectrum "grow lux" fluorescent lights.

Axenic Culture Techniques

Non-axenic cultures of S. obliquus had single-celled, gram-negative, rod-shaped bacteria associated with them. These nonflagellated bacteria were found free in the medium and adherent to the S. obliquus cells. To eliminate bacterial effects from the growth and uptake studies, an attempt was made to isolate S. obliquus from its associated bacteria.

A 2% agar suspension with 0.5 mg/l penicillin was made up in full strength AAP medium, poured into petri dishes and autoclaved. Scenedesmus obliquus cells were washed several times with sterile 50 mg/l $NaHCO_3$ and a dilute suspension of these washed cells was plated onto a nutrient-penicillin agar plate and incubated at 23 C, under continuous fluorescent illumination. After 3 days small green clones of S. obliquus and cream colored clones of bacteria began appearing on the surface of the agar.

Sterile micro-pipettes, drawn to very fine tips from Pasteur pipettes, were used to isolate single clones of S. obliquus from the agar. Each clone was suspended in 10.0 ml of sterile AAP medium with 2.0 ml aliquots being plated on fresh nutrient-penicillin agar medium. This procedure was repeated until 3 transfers had been made without any

bacterial colonies growing on the agar. Clones of the axenic Scenedesmus obliquus were transferred to 35.0 ml of sterile AAP medium in serum bottles. Each serum bottle had a rubber diaphragm top so that samples of the cell suspension could be removed with a sterile syringe without contaminating the culture. The culture bottles were placed on glass supports over 375 ft-c of continuous illumination (Figure 29f).

There was no growth observed in the 7 culture bottles. This was thought to be due to carbon limitation so sterile air was delivered to each serum bottle by a 2-inch, 18 guage hypodermic needle and allowed to escape through a ½-inch, 21 guage hypodermic needle (Figure 29f). A piece of tygon tubing containing a cotton plug on the exit vent minimized evaporation and prevented contamination of the culture. The air delivery system consisted of a series of five ml plastic syringe barrels on glass -T's (Figure 29f). The T's were connected with tygon tubing leading to the piston pump which supplied the air. To protect against contamination, the entire air delivery system was autoclaved and the air passed through 0.45 μ membrane filters in the delivery line immediately after the pump.

Aerating the cultures did not stimulate growth, indicating the S. obliquus was being limited by something other than inorganic carbon. The addition of 2.0 mg/l vitamin B_{12} and biotin also failed to promote algal growth.

When the normally associated bacteria were reintroduced to the axenic cultures, the S. obliquus grew. Scenedesmus obliquus could be grown axenically on agar, but could not be grown axenically in aqueous AAP medium. This indicates that something required by S. obliquus is supplied by bacteria when the alga is grown in an aqueous medium. Working with the blue-green alga Microcystis aeriginosa, Reilly (1972) found that when the alga was separated from its associated bacteria there was little or no algal growth.

Because Scenedesmus obliquus could not be grown in axenic media, all of the growth and uptake studies were done in non-axenic systems. It is difficult to separate the algal and bacterial effects on algal growth and nutrition if cultures are not grown axenically. Although it is desirable to separate out bacterial effects for academic reasons, studying algal growth in the presence of their naturally associated is acceptable from an ecological point of view because in nature the bacteria are always found associated with algal cells. Because of this association bacteria and algae often work as an inseparable ecological unit and should be studied as such.

Measurement of Algal Response

There are many ways to measure and report algal growth responses in batch culture. These include optical density,

cell counts, cell volumes, extracted chlorophyll, *in vivo* chlorophyll fluorescence, and dry weight. Most results are reported as cells/ml or dry weight/l no matter how the response is measured.

Optical density and fluorescence are both easy to measure, but because varying concentrations of humic acids, which absorb in the visible region and also fluoresce, were used in these studies, these 2 methods were inappropriate and the small volume cultures used did not provide enough material for chlorophyll extraction or dry weight determinations.

Direct microscopic cell counts were chosen as the best method of measuring the algal growth responses in this study. Samples were counted in 0.16 ml glass algal counting cells and by knowing the total surface area of the counting cell, volume of the counting cell and area of an ocular micrometer grid field, a constant was derived to convert raw *S. obliquus* cell count data to cells/ml.

Several pilot culture experiments were conducted to determine where the greatest amount of experimental error lay. A nested analysis of variance showed the greatest source of error to be the ocular micrometer field counts. There was little error between flasks and less error between samples drawn from one culture flask. To minimize sampling error, the greatest sampling effort was put on micrometer

count replication and culture flask replication. Fifty random micrometer counts were made on a single sub-sample drawn from the 5 ml preserved sample taken from each culture flask. Treatments were always replicated at least twice and generally in 3 or 4 flasks.

Scenedesmus obliquus exists as tetrads as well as single cells. This tendency to remain together in groups of 4 means that the assumption of random distribution is not strictly fulfilled for single cells even though the tetrads may be randomly distributed. This effect was assumed to be minimal and no correction was made for it (Cress, personal communication). The occular micrometer counts followed a poisson distribution when the cell density was low because finding a S. obliquus cell or tetrad of cells was a rare event, but as the cell density increased, the distribution became normal. This should be considered when interpreting the data from the first few days of each experiment, before a cell density of $1X10^4$ cells/ml had been reached.

Because S. obliquus tended to adhere to the bottom of the culture flasks or simply sink to the bottom of each flask, a magnetic stirrer was used to suspend all of the cells before samples for counting were taken (Figure 29e). Five ml samples were taken from each flask and placed in 3 dram screw top sample bottles with 1.0 ml of 37% formalin. Before counting, the content of each sample bottle was drawn

up into a syringe and forced out against the side of the sample bottle to break up aggregations of cells. This procedure did not break up the typical Scenedesmus obliquus tetrads, but did break up clumps of cells. From this homogeneous cell mixture an aliquot was pipetted into the counting cell (0.16ml), covered with a coverslip and counted at 430X. The micrometer counts were converted to cells per ml of original culture after correcting for the dilution due to the preservative.

Expression of Growth Response

There are several ways to express the response of algae to nutrient availability. One of the most widely used parameters in batch culture is the maximum cell concentration (X) or maximum standing crop. This is a relatively easy parameter to measure but does not contain all of the information that can be expressed by growth curves.

Another frequently used measure of algal growth response is the maximum relative growth rate (Anon., 1971; Fogg, 1971; Toerien et al., 1971). Environmental effects are most frequently expressed as rates, so the maximum relative growth rate is a logical measure of biostimulatory responses. Relative growth rates of different species under different environmental conditions are important in algal competition and succession and the maximum relative growth rate is an

important parameter in understanding interspecific interactions under various conditions.

The maximum relative growth rate for algal cells can be determined by batch or continuous cultures (Pearson et al., 1968; Borchardt, 1968). The relative growth rates (k') were calculated for set time intervals of each treatment in each growth experiment on a $\log_{10}$ base using the following equation (Hoogenhout and Amesz, 1965; Fogg, 1971):

$$k' = \frac{\log_{10}N - \log_{10}N_o}{t} \quad ;$$

where

k' = relative growth rate
N = number of cells per ml at time t
N_o = initial number of cells per ml
t = time interval.

The generation time (G), or the length of time it takes for the population to double, was also calculated for each segment of the growth curves using the following equation:

$$G = \frac{0.301}{k'}$$

The constant 0.301 is derived in the following way:

$$k' = \frac{1}{T} (\log_{10}N - \log_{10}N_o)$$

Since the generation time is the time to double the population;

$$k' = 1/T \ (\log_{10}2N - \log_{10}N_o)$$

where now, $T = G$

$$k' = 1/G \log_{10}2$$

$$\therefore = \frac{G = 0.301}{k'}$$

The relative growth rates and generation times for each experiment are reported in Appendix V. Although the maximum relative growth rate is an important ecological parameter for algae, it may be misleading. Two populations of algae under different regimes of nutrient availability can show the same relative growth rate when both populations are in the log growth phase. Much information can be gleaned from the other growth phases. Two populations may show the same maximum relative growth rate but the population where nutrients are more available will show a continuation of the log growth phase to a greater standing crop. This is true because algae are saturated by low concentrations of mineral salts (Fogg, 1971) and all of the available nutrients may be taken up in a very short period of time.

The relative growth rate (k') is related to nutrient concentration by the following expression (Heinshelwood, 1946):

$$\frac{k'}{k'_\infty} = \frac{c}{c+c_1}$$

where

c = the limiting level of a nutrient

c_1 = the half saturation level of the same nutrient.

It follows that as long as the cell density is low enough not to alter the concentration of the nutrient being studied, k' remains constant even though the concentration of the limiting nutrient may vary.

The lag time, an initial period of low or no growth, is also an important ecological parameter (Fogg, 1971). There may be an apparent lag phase caused by inoculation with inviable cells or cells not in a condition to divide immediately. This is why stock cultures were kept in the actively growing log phase. Even actively growing cultures have been found to exhibit a lag phase when transferred to new media (Fogg, 1971). The lag phase has been suggested as the time needed for a culture to acclimate to a new environment or prepare that environment for growth by actively or passively secreting extracellular by-products. This is especially important when studying the effects of chelaters and growth promoting substances.

Two other important growth phases are the stationary phase and death phase. These phases may be only partly determined by environmental parameters but still must be considered when studying the entire effects of the availability of nutrients and effects of growth stimulators on algal populations. Because all of the growth phases of an algal population are important in understanding the effects of growth stimulators on an algal population, all of the growth phases were reported for each experiment as growth curves.

Most of the growth experiments in this study were of factorial designs. This enables one to study the complex interactions of nutrients as well as eliminate the problem of comparing growth responses from experiments run at different times. Standing crops at each sampling are reported with 95% confidence intervals and the results of appropriate statistical tests reported. Only cell numbers were compared statistically. No statistical analysis was done on the growth rates.

APPENDIX II

Purification and Characterization of Humic Acids

APPENDIX II

Purification and Characterization of Humic Acids

The fraction of those naturally occurring organic molecules known as humic substances which are soluble in alkaline solution but precipitated by mineral acids are referred to as humic acids (Schnitzer and Khan, 1972). Because of their ubiquity and importance in soil and aquatic science, they have been much studied and numerous books have been written on their chemical composition, structure and characteristics.

Humic substances are some of the most widely distributed natural products on earth, making up the bulk of the organic matter in most soils and sediments (Schnitzer and Khan, 1972). Although there is some variation from location to location, most humic acids are very similar in their structure and chemical nature. The greatest variation is in the length of side chains and proportions of various active sites (Steelink, 1963). Nissenbaum and Kaplan (1972) suggested that humic and fulvic acids are of the same general structure, with humic acids being more highly polymerized and containing a smaller number of -COOH groups per unit weight.

Humic acids are a diverse group of 3-dimensional, polymorphic substances of high molecular weight, which are resistant to decomposition (Gillam, 1940; Ishiwatari, 1960; Murphy and Moore, 1960; Felbeck, 1965; Christman and Ghassemi, 1966a; Guminski, 1968). These compounds are discussed as having definite molecular weights, although they may exist as colloids in nature.

There are 4 hypotheses for the mechanism of humic acid synthesis. These include the plant alteration hypothesis, the chemical polymerization hypothesis, the cell autolysis process and the microbial synthesis hypothesis (Knonoova and Sandrova, 1958, 1959; Kononova et al., 1960; Flaig, 1964; Felbeck, 1971). The actual process is probably a combination of all 4 of these mechanisms, in which humic compounds are synthesized from a multiplicity of naturally occurring organic compounds. The main precursor of all humic substances is lignin (Christman and Ghassemi, 1966a, 1966b). To this backbone of lignin may be added many organic compounds and functional groups.

These functional groups may include carboxylic, methoxyl, acetyl, hydroxyl and amine groups as well as numerous less common groups and various length alkyl groups (Forsyth, 1947; Murphy and Moore, 1960; Chalupa, 1966; Schnitzer and Khan, 1972). There may also be sugars, alcohols, aldehydes, ketones, polypeptides and amino acids

integrated as part of the structure (Ishiwatari, 1966; Guminski, 1968). Polyvalent metals may also be structural components of the humic acids.

Humic acids may be formed in lake and marine sediments as well as soils. The humic acids formed in lake and marine sediments may differ from those formed in soils although the general chemical and structural characteristics are the same (Ishiwatari, 1966). The humic acids found in lake sediments are probably of both autochthonous and allochthonous origin, while those present in unpolluted streams are predominately allochthonous (Flaig, 1960).

Purification of Humic Acids

Humic acids used in these studies were obtained from the Aldrich Chemical Company (H 1675-2; lot # 082091). These humic acids were prepared from European sources and supplied in powdered form. The assayed melting point reported was 300 C or greater.

The most widely used method of extracting and separating humic acids is by aqueous alkaline extraction (Bremner, 1954; Shapiro, 1957; Murphy and Moore, 1960; Ishiwatari, 1966; Christman, 1966; Martin, 1971). Other methods have been used with success but since the definition of humic acids is based on solubility properties, a dilute alkaline extraction was used to solubolize the humic acids for

purification. Extraction with strong NaOH may create new exchange sites and cleave the humic acids, thus changing the exchange capacity (Lewis and Broadbent, 1961) and there has been some evidence that some autoxidation of humic acids may occur under alkaline conditions (Bremner and Lees, 1949; Bremner, 1950, 1951; Tinsley and Solam, 1961). Work by Choudhiri and Stevenson (1957) showed that extraction with 0.5 N NaOH did not change the characteristics of humic materials. Humic acids extracted with NaOH, H_2O or $Na_3P_2O_7$ were the same (Forsyth, 1947a; Schnitzer and Skinner, 1968a).

A 5 gram sample of the Aldrich humic acids was dissolved in 25.0 ml of 0.25 N NaOH and made up to 500.0 ml with distilled, doubly deionized water. This was sufficient to dissolve all of the humic acids. The humic acid solution was then acidified (pH = 6.6) with concentrated HCl, causing the humic acids to precipitate. The brown, humic acid precipitate was alternately centrifuged, at 5,000 RPM, and washed. The first 3 washes were 100 ml volumes of 0.05 N HCl and the final 4 washes were with 200 ml of distilled water. The supernatents from the first 2 washes were light brown indicating the presence of some impurities. This may have been due to the presence of a small quantity of fulvic acids in the commercially prepared humic acids or due to fragmentation of the humic acids by the purification process. The discarded supernatent from all successive washes was

clear and colorless. Humic acids have not been found to be hydrolized by dilute HCl (Forsyth, 1947) but there may have been some fragmentation of side chains by the alkaline extraction. It was thought that the color in the first 2 washes was due to impurities in the original mixture rather than humic acid fragments. The washed precipitate was then washed with two 50.0 ml volumes of 95% ethanol to remove impurities of hymatomelanic acids. There was no color extracted by these 2 washes, indicating that there were no hymatomelanic acids in the original humic acid mixture.

After desiccation over $CaSO_4$, 250.0 mg of the purified humic acids were redissolved in 5 ml of 0.25 N NaOH and made up to 1,000 ml with distilled water. It has been suggested that drying with heat may destroy some of the properties of humic acids but changes seem to be minimal for acids dried at room temperature (Forsyth, 1949; Chalupa, 1966). Shapiro (1966b) found no difference in molecular weight between dried and undried humic acid samples from lake sediments.

The 250.0 mg/l stock humic acid solution was dialyzed against distilled water in a Dow "beaker ultrafilter" with a molecular weight exclusion of 30,000 for 2 hours, to remove free inorganic ions as well as lower molecular weight organics. The nominal molecular weight exclusion is listed by the manufacturer for the hollow fiber apparatus as the

molecular weight which will be 85% retained by the cellulose acetate fibers. This molecular weight designation may not be completely applicable to humic acids since they may exist as colloids instead of dissolved molecules. Even the steric considerations of the polymorphic humic acids makes the molecular weight exclusion a crude approximation. Although the dialysis procedure was not an exact method of qualifying the molecular weights of the humic acids used, it did assure that the humic acids used for experimentation were of high molecular weight. This purified and dialyzed stock was then used to make up media for each experiment.

No effort was made to remove the ash content from the purified humic acids. It was thought that since the humic acids occur in association with clays and other minerals in natural systems that the ash content of the humic acids was acceptable. Treatment with HF-HCl to reduce the ash content may also have adverse effects on the structure of the humic acids.

The extraction and purification method should always be considered when comparing results of experiments using purified humic acids and when extrapolations to natural systems are made.

Characterization of Humic Acids

There are many physical and chemical parameters used to characterize humic acids. A partial list of these includes:

ultimate analysis, nitrogen distribution (Bremner, 1965), functional group analysis, absorption in the visible, ultraviolet and infrared regions of the electromagnetic spectrum (Sato and Kumada, 1967), nuclear magnetic resonance, electron spin resonance, X-ray analysis, electrometric titrations, gas chromatography, gel permeation, paper and thin layer chromatography and electrophoresis. All of these techniques have been employed with greater or lesser success in the study of humic acids. Because humic acids are not made of a pure compound but rather a mixture of similar compounds of varying structure and molecular weight, many of these techniques to estimate character are not very useful. Although some of the techniques are not useful in determining structure or composition they are still useful in gross characterization of the humic acids. Since the structure and empiricle formulae of these compounds can not be accurately determined, their characterization takes on a greater importance.

Molecular Weight

Many methods have been described for the determination of the molecular weights of humic acids (Clark, 1941; Hanson and Schnitzer, 1969; Wershaw et al., 1970; Brogden, 1971). One of the most widely used methods is gel permeation (Posner, 1963; Dubach et al., 1964; Gjessing, 1965; Gjessing

and Lee, 1967; Bailly and Margulis, 1968; Schnitzer and Skinner, 1968b). Gjessing (1965) using gel permeation, found the surface waters of Norway to have 2 distinct molecular weight fractions, one with molecular weights of about 10,000 and the other with molecular weights of between 100,000 and 200,000. Gjessing (1970) also found that less than 10% of the organic carbon and 1% of the color of aquatic humus from Norwegian lakes was due to the fraction with a molecular weight of less than 1,000, while more than 50% of the organic carbon and 90% of the color was due to the fraction with a molecular weight of 20,000 or greater. Steelink (1963) found some humic acid fractions to have a molecular weight as high as 500,000, while Rashid (1971) reported some humic acid fractions with molecular weights less than 700.

Shapiro (1966b) reported that the humic acids of some Minnesota lakes could be grouped into 4 ranges. Marine sediments also seem to be grouped into 4 distinctly different fractions: less than 700; 700-10,000; 10,000-100,000 and greater than 100,000 (Rashid, 1971). Schnitzer and Khan (1972) reported that some humic acid fractions may have molecular weights as great as several millions.

The molecular weights of humic acids are quite variable (Schnitzer and Khan, 1972) and may not be a valid measure of the true size of humic acids. The various molecular

weight fractions probably are caused by different length chains of the basic polyphonelic backbone of the humic acid structure. As was pointed out before, humic acids may exist naturally as colloids so that a molecular weight determination is meaningless. Because humic acids probably differ in charge and structure from the dextrans normally used as standards in gel permeation molecular weight determinations, it is difficult to assign meaningful molecular weights to humic acid fractions. Since molecules in the fractionation range of the gel enter the pores of the polysacharide spheres, molecular shape plays a large role in the way a particular set of molecules fractionate. A better characterization method might be a size determination using a membrane technique with known pore sizes. Although the exact molecular weights of various humic acids may not be determined by the gel permeation technique, the method may be used successfully to determine ranges of approximate molecular size and shape.

The molecular weights of the humic acids used in these studies were not determined, but the size-shape ranges were characterized using "Sephadex G-200". This particular polysacharide gel has a fractionation range of 5,000-800,000 for peptides and globular proteins and a fractionation range 1,000-200,000 for dextrans which was assumed to cover the "molecular weight" range of the purified humic acids.

A 40 cm glass column (½ cm I.D.) was filled with swollen gel and allowed to pack for 24 h, at a flow rate of 1.0 ml/min with pH 6.85 Sorenson's buffer. After layering 0.2 ml of a 1.0 g/l humic acid solution onto the top of the column, the column was eluted with Sorenson's buffer at a rate of 1.0 ml/min. One ml fractions were collected by a fraction collector and the humic acid concentration determined fluorometrically (see section of fluorometric determinations). The fluorescence, as read, was plotted as a function of the fraction number (ml) to give a "molecular weight" distribution (Figure 31b).

The column was allowed to elute for more than 100 fractions with all of the humic acids being eluted in the first 25 ml. The eluted humic acids were diluted by a factor of 125. There was only one maximum in the size distribution, although the distribution was spread out in the later fractions. This spreading was probably due to pibonding to the gel particles by the functional groups of the humic acids. All of the humic acids seem to be of similar "molecular weights", although the exact molecular weights were not determined because of the above mentioned difficulties. The apparent bimodal distribution is probably an artifact due to adsorption or fraction collection.

Figure 31.--(A) Elution of humic acids and bound ^{59}Fe. The dotted line represents actual activity determinations for each fraction, while the histogram represents the area under the curve (total CPM) used to calculate the total bound to the humic acids.

(B) Distribution of humic acids eluted from "Sephadex G-200" gel filtration column.

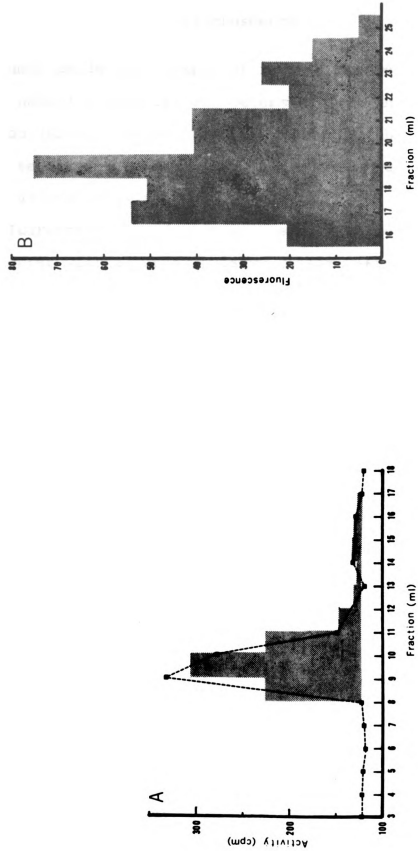


Figure 31

Fluorescence

Humic acids are capable of intense fluorescence which can be used for quantitative determinations in an aqueous solution (Christman and Ghassemi, 1966a, 1966b). Buck (1968) reported a peak of maximum fluorescence at 4700 Å for an activation wavelength of 3350 Å.

Fluorescence was determined using a Turner model 111 fluorometer with a Turner primary filter 7-60 and a secondary filter combination of Turner filters 2A and 48. All determinations were made in 12X75 mm, 8 cc, "Pyrex" cuvettes using a 3660 Å mercury line as an excitation source. With the fluorometer connected to a Sargent model SR strip chart recorder, responses were expanded to 12.5 mv full scale.

Because the fluorescence of organic compounds is sensitive to pH and temperature fluctuations, all fluorometric determinations were made at pH 8.3 and 23 C.

The standard curves for the 3X and 1X sensitivity settings for concentrations of humic acids between 10 and 100 mg/l showed that the relationship was linear only for the concentrations below 20 mg/l (Figure 32). Standard curves were prepared for humic acid concentrations between 1.0 and 10.0 mg/l at the 3X sensitivity setting and from 1.0 to 5.0 mg/l for the 10X sensitivity setting (Figure 33).

The linear regression prediction equation for the 3X sensitivity setting was determined to be:

Figure 32.--Humic acid fluorescence as a function of concentration from 0 to 100 mg/l humic acids for sensitivity settings of 1X and 3X. All determination made at 23 C and a pH of 8.3.

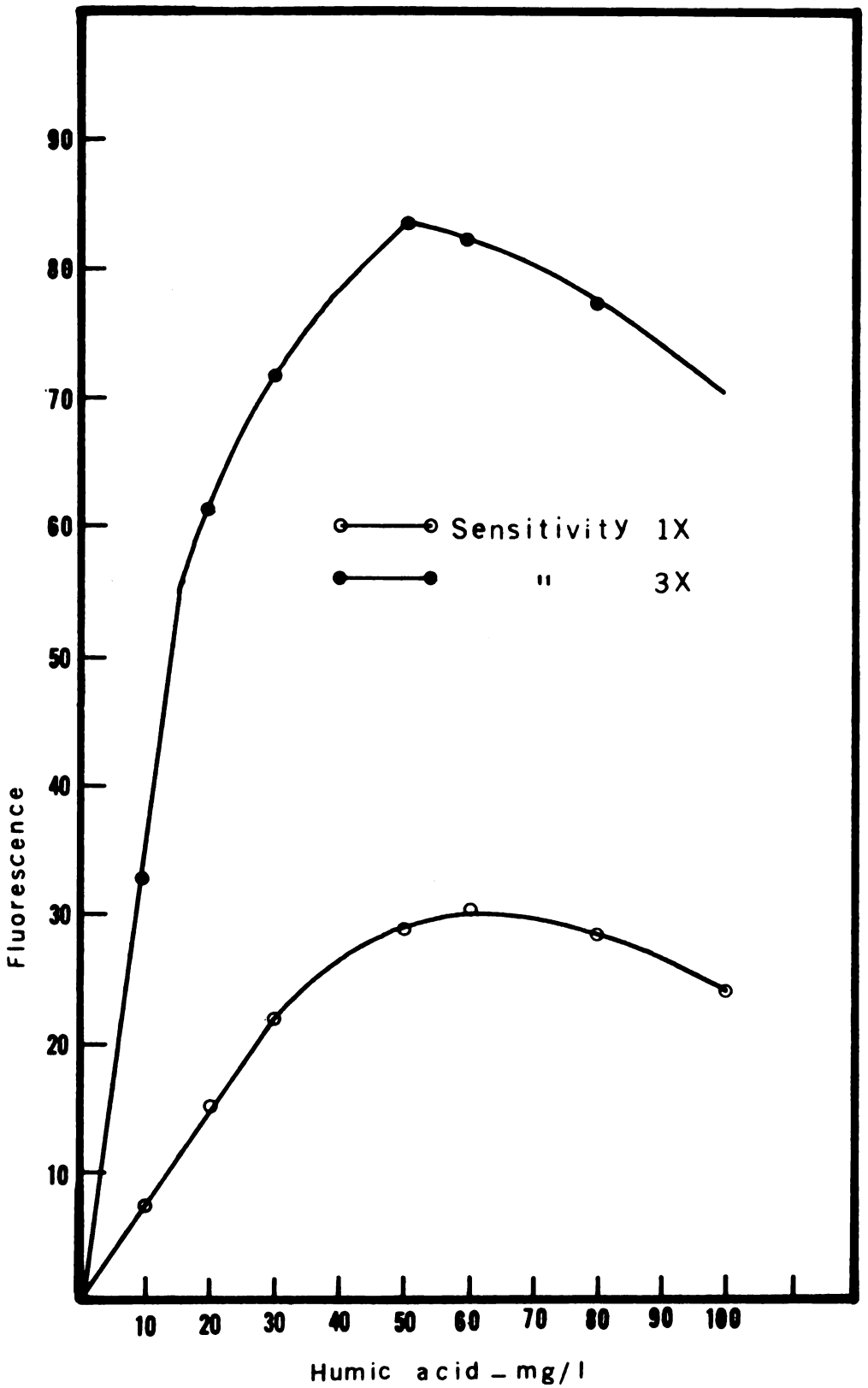

90
80
70
60
50
40
30
20
10
Fluorescence
Sensitivity 1X
" 3X
10
20
30
40
50
60
70
80
90
100
Humic acid - mg/l

Figure 32

Figure 33.--Humic acid fluorescence as a function of concentration from 0 to 10 mg/l humic acids for a sensitivity setting of 3X and from 0 to 5 mg/l humic acids for a sensitivity setting of 10X. All determinations were made at 23 C and a pH of 8.3.

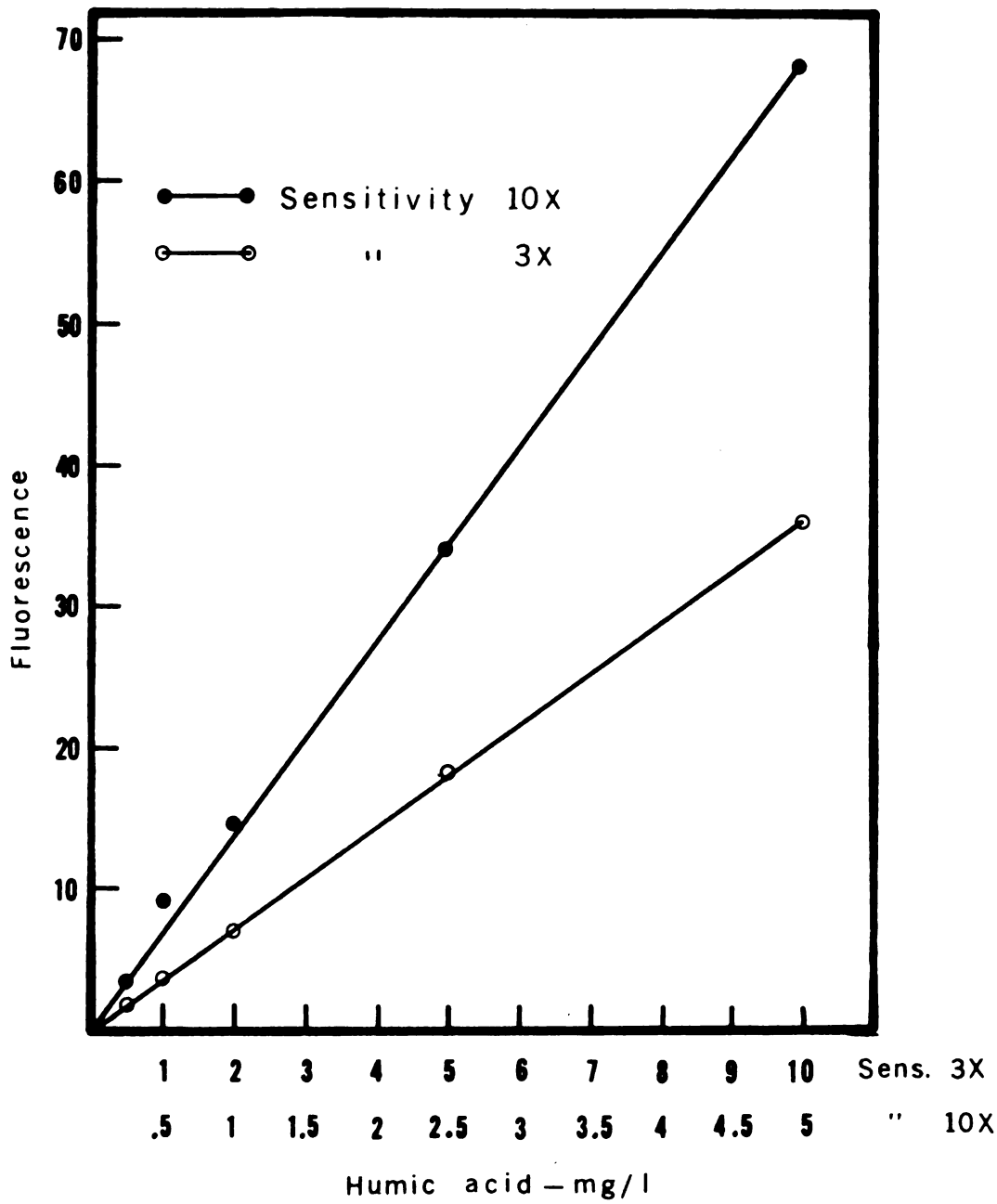

70
60
50
40
30
20
10
Fluorescence
Sensitivity 10X
" 3X
1 2 3 4 5 6 7 8 9 10 Sens. 3X
.5 1 1.5 2 2.5 3 3.5 4 4.5 5 " 10X
Humic acid — mg/l

Figure 33

$$\hat{Y} = 0.282X-0.197.$$

The coefficient of regression of fluorometer units on humic acid concentration was 0.999.

From this equation, the humic acid concentrations could be calculated but most of the laboratory studies required only relative measurements in fluorometer units. The use of fluorometry to detect humic acids in natural waters is limited because of interferences from other organics in the humus group as well as from dissolved organics such as chlorophyll, proteins and the whiteners added to detergents (Wright and Collings, 1964).

Visible Spectrophotometry

Visible spectral characteristics have long been used in the study of humic acids. The color of natural waters has been one of the routinely measured parameters in limnological studies and initially was measured by comparison to some standard such as platinum cobalt. The color characteristics are now determined spectrophotometrically.

The absorbance (optical density) of a 250.0 mg/l solution of purified humic acids was determined at 20 mμ intervals between 350 and 950 mμ using a Beckman DB-G spectrophotometer (Figure 34b). The featureless visible spectrum, which decreased continually with increasing wavelength, closely resembles those found by Schnitzer and Khan (1972) for humic acids.

Figure 34.--(A) Visible spectrum of humic acids of molecular weight 30,000 or greater used in this study.

(B) Ultraviolet spectrum.

(C) Infrared spectrum with polystyrene calibration peak at 1944 cm^{-1}.

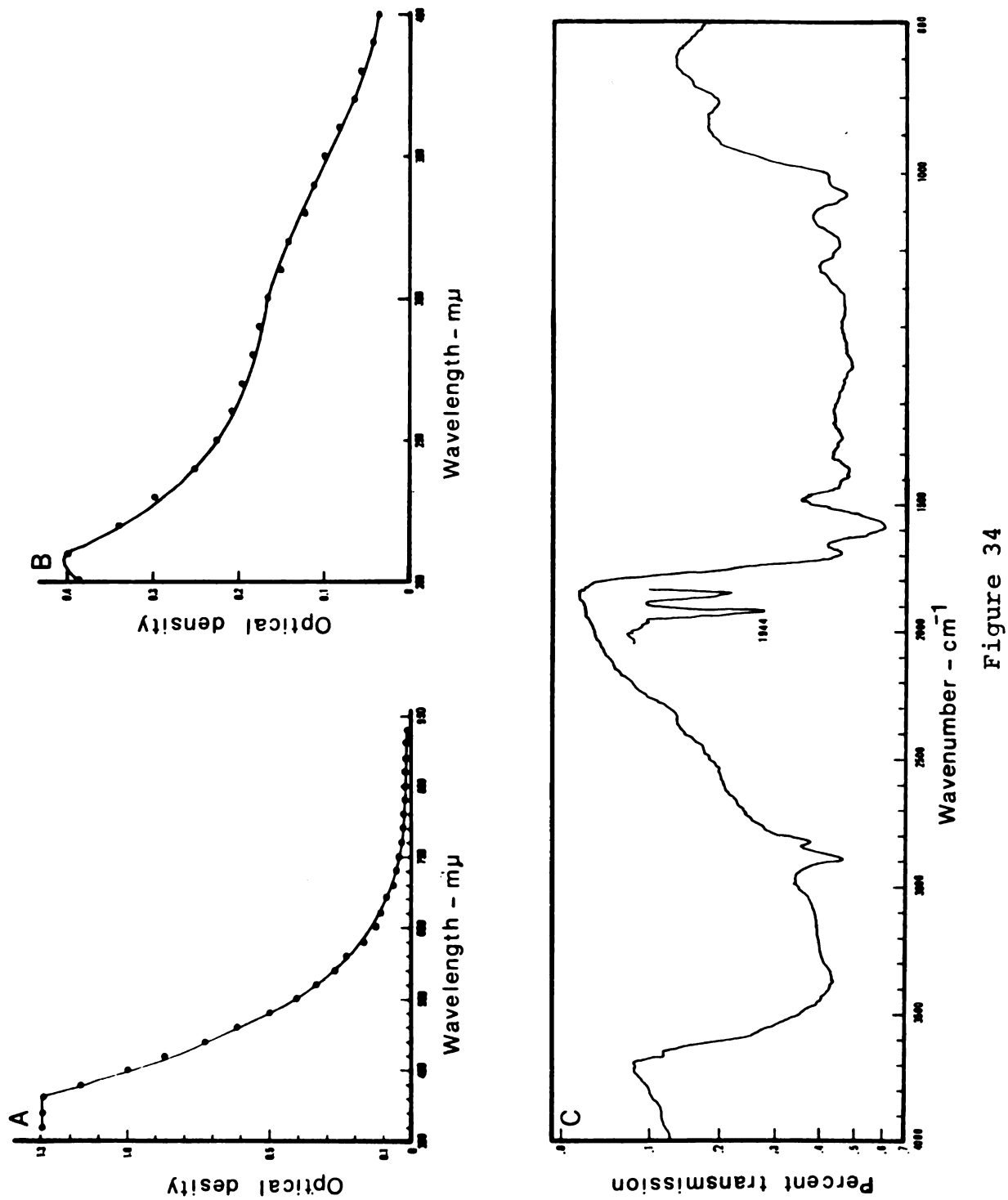

A
Optical desity
Wavelength - mμ
B
Optical density
Wavelength - mμ
C
Percent transmission
Wavenumber - cm-1
1944

Figure 34

The E_4/E_6 ratio, which is the ratio of the absorbances at 465 and 665 mμ respectively, is often used for the characterization of humic acids (Schnitzer and Khan, 1972). This ratio is independent of concentration but varies for humic acids from different sources (Konova, 1966). The E_4/E_6 ratio for the humic acids used in this study was calculated to be 9.9. Schnitzer and Khan (1972) reported E_4/E_6 values of about 5.0 for brown humic acids while Kononova (1966) found grey humic acids to have E_4/E_6 ratios of between 2.2 and 2.8. Increased humification and condensation generally causes a decrease in the E_4/E_6 ratio. This indicates that the humic acids used in this study were less aromatic and thus of more recent origin than most of the soil humic acids which have been studied. Fulvic acids have been reported to have E_4/E_6 ratios of between 6.0 and 8.5 (Kononova, 1966).

Direct spectral analysis is only valid for humic acids of similar origin so the molar absorbtivity (extinction coefficient, $E_1^{0.001\%}$ cm) is often used to characterize humic acids (Orlov, 1967). The molar absorbtivity for the humic acids used in this study was calculated to be 0.04 when measured at 465 mμ, which corresponds to the value found by Orlov (1967) for humic acids extracted from a sod podzolic soil.

Ultraviolet Absorption Spectra

The multiple bonds and unshared electron pairs which confer color to organic molecules also absorb energy in the ultraviolet region of the electomagnetic spectrum (200-400 mμ). This absorption may be used to characterize the structure of organic compounds.

An ultraviolet absorption spectrum (Figure 34a) was determined using a Beckman DB-G spectrophotometer. The large number of double bonds and unshared electrons in the humic acid functional groups are probably responsible for the smooth curve obtained. When more than one chromophoric group is present, the interpretation of spectra becomes difficult. Since humic acids occur as mixtures and not as pure compounds, they generally show a smooth curve of decreasing absorbance with increasing wavelength. Because of these problems, ultraviolet spectra are of little use in characterizing humic acids and identifying functional groups, although they may be useful in identifying fragments and decomposition products.

Infrared Spectrophotometry

Infrared spectrophotometry can be used for the gross characterization of humic acids of diverse origins as well as for determining structure (Ceh and Hadzi, 1956; Theng et al., 1967). Infrared spectra are generally used to

detect the presence of particular functional groups of structural configurations or the effect of treatments such as oxidation and reduction on humic acid molecules (Farmer, 1960). Because of the uncertainty of peak identification, the characterization of humic acids by infrared analysis should be substantiated by other methods (Schnitzer and Skinner, 1963).

Desiccated, purified humic acids were pressed into a pellet with KBr as the matric material and the spectrum determined with a Perkin-Elmer model 337 infrared spectrophotometer.

The resulting spectrum (Figure 34c) was very similar to the spectra obtained by others working with humic acids (Elofsen, 1951; Kumada and Aizawa, 1958; Schnitzer, 1971). The spectrum was a series of broad bands caused by the many functional groups and heterogeneity of the humic acid mixture. The large bands between 2900 cm^{-1} and 3500 cm^{-1} may be due to many groups including the absorptions of C-H stretching, N-H stretching, O-H stretching and aromatic C-H stretching, all of which may be present in the purified humic acids. The large peak at 1100 cm^{-1} is undoubtedly due to the C-O stretching of the numerous carboxyl groups associated with the humic acids. The fingerprint region (less than 1000 cm^{-1}) is one broad band, again due to the multiplicity of functional groups and is not of much use qualitatively.

Because of their complexity, humic acids absorb at almost all of the infrared wavelengths, causing all humic acids to have similar infrared spectra. Rice et al. (1962) found that humic acids all had similar infrared spectra regardless of where they were collected. The fact that all humic acids infrared spectra are so similar they are of almost no use for humic acid characterization.

Nutrient Analysis of Humic Acids

Humic acids are large organic molecules composed mainly of carbon, oxygen, nitrogen, hydrogen and sulfur with small quantities of numerous other elements integrated into their structures and associated as chelates, complexes or surface sorbed.

Schnitzer and Khan (1972) compiled an inventory of humic acid ultimate analyses. They found that the percent carbon for soil humic acids ranged from 53.7% to 60.4% while the carbon content of humic acids from lake sediments was 53.7%. They also reported soil humic acids as being between 3.7% and 5.8% hydrogen, 1.6% to 4.1% nitrogen, about 0.4% sulfur and 31.9% to 36.8% oxygen. Lake sediment humic acids were very similar with a composition of 5.8% hydrogen, 5.4% nitrogen and 35.1% oxygen.

The humic acids used in this study were analysed to determine their phosphorus and metal content. Five grams of

purified and unpurified humic acids were both refluxed with 400 ml of concentrated HNO_3 and 50 ml of concentrated $HClO_4$ for 12 h in a 500 ml glass distillation flask. The HNO_3 was then distilled off and the $HClO_4$ allowed to reflux until the reaction mixture reached 200 C. This resulted in a 60 ml sample which was diluted to 100 ml with distilled water and stored in polyethylene bottles.

Sulfur, released by the digestion, floated to the top of the acid mixture but was not assayed. There was also an oily residue, perhaps due to the very resistant phenolic fractions of the humic acids and a precipitate which was probably due to clay.

Metals were measured by atomic absorption spectrometry within 24 h of digestion. The Jarrel Ash model 800 unit was zeroed with dilutions from refluxed acid blanks and standard curves were used to compare with the metal concentrations in samples of the original solution diluted 1 to 10 with distilled water.

No detectable concentrations of the following metals could be found in the digested, unpurified humic acids: Ni, Co, Cu, Zn, As, Pb, Mn, Si, Cd, V and Mo. Fe, Ca and Mg were the only metals that were detected. Fe was in the greatest abundance, with the purified and unpurified humic acids having about 20.0 mg Fe/g humic acids. This Fe was thought to be structural Fe and not present as a complex or chelated form because the acid purification procedure would

remove most of the Fe bound in these forms. The binding of metals to the humic acids may have involved the associated clays but this was not studied here.

The digested unpurified humic acids had 2.5 mg Mg/g humic acids and 1.1 mg Ca/g humic acids. No detectable concentration of either of these elements could be found in samples of the digested, purified humic acids. These elements were probably present in the raw humic acids as chelates, complexes or surface bound by sorption phenomena and were removed by the purification process.

PO_4 in the undigested purified and digested samples was analysed using the ammonium molybdate-stannous chloride method. No PO_4 was detected in either sample.

Ash Content of Humic Acids

Humic acids not only interact with metals but also form complexes with clays and metal oxides. Humic acids are almost always found associated with ash in natural systems (Mortland, 1970). Because of this natural association, it was believed valid to study humic acids in the presence of their associated ash. Humic acids and their associated ash, including clays and metals, act as a functional unit in nature and should be studied as such. The humic acids used in all of the experiments reported here were not freed from their associated ash because it was also believed that the

usual HCl-HF treatment generally used for the removal of ash from humic acids would be detrimental to the humic acids themselves as well as destroying the natural association.

The ash content of the purified humic acids was determined by ashing at 600 C for 6 h. Five gram samples of the humic acids were placed in tared porcelain casseroles and ashed in a muffle furnace. The ashing was done under an air atmosphere. This may have caused some oxidation of the metals associated with the humic acids, which would cause an increase in the estimated ash content. Schnitzer and Khan (1972) reported that the combustion of humic substances occurs between 350 and 550 C and weight losses above this temperature range are due to reorganization of the ash materials and loss of waters of hydration from the clay materials.

The ash content of the purified humic acids was found to be 15.9%. Schindler (personal communication) found humic acids from the South Eastern United States to have an ash content of about 15% after an HCl-HF treatment.

The ash associated with the humic acids was not assayed for elemental content but an ocher color indicated the presence of Fe.

Iron Binding Capacity of Humic Acids

The interactions of metal ions with humic acids may be ion exchange (Basu, 1964), surface sorption, complexation

(Schnitzer and Khan, 1972), chelation, coagulation or peptization reactions (Mortland, 1970). The metals bound to humic acids may be held in part by all of the above mechanisms. The numerous carboxyl, phenolic and carbonly groups are probably responsible for the complexing and chelation of metals by humic acids and the fact that the humic acids may also act like colloids explains the possible surface reactions of humic acids and metals. There has been much work done on the determination of metal-organic matter chelates (Martell and Calvin, 1952; Chaberek and Martell, 1959; Rossatti and Rossatti, 1961).

Humic acids have a particular binding capacity for metal ions such as Fe. This capacity is generally measured as a stability constant calculated either thermodynamically or stochiometrically (Stevenson and Ardakani, 1972). The methods of calculating stability constants have been successful for metal complexes and chelates with ligands of known molecular weight structure and exchange capacity (Stevenson and Ardakani, 1972), but may not be suitable in cases where the molecular weight and structure are not known or the ligand is a mixture of compounds or heterogeneous structures or exchange capacities.

Since the calculation of stability constants is not applicable to humic acids, an Fe binding capacity was calculated for the purified humic acids used in the experiments reported here. This is simply the amount of Fe that will be

bound by the humic acids at a particular temperature and pH regime, expressed as mg Fe/mg humic acids. Two methods, a gel permeation method and a dialysis method, using an ^{59}Fe tracer, were used to determine the Fe binding capacity of the purified humic acids.

Gel Permeation Technique

The Fe binding capacity of purified humic acids was determined using a gel permeation column of "Sephadex G-15". After swelling in distilled water for 24 h, the gel was slurried into a 40 cm glass column with an inside diameter of ½ cm and packed at a rate of one ml/min for 3 hours with a solution of 1.0 mg/l stable Fe and $6X10^{-8}$ mg/l ^{59}Fe (202 CPM/ml). A 500.0 mg/l solution of humic acids was made up to have the same specific activity as the eluent. After both the humic acid and eluent had come to equilibrium with their mixing flask, their activities were found to be 173 and 175 CPM/ml respectively.

The eluent was passed through the column at a rate of 1.0 ml/min until the fractions collected showed a constant activity (Figure 31a). A 0.1 ml sample of the humic acids was then layered onto the top of the column and eluted at a rate of 1.0 ml/min. One ml fractions were collected with a fraction collector and their activity determined with a well type, No. I, single channel scintillation counter.

Because the half life of ^{59}Fe is 45 days and the activities were all determined immediately, no correction was made for decay. Since all determinations were relative, all of the activities were reported as counts per minute (CPM) and not corrected for counter efficiency or converted to mg/l Fe.

The fractionation range of the "Sephadex G-15" gel is 0-1,500 molecular weight units. Since the purified humic acids had a molecular weight of 30,000 or greater, they were not in the fractionation range of the gel and were excluded from the pores of the gel. The larger humic acid molecules were eluted in the void volume of the gel (V_o), while the Fe atoms in solution were fractionated. Because the free Fe atoms were entering the pores of the gel, they were eluted in a volume greater than V_o. The eluent and humic acid mixture had the same specific activity, so as the humic acid molecules with their bound Fe moved ahead of the unbound Fe atoms that they were added with, the activities of the fractions containing humic acids were increased by the amount of ^{59}Fe bound to them. By subtracting the activity of the eluent from the fractions containing humic acids, the activity of the ^{59}Fe bound to the humic acids was calculated. The activity of each fraction was plotted as a function of the fraction number and the area under the curve computed (Figure 31a). A total of 517 CPM or $9.30X10^{-10}$ mg of ^{59}Fe was bound to the humic acids. By knowing the specific activity of the samples, the bound stable Fe was calculated to be

$1.5X10^{-2}$ mg Fe/mg humic acids. Replications of this determination produced values of $1.6X10^{-2}$, $1.7X10^{-2}$ and $1.4X10^{-2}$ mg Fe/mg humic acids.

Fluorometric measurements of the fractions indicated that all of the humic acids were eluted with a dilution factor of 45.

Dialysis Technique

A 50 ml solution containing 10 mg humic acids, 0.5 mg stable Fe and $6.3X10^{-4}$ mg ^{59}Fe (34,860 CPM), giving a specific activity of $6.98X10^{-4}$ CPM/mg Fe was allowed to react for 30 min and placed in a 1.0 in diameter dialysis bag, which was suspended in a flask containing 3 liters of distilled water. The system was allowed to stand for 7 days, after which time the activity inside and outside the dialysis bag was determined and corrected for decay to the start of the experiment. The distilled water outside the dialysis bag was acidified with concentrated HCl to remove absorbed Fe from the glass sides of the reaction flask. The activity inside the dialysis bag was 452 CPM/ml while that outside the bag was 3.2 CPM/ml. Assuming that the solution inside the dialysis bag also had an activity of 3.2 CPM/ml unbound Fe, the activity of the bound Fe was 449 CPM/ml. Using the specific activity, the amount of bound stable Fe was calculated to be $3.2X10^{-2}$ mg Fe/mg humic acids,

which is very near the value obtained with the gel permeation method. Shapiro (1966b) found that the Fe binding capacity of dissolved organic acids varied from one lake to another and from one pH regime to another. The Fe binding capacity of the humic substances in the lakes studied by Shapiro (1966b) ranged from 1.5 mg Fe/mg humic substance to 2.6 mg Fe/mg humic substance at a pH of 9.0, which are higher than the values determined here. The pH of the solutions used to determine the Fe binding capacity reported here were all 8.3. The greater binding capacity reported by Shapiro (1966b) may be due to differences in the organic acids studied or to the initial Fe concentration associated with the organic acids. There also may have been further differences caused by the extraction methods used. Rashid (1971) found that humic acids of molecular weight less than 700 had the greatest binding capacity for divalent metals on a weight per weight basis with binding capacity decreasing as the molecular weight of the humic acids increased. The molecular weight distribution of the humic substances in the lakes studied by Shapiro (1966b) may have been composed of smaller molecular weights or the extraction procedure used by Shapiro may have selected for the lower molecular weight fractions in the lake waters. It has been determined that about a third of the exchange sites of humic acids exist in the nonchelating form, although this number would change with molecular size and structure.

Release of Bound Fe From Humic Acids

When studying the Fe binding effects of humic acids on the availability of Fe to algae, it is important to know something about the rate of release of bound Fe from the ligand. It is also necessary to know the ability of common ions to displace Fe from the organically bound forms.

Several experiments were conducted to determine the rate of Fe release from the purified humic acids used in the Algal growth and uptake studies. The ability of Ca to displace bound Fe was also studied. Since a stability constant of the traditional type is inappropriate in the humic acid metal system (Stevenson and Arakani, 1972) a simple release rate was determined using a ^{59}Fe dialysis technique. The humic acid-Fe complex described in the section on binding capacity was used to study the rate of release of Fe from humic acids. The humic acids studied had their binding sites completely saturated with Fe ($1.6X10^{-2}$ mg Fe/mg humic acids and a specific activity of $6.98X10^{-4}$ CPM/mg Fe). The pH of the humic acid solution was 6.5 while that of the distilled water was 6.7.

A 10.0 ml aliquot of the humic acid-Fe complex was placed in a dialysis bag and suspended in 20.0 ml of distilled water in a 9 dram plastic counting vial. The dialysis bag was periodically removed and the activity in the distilled water determined. By counting the whole vial there

was no error due to adsorption losses of Fe to the sides of the vial.

After 4 days the activity of the distilled water was 0.3 CPM/ml. This is about the activity that would be expected from partitioning of the 3.0 CPM/ml due to the free ^{59}Fe in the humic acid solution. This free Fe alone would be expected to produce an activity of 0.5 CPM/ml in the distilled water. This indicates that Fe bound to humic acids of molecular weight 30,000 or greater is bound very tightly, making it essentially unavailable. Stevenson and Ardakani (1972) suggested that this is due to the fact that the larger humic acid molecules have basic and acidic functional groups such as phenolic and carboxylic groups arranged such that chelation is highly favored. This is especially true in alkaline solutions where the humic acids are ionized.

Replacement of Fe by Ca

Since meaningful equations could not be derived to express the complex competition of Fe and Ca ions for binding sites on the humic acids, a simple tracer study was done to determine if Ca ions were able to displace humic acid-bound Fe.

Two 10.0 ml aliquots of the humic acid-bound Fe used in the release experiment were placed in dialysis bags and suspended in 300 ml Erlenmeyer flasks. Two hundred ml of distilled water were added to one flask while 200 ml of a 100

mg/l Ca solution were added to the other flask. The pH of the Ca and distilled water solutions were 7.1, while the humic acid solutions had a pH of 6.3.

After 5 days, the dialysis bags were removed and the solutions in the flasks acidified with concentrated HCl to remove adsorbed Fe from the walls of the reaction flasks and 10.0 ml samples were counted for ^{59}Fe activity. Samples from the distilled water had an activity of only 0.4 CPM/ml, while the 100 mg/l Ca solution had an activity of 26 CPM/ml indicating that the Ca ions were able to displace humic acid-bound Fe.

This may explain why Fe bound to humic acids has been found to be available to marine phytoplankton (Martin, 1971). Basu (1964) determined that the great binding ability of humic acids was due to the higher exchange capacity of colloidal humic acids. He also indicated that adsorbed cations were readily released by exchange reactions. The Fe bound to humic acids washed into sea water could be released by the high ionic strength solution, thus making it available to phytoplankton.

APPENDIX III

Humic Acid Removal from Water, Using Ion Exchange Resins

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Introduction

Ion exchange resins have been used for many years to remove humic acids from industrial feed waters (Leclerc and Samuel, 1956; Jong and Jansen, 1958; Bogers, 1958; Van Beneden, 1959). Strongly basic anionic exchange resins of the tertiary amine type have been used to remove humic materials from the highly colored waters of Northern Europe (Jong and Jansen, 1958; Van Beneden, 1959). These resins have proven to effectively remove the organic color in water but the humic substances are difficult to elute from the resins. In many cases the organics seem to be irreversibly bound to the resins (Wilson and Collier, 1955). This phenomenon, known as organic fouling, makes regeneration impossible and decreases the effectiveness of these resins for water purification purposes. The difficulty in eluting humic acids from strongly basic anionic exchange resins also makes them of little use in concentrating humic acids for scientific study (Hissel, 1955). Shapiro (1957) also found that anionic exchange resins were good scavengers for humic acids but the elution recovery was low.

Other exchange matrices, such as activated charcoal and magnesia-silica gels have been used to extract humic

acids from water (Shapiro, 1957). Many workers have extracted humic acids by freeze-drying (Shapiro, 1961) and liquid-liquid partitioning techniques. These methods are acceptable when small quantities of the organic acids are needed for analysis but impractical when larger quantities are needed for biological experiments.

Buck (1958) used the chlorine form of "Duolite A-4" (a weakly basic polystyrene exchange resin) to remove colored, dissolved organics from Michigan streams. Moreau (1972) also used a strongly basic ionic exchange resin ("Dowex 1-8X") to remove humic acids from natural water. Both authors reported good elution with NaOH or NaCl or a combination of both.

Experiments were conducted to determine the scavenging and regeneration capacities of 4 anionic exchange resins for known humic acid solutions as well as complex colored organics from natural waters. Most ion exchange procedure for removing humic acids from water have been done with complex naturally occurring compounds and not tested for humic acids of a relatively pure form.

Strongly Basic Anionic Exchange Resin

"Dowex-1" is a strongly basic anionic exchange resin with a trimethyl benzyl ammonium active group on a polystyrene divinyl benzene cross-linked matrix formed into spheres. The 50-100 mesh analytical grade chloride form of

this resin has a total exchange capacity of 1.4 meq/ml swollen resin.

A 30 ml polyethylene exchange column was packed with swollen resin and conditioned with 5 bed-volumes of 2.0 N NaCl, followed by 5 bed-volumes of distilled water.

The removal efficiency for removing humic acids from water was tested with 1.0 liter of a 10.0 mg/l solution of the purified humic acids used in the growth and uptake experiments (Appendix II). The humic acid solution was passed through the column at a rate of 30 ml/min with 30 ml fractions collected for fluorometric analysis to determine the column effeciency (Appendix II).

There was no breakthrough of humic acids as the humic acid solution was passed through the column which took on a dark brown color.

The column was eluted with 100 ml of 3.0 N NaCl which removed about 0.3 mg humic acid or about 3% of the total added. Batch elution of the resin with 100 ml hot (80 C), 3.0 N NaCl removed no more humic acid.

Column elution with 100 ml of 5.0 N NaOH removed about 6% of the humic acid, while a 24 h batch elution with 200 ml 5.0 N NaOH removed 7% of the humic acids from the resin.

"Dowex-1" was also used to remove dissolved, colored organics from the highly colored waters of the Red Cedar River on the Michigan State University campus, Ingham County,

Michigan (Meridian Township, SW¼ Sec 18, T3N, R1W). Samples were taken during the high water period of October, when the river was swollen by runoff. Swollen "Dowex-1" was packed into a 30 cm long, 4.5 cm inside diameter glass column with a volume of 475 ml. This column was fitted at each end with #6, one-hole, rubber stoppers. Each stopper had a piece of glass tubing inserted in it and these were attached to tygon tubing.

Screw clamps were attached to the inlet and exit tubing to control the water flow and prevent desiccation of the resin when not in use. Porous, polyethylene dishes were located above and below the resin to hold it in place. In addition, a glass wool plug was positioned at the top of the column. This as well as glass wool and polyethylene in-line filters removed silt and debris from the feed water before it passed through the column.

The column was conditioned with 5 bed-volumes of 2.0% NaCl followed by 5 bed-volumes of distilled water. River-water was passed by a metering pump (Seelye, 1971) to a tank equipped with a floating microswitch which maintained a constant head-height ($\pm$ 1 in). Over a period of 6½ h 200 liters of water were passed from the river through the column. This gave a dark brown color to the top 75% of the column, but no discoloring of the bottom quarter.

Because of the complexity of the organic mixture, the concentrations could not be determined by fluorometry

(Appendix II). Determinations of elution efficiency were made visually by observing the change in the color of the resin and the color intensity of the eluent. The column was moved to the laboratory where it was eluted with 500 ml of 2.0 N NaCl. Very little of the color was taken from the resin. A batch elution with 500 ml of 2.0 N NaOH also failed to remove much of the color from the resin, although the eluent did have a slight yellow color.

The two 500 ml elution volumes were combined and freeze-dry concentrated to 1/10 of their original volume yielding a clear yellow solution.

This concentrated fraction was acidified with concentrated HCl to a pH of 6.3 and centrifuged at 5,000 rpm for 30 min. No precipitate was formed. According to Christman and Ghassemi (1966a), this indicates that the color removed from the resin was due to the fulvic acid fraction of the dissolved, organic, colored substances. The yellow color of the eluent is further support for the supposition that the fulvic acids were eluted from the resin, leaving the dark brown humic acid fraction behind.

Weakly Basic Anionic Exchange Resin

Because the humic acids could not be eluted from the strongly basic anionic exchange resin, a weakly basic resin was tested for its ability to remove them from water and be regenerated. The resin used was Rohm and Haas, 50-100 mesh,

"IR-45". This resin has a polyamine functional group on a polystyrene-DVB matrix.

The resin was swollen in distilled water, packed into a 30 ml polyethylene exchange column and conditioned with 5 bed-volumes of 2.0 N NaOH followed by 5 bed-volumes of distilled water.

A 1.0 liter sample of 10.0 mg/l purified humic acid solution was passed through the column at a rate of 10 ml/min.

The resin was batch eluted with 200 ml of 2.0 N NaCl after which the resin was transferred to 200 ml of 2.0 N NaOH. Only a small amount of the humic acid was exchanged off the resin, which remained dark brown. The concentration of acids in the eluent was determined by fluorometry and the percent recovery determined. About 6% of the humic acid added to the column was eluted from it. As with the strongly basic exchange resin, the weakly basic resin effectively removed the humic acids from water, with no detectable humic acid breakthrough. Only a slightly larger percentage of the humic acid could be eluted from the weakly basic anionic exchange resin than from the strongly basic anionic exchange resin.

These results, and those with the strongly basic anionic resin differ from those of Buck (1968) and Moreau (1972) and indicate that ion exchange resins of the type tested are

very ineffective for the concentration of humic acids from water.

Attempts have been made to elute humic acids from anionic exchange resins with very stringent methods, such as eluting with hypochlorite, concentrated mineral acids, and hot solvents (Leclerc and Samuel, 1955); Harrington, personal communication). Rapid heating and cooling of the resins in combination with strong eluents has also been used to expand and contract the pores so that the organics are released. The larger molecular weight humic acids are not exchangeable from anionic resins without destroying the integrity of the exchange resin or that of the humic acids when these techniques are employed.

Since humic acids are large molecules and highly branched with many active functional groups (Appendix II), they are difficult to elute from exchange resins. The humic acids tend to become lodged in the pores and since they have a large number of exchange sites, it is difficult to have them all exchanged at any one time. These difficulties, combined with the high bond strengths of the humic acids with the anionic exchange resins are the principal reasons for the low recovery rate.

Large Pore and Macroporus Strongly Basic Ion Exchange Resins

Since the size and complex geometry of humic acid molecules may be the reason they are not easily eluted from

exchange resins, both a large pore and a macroporus anionic exchange resin were used to try to lessen the effect of molecular size and geometry.

The large pore anionic exchange resin used in this study was 20-50 mesh "Dowex-11" in the hydroxide form. This is a strongly basic resin consisting of trimethyl benzyl ammonium active groups on a polystyrene-DVB matrix (Wheaton and Seamster, 1966).

Thirty milliliters of the swollen resin were placed in a 30 ml polyethylene column and conditioned with 3 bed-volumes of distilled water, 3 bed-volumes of 4% NaOH and finally washed with 3 bed-volumes of distilled water. A 1.0 l sample of a 10.0 mg/l solution of purified humic acids was passed through the column at a rate of 30 ml/min with a hydrostatic head of 12 inches. As in previous experiments, 30 ml samples were taken for fluorometric analysis to determine the removal efficiency of the resin.

The removal efficiency was initially about 90% but decreased rapidly to about 50% after only 150 ml of humic acid solution had been passed through the column. After retaining this efficiency for the next 300 ml the removal efficiency decreased to 20%, at which time the experiment was terminated. This low removal efficiency may be due to the large pores and large void volume letting humic acids pass through without being exchanged.

Although "Dowex-11" had a low removal efficiency, once the humic acids were bound to the resin they were not easily exchanged. The humic acids could not be eluted by batch elution with 4% NaOH, 4% HCl or 5% LiCl.

The chloride form of 20-40 mesh Dow "MSA-1" macroporous, strongly basic anionic exchange resin was tested to determine its removal efficiency and elution capacity for purified humic acids in aqueous solution. This resin is also a polystyrene-DVB resin with trimethyl benzyl ammonium active groups (Wheaton and Seamster, 1966). The resin is formed into large spherical beads (0.5 mm), giving a large void volume between beads.

As with the other resins tested, a 30 ml portion of the swollen resin was packed in a polyethylene exchange column and conditioned with 5 bed-volumes of 4% NaOH, followed by 5 bed-volumes of distilled water. One liter of 10.0 mg/l purified humic acid was passed through the column at a rate of 30 ml/min with 30 ml fractions collected to determine the resin's removal efficiency.

The removal efficiency was about 99% for the entire liter, with no breakthrough. Batch elution with 4% NaOH for 24 h did not elute a detectable amount of humic acid and the resin remained a dark brown color.

Conclusions

Three of the anionic exchange resins studied ("Dowex-1", "Amberlite IR-45" and "MSA-1") were found to be highly effective scavengers for humic acids in aqueous solution. Of the anionic exchange resins studied only "Dowex-11" was ineffective for removing humic acids from water.

All of the resins bound humic acids very tightly, making their elution efficiency very low. Some drastic elution schemes have been proposed but these destroy the integrity of the exchange resins and probably the humic acids as well.

A portion of the organic color exchanged from natural water was eluted from the columns. The experiments with purified humic acids of molecular weight 30,000 or greater indicate that the color eluted from the "Dowex-1" used to scavenge Red Cedar River water was probably not due to humic acids but due to the fulvic acid fraction of the dissolved organic acids. This hypothesis is further supported by the fact that when the eluents of the Red Cedar River column were combined, concentrated, and acidified no precipitate resulted. The fulvic acids are soluble in acid solution while the humic acids would be precipitated. The Red Cedar River colored organics eluted from the anionic exchange resin were the yellow color typical of fulvic acids.

The dissolved organic color extracted by Buck (1968) was probably of the fulvic fraction although it could also have been tannins or lignins. Moreau (1971) reported successfully concentrating dissolved, colored, organic acids from a Michigan stream with 50-100 mesh "Dowex-1" in the chloride form. The acids that he was able to elute with 2.0 M NaCl were probably only the fulvic acid portion of the dissolved, organic, colored acids and not humic acids as he reports.

It was concluded that the types of anionic exchange resins tested are not an effective tool for concentrating humic acids for scientific study.

APPENDIX IV

pH Data for Growth Experiments

Table 11.--pH values for cultures of Fe-starved S. obliquus grown in AAP media containing 6 different Fe concentrations. Each pH value is the mean of 4 replications.

TREATMENT	DAY			
	7	9	12	16
0.1 mg/l Fe	7.6	9.2	8.9	8.4
0.2 mg/l Fe	7.4	9.2	8.9	8.2
0.3 mg/l Fe	7.5	9.4	8.9	8.6
0.4 mg/l Fe	7.4	9.0	9.0	8.4
0.5 mg/l Fe	7.6	9.1	8.3	8.1
1.0 mg/l Fe	7.5	9.5	8.2	8.2

Table 12.--pH values for cultures of PO_4-starved S. obliquus grown in AAP media containing 6 different concentrations of PO_4. Each pH value represents the mean of 4 replications.

TREATMENT Mg/l PO_4	DAY		
	5	7	9
2×10^{-4}	7.5	7.6	7.3
1×10^{-3}	7.4	7.4	7.3
5×10^{-3}	7.8	7.6	7.5
1×10^{-2}	7.4	7.3	7.4
1×10^{-1}	7.5	8.4	8.6
5×10^{-1}	9.1	9.4	8.8

Table 13.--pH values for cultures of PO_4-starved S. obliquus grown in AAP media containing 6 different concentrations of PO_4. Each value represents the mean of 4 replications.

TREATMENT (mg/l PO_4)	DAY			
	3	5	7	9
0.05	7.4	8.0	7.7	7.6
0.10	7.4	7.9	7.7	7.6
0.25	7.4	8.2	7.8	7.7
0.50	7.4	9.3	7.8	7.6
0.75	7.4	9.6	7.8	7.6
1.00	7.4	10.0	7.9	7.7

Table 14.--pH values for cultures of PO_4-Fe-starved S. obliquus grown in AAP media containing 12 different treatment combinations of Fe and PO_4. Each value represents the mean of 4 replications.

TREATMENT PO_4 (mg/l)	TREATMENT Fe (mg/l)	DAY 4	DAY 5	DAY 6	DAY 8
0.5	0.3	6.6	6.9	7.2	7.3
0.5	0.5	6.9	7.6	7.4	7.2
0.5	1.0	6.8	7.5	7.0	7.2
0.5	1.2	6.7	7.4	7.1	7.2
0.7	0.3	7.0	8.0	7.4	7.3
0.7	0.5	6.9	7.2	7.2	7.3
0.7	1.0	7.0	7.8	7.3	7.2
0.7	1.2	7.0	7.1	7.3	7.3
0.9	0.3	6.6	6.6	7.3	7.4
0.9	0.5	7.0	7.9	7.4	7.3
0.9	1.0	7.2	9.1	7.3	7.3
0.9	1.2	7.1	7.5	7.2	7.2
1.1	0.3	6.8	7.5	7.1	7.2
1.1	0.5	7.0	7.7	7.4	7.2
1.1	1.0	6.9	7.5	7.1	7.2
1.1	1.2	7.0	7.7	7.2	7.2

Table 15.--pH values for cultures of Fe-starved S. obliquus grown in AAP media containing 6 concentrations of humic acids. Each value represents the mean of 4 replications.

TREATMENT	DAY					
	5	7	9	11	12	15
0 mg/l H.A.	7.4	7.5	7.5	7.4	7.4	7.4
2 mg/l H.A.	7.4	7.6	7.6	7.5	7.6	7.6
5 mg/l H.A.	7.5	7.7	7.7	7.8	7.8	7.9
10 mg/l H.A.	7.7	7.7	7.8	7.7	7.8	8.0
15 mg/l H.A.	7.7	7.8	7.9	7.9	8.0	8.2
25 mg/l H.A.	7.9	8.0	8.2	8.3	8.3	8.5

Table 16.--pH values of cultures of Fe-starved S. obliquus grown in AAP media containing 8 treatment combinations of humic acids and EDTA. Each value represents the mean of 4 replications.

TREATMENT H.A. (mg/l)	EDTA		DAY 2	3	4	5	7	9
5	0.0	S**	7.4	7.9	8.1	8.3	10.1	8.4
5	0.0	U***	7.3	7.8	7.8	8.0	8.1	7.9
5	0.3	S	7.4	8.0	7.9	8.0	8.0	8.0
5	0.3	U	7.4	7.9	8.4	8.1	8.0	7.8
15	0.0	S	7.5	8.2	9.1	8.1	8.1	8.0
15	0.0	U	7.5	8.0	8.9	8.1	8.4	8.0
15	0.3	S	7.5	8.1	8.6	9.3	8.3	8.0
15	0.3	U	7.6	8.0	8.1	8.3	8.1	8.0

** Starved Stock Scenedesmus obliquus
*** Unstarved Stock Scenedesmus obliquus

Table 17.--pH values for cultures of Fe-starved S. obliquus grown in AAP media containing 12 treatment combinations of humic acids and EDTA. Each value represents the mean of 2 replications.

TREATMENT H.A. (mg/l)	TREATMENT EDTA (mg/l)	DAY 2	4	5	7	9	12	16
0	0.0	7.3	7.4	7.4	7.4	7.4	7.5	7.8
2	0.0	7.3	7.5	7.4	7.5	7.4	7.8	8.2
5	0.0	7.4	7.5	7.4	7.5	7.5	8.0	8.2
10	0.0	7.5	7.5	7.6	7.7	7.7	8.0	8.2
15	0.0	7.5	7.6	7.5	7.8	7.7	8.0	8.2
25	0.0	7.6	7.6	7.7	7.9	8.0	8.2	9.0
0	0.3	7.4	7.5	7.5	7.4	7.4	7.5	7.7
2	0.3	7.3	7.5	7.5	7.6	7.6	8.0	7.8
5	0.3	7.4	7.5	7.5	7.6	7.7	7.9	8.1
10	0.3	7.5	7.5	7.5	7.7	7.7	8.0	8.9
15	0.3	7.5	7.6	7.6	7.7	7.8	8.1	8.4
25	0.3	7.6	7.7	7.8	8.9	8.0	8.9	8.2

Table 18.--pH values for cultures of Fe-PO_4 starved S. obliquus grown in AAP media containing 8 treatment combinations of Fe, PO_4 and humic acids. Each value represents the mean of 3 replications.

TREATMENT			DAY				
H.A.	Fe	PO_4					
(mg/l)			2	3	4	5	7
0	0.5	0.5	7.5	8.4	8.5	8.3	8.4
15	0.5	0.5	7.7	8.5	8.3	8.1	8.2
0	1.2	0.5	7.3	8.1	8.1	7.9	8.1
0	0.5	0.9	7.3	8.9	9.1	8.7	8.5
0	1.2	0.9	7.2	8.1	8.9	8.6	8.4
15	0.5	0.9	7.6	8.9	9.3	9.2	8.1
15	1.2	0.5	7.6	8.9	9.1	8.6	8.4
15	1.2	0.9	7.8	9.6	9.4	8.7	8.2

Table 19.--pH values for cultures of unstarved S. obliquus grown in AAP media containing 8 treatment combinations of Fe, PO_4 and humic acids. Each value represents the mean of 3 replications.

TREATMENT			DAY				
H.A.	Fe	PO_4					
(mg/l)			2	3	4	5	7
0	0.5	0.5	8.4	8.8	9.1	8.7	8.4
15	0.5	0.5	8.4	8.9	9.2	9.1	8.6
0	1.2	0.5	8.2	8.4	8.8	8.7	8.5
0	0.5	0.9	8.4	8.9	8.9	9.1	8.7
0	1.2	0.9	8.5	9.2	9.1	9.2	8.8
15	0.5	0.9	8.5	8.9	9.2	9.1	9.0
15	1.2	0.5	8.5	8.9	9.3	9.0	8.6
15	1.2	0.9	8.5	9.0	8.9	9.2	8.8

Table 20.--pH values for cultures of Fe-starved S. obliquus grown in AAP media containing 9 treatment combinations of Ca and humic acids. Each value represents the mean of 2 replications.

TREATMENT		DAY			
Ca	H.A.				
(mg/l)		2	3	4	5
0.0	0.0	8.0	9.4	10.1	9.4
20.0	0.0	8.0	9.9	9.9	8.0
40.0	0.0	8.3	10.0	9.9	9.2
0.0	10.0	7.9	8.0	8.7	8.3
20.0	10.0	7.8	8.0	9.9	8.4
40.0	10.0	7.9	9.0	9.9	9.2
0.0	15.0	8.0	8.7	10.1	8.5
20.0	15.0	8.0	8.5	9.8	10.0
40.0	15.0	8.0	8.4	10.0	9.0

APPENDIX V

Relative Growth Rates and Generation Times for Growth Experiments

Table 21.--Relative growth rates (k') and generation times (G) for Fe-starved S. obliquus grown in AAP media containing 6 different concentrations of Fe. Values are tabled for each treatment by segments of the growth curves.

TREATMENT Fe (mg/l)		SEGMENT (DAYS) 0-3	3-5	5-7	7-10	10-12
0.0003	k'	0.0	0.0	0.18	0.13	- 0.11
	G	undef.	undef.	1.67	2.32	- 2.74
0.001	k'	0.0	0.0	0.24	0.40	- 0.53
	G	undef.	undef.	1.25	0.75	- 0.57
0.01	k'	0.0	0.0	0.21	0.41	- 0.51
	G	undef.	undef.	1.43	0.73	- 0.59
0.1	k'	0.0	0.0	0.45	0.11	- 0.01
	G	undef.	undef.	0.67	2.74	-30.10
0.5	k'	0.36	0.17	0.04	0.01	- 0.03
	G	0.84	1.77	7.53	30.10	-10.03
1.0	k'	0.37	0.09	0.03	0.01	- 0.07
	G	0.81	3.34	10.03	30.10	- 4.30

Table 22.--Relative growth rates (k') and generation times (G) for Fe-starved S. obliquus grown in AAP media containing 6 different Fe concentrations. Values are tabled for each treatment by segments of the growth curves.

TREATMENT Fe (mg/l)		SEGMENT (DAYS) 0-7	7-9	9-12	12-16
0.1	k'	0.11	0.08	0.08	- 0.02
	G	2.74	3.76	3.76	-15.05
0.2	k'	0.12	0.10	0.03	- 0.02
	G	2.51	3.01	10.03	-15.05
0.3	k'	0.12	0.11	0.08	- 0.05
	G	2.51	2.74	3.76	- 6.02
0.4	k'	0.10	0.10	0.08	- 0.05
	G	3.01	3.01	3.76	- 6.02
0.5	k'	0.16	- 0.01	0.04	- 0.01
	G	1.89	-30.10	7.53	-30.10
1.0	k'	0.21	- 0.15	- 0.04	- 0.02
	G	1.43	- 2.01	- 7.53	-15.05

Table 23.--Relative growth rates (k') and generation times (G) for PO_4-starved S. obliquus grown in AAP media containing 2 PO_4 concentrations. Values are tabled for each treatment by segments of the growth curves.

TREATMENT PO_4 (mg/l)		SEGMENT (DAYS) 0-5	5-7	7-9
0.5	k'	0.35	0.18	0.01
	G	0.86	1.67	30.10
0.1	k'	0.21	0.25	0.0
	G	1.43	1.20	undef.

Table 24.--Relative growth rates (k') and generation times (G) for PO_4-starved S. obliquus grown in AAP media containing 6 concentrations of PO_4. Values are tabled for each treatment by segments of the growth curves.

TREATMENT PO_4 (mg/l)		SEGMENT (DAYS) 0-3	3-5	5-7	7-9
0.05	k'	0.09	0.37	0.04	- 0.06
	G	3.34	0.81	7.53	- 5.02
0.10	k'	0.50	0.11	- 0.04	- 0.01
	G	0.60	2.74	- 8.53	-30.10
0.25	k'	0.47	0.29	0.03	- 0.04
	G	0.64	1.04	10.03	- 7.53
0.50	k'	0.43	0.43	0.06	0.08
	G	0.70	0.70	5.02	3.76
0.75	k'	0.57	0.33	- 0.02	0.08
	G	0.53	0.91	-15.05	3.76
1.00	k'	0.49	0.48	- 0.05	0.06
	G	0.61	0.63	- 6.02	5.02

Table 25.--Relative growth rates (k') and generation times (G) for Fe-PO_4-starved S. obliquus grown in AAP media at 16 different treatment combinations of Fe and PO_4. Values are tabled for each treatment combination by segments of the growth curves.

TREATMENT			SEGMENT (DAYS)			
PO_4 (mg/l)	Fe (mg/l)		0-4	4-5	5-6	6-7
0.5	0.3	k'	0.38	0.10	0.04	0.27
		G	0.79	3.10	7.53	1.11
0.5	0.5	k'	0.0	1.60	0.24	1.12
		G	undef.	0.19	1.25	0.27
0.5	1.0	k'	0.36	0.03	0.05	- 0.03
		G	0.84	10.03	6.47	-10.03
0.5	1.2	k'	0.37	0.15	0.02	0.24
		G	0.82	2.01	15.05	1.25
0.7	0.3	k'	0.0	1.49	0.49	0.87
		G	undef.	0.20	0.61	0.35
0.7	0.5	k'	0.37	0.42	0.05	0.20
		G	0.81	0.72	6.02	1.51
0.7	1.0	k'	0.36	0.21	0.48	0.14
		G	0.84	1.43	0.63	2.15
0.7	1.2	k'	0.40	0.47	0.12	0.04
		G	0.75	0.64	2.51	7.53
0.9	0.3	k'	0.0	1.49	0.17	0.36
		G	undef.	0.20	1.77	0.84
0.9	0.5	k'	0.0	1.79	0.29	- 0.25
		G	undef.	0.17	1.04	- 1.30
0.9	1.0	k'	0.37	0.40	0.30	- 0.13
		G	0.81	0.75	1.00	- 2.32
0.9	1.2	k'	0.42	0.39	0.12	- 0.08
		G	0.72	0.77	2.51	- 3.76
1.1	0.3	k'	0.0	1.46	0.61	- 0.25
		G	undef.	0.21	0.49	- 1.20
1.1	0.5	k'	0.32	0.24	0.43	0.46
		G	0.94	1.25	0.70	0.65
1.1	1.0	k'	0.0	2.06	0.09	- 0.50
		G	undef.	0.15	3.34	- 0.60
1.1	1.2	k'	0.38	0.70	0.03	- 0.07
		G	0.79	0.43	10.03	- 4.30

Table 26.--Relative growth rates (k') and generation times (G) for Fe-starved S. obliquus grown in AAP media containing 6 concentrations of humic acids. Values are tabled for each treatment by segments of the growth curves.

TREATMENT H.A. (mg/l)		SEGMENT (DAYS) 0-5	5-7	7-9	9-11	11-12	12-15
0.0	k'	0.0	0.14	0.26	0.01	0.05	0.02
	G	undef.	2.15	1.16	30.10	5.02	15.05
2.0	k'	0.07	0.10	0.10	0.12	- 0.01	0.14
	G	4.30	3.01	3.01	2.51	-30.10	2.15
5.0	k'	0.89	0.04	0.19	0.08	0.05	0.11
	G	0.34	7.53	1.58	3.76	6.02	2.74
10.0	k'	0.08	0.15	0.23	0.08	- 0.02	0.04
	G	3.76	2.01	1.31	3.76	-15.05	7.53
15.0	k'	0.13	0.11	0.16	0.10	0.03	0.06
	G	2.32	2.74	1.88	3.01	10.03	5.02
25.0	k'	0.14	0.12	0.23	0.04	0.01	0.04
	G	2.15	2.51	1.31	7.53	30.10	7.53

Table 27.--Relative growth rates (k') and generation times (G) for Fe-starved S. obliquus grown in AAP media containing 8 treatment combinations of humic acids and EDTA. Values are tabled for each treatment combination by segments of the growth curves.

TREATMENT				SEGMENT (DAYS)			
H.A. (mg/l)	EDTA (mg/l)	ALGAE		0-3	3-4	4-5	5-7
5	0.0	S*	k'	0.52	0.70	-0.07	0.51
			G	0.58	0.43	-4.30	0.59
5	0.0	U**	k'	1.78	0.55	0.51	0.18
			G	0.17	0.55	0.59	1.67
5	0.3	S	k'	1.90	0.63	0.58	0.12
			G	0.16	0.48	0.52	2.51
5	0.3	U	k'	2.00	0.43	0.49	0.18
			G	0.15	0.70	0.61	1.67
15	0.0	S	k'	1.34	1.07	0.59	0.0
			G	0.22	0.28	0.51	undef.
15	0.0	U	k'	1.97	0.45	0.62	0.05
			G	0.15	0.67	0.49	6.02
15	0.3	S	k'	1.77	0.72	0.49	0.18
			G	0.17	0.42	0.61	1.67
15	0.3	U	k'	1.73	0.79	0.46	0.12
			G	0.17	0.38	0.65	2.51

* Starved stock algae
** Unstarved stock algae

Table 28.--Relative growth rates (k') and generation times (G) for Fe-starved S. obliquus grown in AAP media containing 12 treatment combinations of humic acids and EDTA. Values are tabled for each treatment combination by segments of the growth curves.

TREATMENT H.A. (mg/l)	EDTA (mg/l)		SEGMENT (DAYS) 0-2	2-4	4-5	5-7	7-9	9-12	12-16
0	0.0	k'	0.58	0.17	0.23	0.21	0.11	0.11	- 0.02
		G	0.52	1.77	1.31	1.43	2.74	2.74	-15.05
2	0.0	k'	0.67	0.10	0.41	0.19	0.16	0.09	0.02
		G	0.45	3.01	0.73	1.58	1.88	3.34	15.05
5	0.0	k'	0.71	0.21	0.22	0.21	0.19	0.12	- 0.03
		G	0.42	1.43	1.37	1.43	1.58	2.51	-10.03
10	0.0	k'	0.67	0.22	0.07	0.29	0.16	0.14	0.01
		G	0.45	1.37	4.31	1.04	1.88	2.15	30.10
15	0.0	k'	0.67	0.21	0.18	0.28	0.17	0.09	0.02
		G	0.45	1.43	1.67	1.08	1.79	3.34	15.05
25	0.0	k'	0.63	0.39	0.16	0.23	0.14	0.10	- 0.03
		G	0.48	0.77	1.88	1.31	2.15	3.01	-10.03
0	0.3	k'	0.81	0.12	0.31	0.22	0.10	0.06	- 0.02
		G	0.37	2.51	0.97	1.37	3.01	5.02	-15.05
2	0.3	k'	0.67	0.28	0.17	0.29	0.09	0.09	0.0
		G	0.45	1.08	1.77	1.04	3.34	3.34	undef.
5	0.3	k'	0.70	0.18	0.46	0.19	0.21	0.04	- 0.01
		G	0.43	1.67	0.65	1.58	1.43	7.53	-30.10
10	0.3	k'	0.72	0.17	0.23	0.28	0.18	0.07	- 0.01
		G	0.42	1.77	1.31	1.08	1.67	4.30	-30.10
15	0.3	k'	0.71	0.23	0.31	0.20	0.20	0.08	- 0.03
		G	0.42	1.31	0.97	1.51	1.51	3.76	-10.03
25	0.3	k'	0.59	0.38	0.39	0.21	0.17	0.05	- 0.01
		G	0.51	0.79	0.77	1.43	1.77	6.02	-30.10

Table 29.--Relative growth rates (k') and generation times (G) for Fe-PO_4-starved S. obliquus grown in AAP media containing 8 treatment combinations of Fe, PO_4 and humic acids. Values are tabled for each treatment combination by segments of the growth curves.

TREATMENT H.A. (mg/l)	Fe	PO_4		SEGMENT (DAYS) 0-2	2-3	3-4	4-5	5-7
0	0.5	0.5	k'	0.59	0.72	0.82	0.22	- 0.16
			G	0.51	0.42	0.37	1.37	- 1.88
15	0.5	0.5	k'	0.45	1.19	0.80	0.07	- 0.12
			G	0.67	0.25	0.38	4.30	- 2.51
0	1.2	0.5	k'	0.65	0.94	0.56	- 0.01	- 0.11
			G	0.46	0.32	0.54	-30.10	- 2.74
0	0.5	0.9	k'	0.42	1.12	0.63	0.27	- 0.12
			G	0.72	0.27	0.48	1.11	- 2.51
0	1.2	0.9	k'	0.60	0.88	0.79	- 0.01	- 0.12
			G	0.50	0.34	0.38	-30.10	- 2.51
15	0.5	0.9	k'	0.50	0.87	0.81	0.01	- 0.01
			G	0.60	0.35	0.37	-30.10	-30.10
15	1.2	0.5	k'	0.44	1.08	0.92	0.19	- 0.22
			G	0.68	0.28	0.33	1.60	- 1.37
15	1.2	0.9	k'	0.51	1.06	0.81	0.19	- 0.19
			G	0.59	0.28	0.37	1.58	- 1.58

Table 30.--Relative growth rates (k') and generation times (G) for unstarved S. obliquus grown in AAP media containing 8 treatment combinations of Fe, PO_4 and humic acids. Values are tabled for each treatment combination by segments of the growth curves.

TREATMENT H.A. (mg/l)	Fe (mg/l)	PO_4 (mg/l)		SEGMENT (DAYS) 0-2	2-3	3-4	4-5	5-7
0	0.5	0.5	k'	0.36	0.92	0.80	0.03	- 0.02
			G	0.84	0.33	0.38	10.03	-15.05
15	0.5	0.5	k'	0.47	0.71	0.89	- 0.17	0.02
			G	0.64	0.42	0.34	- 1.77	15.05
0	1.2	0.5	k'	0.42	0.80	0.38	0.40	0.0
			G	0.72	0.38	0.79	0.75	undef.
0	0.5	0.9	k'	0.51	0.53	0.87	- 0.04	- 0.03
			G	0.59	0.57	0.35	- 8.47	-10.03
0	1.2	0.9	k'	0.39	0.64	0.58	0.46	- 0.08
			G	0.77	0.47	0.52	0.65	- 3.76
15	0.5	0.9	k'	0.47	0.71	0.62	0.15	0.03
			G	0.64	0.42	0.49	2.01	10.03
15	1.2	0.5	k'	0.49	0.65	0.69	0.16	- 0.04
			G	0.61	0.46	0.44	1.88	- 7.53
15	1.2	0.9	k'	0.56	0.49	0.54	0.26	- 0.03
			G	0.54	0.61	0.56	1.16	-10.03

Table 31.--Relative growth rates (k') and generation times (G) for Fe-starved S. obliquus grown in AAP media containing 9 treatment combinations of humic acids and Ca. Values are tabled for each treatment combination by segments of the growth curves.

TREATMENT Ca (mg/l)	TREATMENT H.A. (mg/l)		SEGMENT (DAYS) 0.2	2-3	3-4	4-5
0.0	0.0	k'	0.46	-0.13	1.40	0.57
		G	0.65	-2.31	0.21	0.53
20.0	0.0	k'	0.51	1.07	0.25	0.36
		G	0.59	0.28	1.20	0.83
40.0	0.0	k'	0.61	0.98	0.12	0.50
		G	0.49	0.31	2.50	0.60
0.0	10.0	k'	0.38	0.95	0.48	0.42
		G	0.79	0.32	0.63	0.71
20.0	10.0	k'	0.61	0.65	0.39	0.54
		G	0.49	0.46	0.77	0.56
40.0	10.0	k'	0.56	1.06	0.08	0.64
		G	0.54	0.28	3.75	0.47
0.0	15.0	k'	0.62	0.66	0.45	0.34
		G	0.48	0.45	0.67	0.88
20.0	15.0	k'	0.55	0.69	0.44	0.52
		G	0.55	0.43	0.68	0.58
40.0	15.0	k'	0.56	0.80	0.33	0.50
		G	0.54	0.38	0.91	0.60

LITERATURE CITED

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Alben, A. O., L. O. Tiffin and R. S. Holmes. 1960. Competition between chelating agents and roots as a factor affecting adsorption of iron and other ions by plant species. Plant Physiol. 35: 878-886.

_____, _____, A. W. Specht and J. W. Resricky. 1961a. Stability and concentration of metal chelates, factors in iron chlorosis in plants. Agron. J. 53: 85-90.

_____, _____, _____ and _____. 1961b. Iron absorption by roots as affected by plant species and concentration of chelating agent. Agron. J. 53: 81-85.

Algeus, S. 1949. Alanine as a source of nitrogen for green algae. Physiol. Plant. 2: 266-271.

Allen, E. R. 1919. Some conditions affecting the growth and activities of Azotobacter and Chroochoccum. Anal. Missouri Bot. Gard. 6: 1-44.

Anon. 1971. Algal assay procedure bottle test. By National Eutrophication Research Program. 82p.

Ashby, E. 1929. The interaction of factors in the growth of Lemna: 4. The influence of minute quantities of organic matter upon growth and reproduction. Anal. Bot. 43: 805-816.

Aso, S. and I. Sakai. 1963. Studies in the physiological effects of humic acid. 1. Uptake of humic acid by crop plants and its physiological effects. Soil Sci. Plant Nutr. Tokyo. 9(3): 1.

Bailey, J. and H. Margulis. 1968. Étude de quelques acides humiques sur gel de dextrane. Plant and Soil. 29: 343.

Baker, H. A. 1935. The metabolism of the colorless alga, Prototheca zopfii Krüger. J. Cell. Comp. Physiol. 7: 73-79.

Barber, R. T. 1968. Dissolved organic carbon from deep waters resists microbial oxydation. Nature. 220: 274-275.

Basu, A. N., D. C. Mukherjee, and S. K. Mukherjee. 1964. Interaction between humic acid fraction of soil and trace element cations. J. Indian Soc. Soil Sci. 12: 311-318.

Beres, T. and I. Kiraly. 1959. Investigation of the reducing effect of peat fulvic acids on ferric ions: I and II. Soils and Fertilizers. 22: 528. 1959.

Bogers, P. F. J. 1958. Déminéralisation et substances organiques. pt. 1. Centre Belge D'etude et de Documentation Des Eaux. No. 40. 1958 1H.

Bold, Harold C. 1967. Morphology of Plants. Harper and Row, New York. 541p.

Borehart, J. A. 1968. Biological Extraction of nutrients. J. Wat. Pollut. Control Fed. 40: 1739.

Bowen, H. J. M. 1966. Trace elements in biochemistry. Academic Press, London and New York. 241p.

Bozniak, E. G. 1969. Laboratory and field studies of phytoplankton communities. Ph.D. Dissertation, Washington Univ. Dept. Botany, St. Louis, Mo. 106p.

Bremner, J. M. 1950. Some observations on the oxidation of soil organic matter in the presence of alkali. J. Soil Sci. 1: 198.

_____. 1951. A review of recent work on soil organic matter I. J. Soil Sci. 2: 67-82.

_____. 1954. A review of recent work on soil organic matter II. J. Soil Sci. 5: 214-232.

_____. 1965. Organic nitrogen in soils. In: Soil nitrogen. (W. J. Bartholomew and F. E. Clark, Eds.) Amer. Soc. Agron. Madison, Wisconsin.

_____ and H. Lees. 1949. Studies on soil organic matter. Part II. The extraction of organic matter from soils by neutral reagents. J. Agr. Sci. 39: 274.

Brogden, William B. 1971. Characterization of fresh water and esturine humic acids by molecular weight distribution. Florida State University Ph.D. Dissertation.

Buck, E. Howard. 1968. The limnological and ecological significance of dissolved yellow organic acids in natural waters. M. S. Thesis. Michigan State University. 160p.

Bulloch, W. 1935. Obituary notice of deceased member Theobald Smith. J. Path. Bact. 40: 621.

Burk, D., H. Lineweaver and C. K. Horner. 1932a. Iron in relation to the stimulation of growth by humic acid. Soil Sci. 33: 413-454.

_____, _____ and _____. 1932b. The physiological nature of humic acid stimulation of Azotobacter growth. Soil Sci. 33: 455-487.

Chaberek, S. and A. H. Martell. 1959. Organic sequestering agents. John Wiley and Sons, Inc., New York. 616p.

Chaminade, R. 1956. Action de l'acide humique sur le development et la nutrition mineral des végétaux. Trans. 6th Int. Cong. Soil Sci. Paris 4: 65.

Ceh, M. and D. Hadzi. 1956. Infrared spectra of humic acids and their derivatives. Fuel. 35: 77-83.

Chalupa. Jiri. 1966. A contribution to the knowledge of humic substances in peat waters. Vern. Int. Verein Limnol. 16: 485-490.

Choudhiri, M. B. and F. J. Stevenson. 1957. Chemical and physiochemical properties of soil humic colloids: III. Extraction of organic matter from soils. Soil Sci. Soc. Amer. Proc. 21: 508.

Christman, R. F. and Masood Ghassimi. 1966a. The nature of organic color in water. Univ. of Washington. College of Engineering, Dept. of Civil Engineering. 45p.

_____ and _____. 1966b. Chemical nature of organic color in water. J. Amer. Water Works Assoc. 58: 723-741.

Clark, E. P. 1941. The signer method for determining molecular weights. Anal. Chem. 13: 820.

Clark, N. A. 1930. "Auximones" and the stimulation of Lemna by organic matter. Science. 71: 268-269.

Clark, N. A. and E. M. Roller. 1931. The stimulation of Lemna minor by organic matter under sterile and non-sterile conditions. Soil Sci. 31: 299-308.

Cooper, L. H. N. 1937. Some conditions governing the solubility of iron. Proc. Royal Soc. (London) Ser (B), 124: 299.

Davies, A. G. 1970. Iron chelation and the growth of marine phytoplankton. I. Growth kinetics and chlorophyll production in cultures of the euryhaline flagellate Dunaliella tertiolecta under iron limiting conditions. J. Marine Biol. Assoc. U. K. 50: 65-86.

Dubach, P., N. C. Mehta, T. Jakab, F. Martin and N. Roulet. 1964. Chemical investigations on soil humic substances. Geochem. Cosmochim. Acta. 28: 1567.

Dvorakova-Hladka, J. 1966. Utilization of organic substances during mixotrophic and heterotrophie cultivation of algae. Biol. Plant. 8: 354-361.

Elofsen, R. M. 1951. The infrared spectra of humic acid and related materials. Can. J. Chem. 35: 926-931.

Eyster, Clyde. 1967. Microorganic and microinorganic requirements for algae. In: D. F. Jackson. Algae, Man and the Environment. 1967. Syracuse University Press. p. 27.

Felbeck, G. T. 1965. Structural chemistry of soil humic substances. Adv. Agron. 17: 327-368.

_____. 1971. Chemical and biological characterization of humic matter. In: Soil Biochemistry, Vol. II (A. D. McLaren and J. Skeyins, eds.) Marcel Dekker, New York. pp. 55-56.

Fitzgerald, George P. 1970. Evaluations of the availability of sources of nitrogen and phosphorus for algae. J. Phycol. 6: 3: 239-247.

_____. 1972. Bioassay analysis of nutrient availability. Nutrients in Natural Waters, Chapter V, pp. 147-169.

_____ and G. F. Lee. 1971. Use of tests for limiting of surplus nutrients to evaluate sources of nitrogen and phosphorus for algae and aquatic weeds. Rep. Wat. Chem. Prog. Univ. of Wisconsin, 1971. 35p.

Flaig, W. 1960. Comparative chemical investigations on natural humic compounds and their model substances. Sci. Proc. Roy. Dublin Soc. Seral. # 4K. pp. 149-162.

_____. 1964. Effects of microorganisms in the transformation of lignin to humic substances. Geochem. cosmochim. Acta. 28: 1523-1535.

_____ and H. Otto. 1951. Humic acids. III. Forsch. 3: 66.

Fogg, C. E. 1965. Algal cultures and phytoplankton ecology. Univ. of Wisconsin Press. Madison, Wisconsin. 126p.

Forsyth, W. G. C. 1947a. The characterization of the humic complexes of soil organic matter. J. Ag. Sci. 37: 132-198.

Giesy, J. P. Unpublished data.

Gillam, W. S. 1940. A study on the chemical nature of humic acid. Soil Sci. 49: 433-453.

Gjessing, E. T. 1964. Ferrous iron in water. Limnol. Ocean. 9: 272-274.

_____. 1965. Use of "Sephadex" gel for the estimation of molecular weight of humic substances in natural waters. Nature, Lond. 208, 1091-1092.

_____. 1970. Ultrafiltration of aquatic humus. Environ. Sci. Technol. 4: 437-438.

_____ and G. F. Lee. 1967. Fractionation of organic matter in natural waters on sephadex columns. Environ. Sci. and Tech. 1: 631-638.

Goldberg, E. D. 1952. Iron assimilation by marine diatoms. Biol. Bull., Mar. Biol. Lab. Woods Hole. 102: 243-248.

Goldman, Charles R. 1972. The role of minor nutrients in limiting the productivity of aquatic ecosystems. Nutrients and eutrophication. Special Symposia. Limnol. Oceanog. VI, pp. 21-33.

Golterman, H. L. 1960. Studies on the cycle of elements in fresh water. Acta Bot. Nedr. 9: 1-58.

Gran, H. H. 1933. Studies on the biology and chemistry of the Gulf of Maine. II. Distribution of the phytoplankton in August, 1932. Biol. Bull. 64: 159-181.

Guminski, S. 1968. The mechanism of physiological effects induced in plants by humus compounds. Pochuovedenie 9: 62-9. Russian.

Hajduhovic, M. and B. Ulrich. 1965. Effect of sodium humate on plant growth and phosphate dynamics of a soil. Bodenkultur 16½: 141-9. German.

Hansen, E. H. and M. Schnitzer. 1969. Molecular weight measurements of polycarboxylic acids and in water by vapor pressure osmometry. Amal. chim. Acta, 46, 247.

Harvey, H. W. 1937a. The supply of iron to diatoms. J. Mar. Biol. Ass. U.K. 22: 205-219.

_____. 1937b. Note on colloidal ferric hydroxide in sea water. J. Mar. Biol. Ass. U.K. 22: 221-225.

Hayward, J. 1968. Studies on the growth of Phaeodactylum tricornutum. III. The effect of iron on growth. J. Mar. Biol. Ass. U.K. 48: 295-302.

Hem, J. D. and W. H. Cropper. 1959. Survey of ferrous-ferric chemical equilibria and redox potentials. Chemistry of Iron in Natural Water. USGS Water Supply Paper. 1459A.

Heinrich, Georg. 1966. The effect of mono and multivalent ions and basic dyes on plant cells in the presence of humic acid. Phyton. Ann. Rei. Bot. 11(3-4) 201-217. German.

Hissel, J. 1955. Relations between iron and humic materials on the waters of the Fagnes district. J. Bull. Centre Belge et Document. Eaux. 28: 127-130.

Hoogenhout, H. and J. Amesz. 1965. Growth rates of photosynthetic microorganisms in laboratory cultures. Arch. Mikrobiol. 50: 10-25.

Horner, R. K., D. Burk and S. R. Hoover. 1934. Preparation of humate iron and other humate metals. Plant Pjysiol. 9: 663.

_____. 1973. Eutrophication: The scientific background of a contemporary practical problem. Am. Scientist. May-June.

Hutchinson, G. Evelyn. 1957. A treatise on limnology. John Wiley, New York. 1015p.

Ishiwatari, Ryashi. 1966. Structural characteristics of humic substances in recent lake sediments. Advances in Organic Geochemistry. Proceedings of Third International Conference. G. D. Hobson (ed.) pp. 285-311.

_____. 1967. Estimation of the aromaticity of a lake sediment humic acid by air oxidation and evaluation of it. Soil Sci. 1969, 107 (1), 53-57.

Iswaran, V. 1960. Effect of humified organic matter on nitrogen fixation by Azotobacter. J. Indian Soc. Soil Sci. 8: 107-113.

Johnston, H. W. 1964. Sea water, the natural medium of phytoplankton. I. General features. J. Mar. Biol. Ass. U.K. 43: 427-456.

Jong, G. J. D. and J. C. Jansen. 1958. Demineralization and organic substances. II. The absorption of humic acids by anionic exchange materials. Bull. Centre Belge Et Document Eaux. No. 40: 119-123.

Kain, Joanna and G. E. Fogg. 1958. Studies on the marine phytoplankton II. Isochysis galbana Parke. J. Mar. Biol. Ass. U.K. 37: 781-788.

Karali, E. F. and C. A. Price. 1963. Iron, porphyrins and chlorophyll. Nature. 198: 708.

Karlander, E. P. and R. W. Krauss. 1966. Responses of heterotrophic cultures of Chlorella vulgares Beijerinik to darkness and light. II. Action spectrum for and mechanism if the light requirement for heterotrophic growth. Plant Physiol. 41: 7-14.

Kawaguchi, K. and K. Kyuma. 1959. On the complex formation between soil humus and polyvalent cations. Soil and Plant Food (Japan). 5: 54-63.

Knezek, B. D. and R. H. Maier. 1971. Influence of level and source of Fe on growth and nutrient content of Euglena gracilis. Soil Science and Plant Analysis. 2 (1): 37-44.

Kononova, M. M. 1966. Soil organic matter: Its nature, its role in soil formation and in soil fertility. Pergamon Press, Oxford. 544p.

Kononova, M. M. and I. V. Alek Sandrova. 1958. Biochemistry of the process of humic formation and certain questions of plant nutrition. Izd. Akad. Nauk. SSSR. Ser. Biol. No. 1.

_____ and _____. 1959. The biochemistry of humus formation and some problems of plant nutrition. Soils and Fertilizers. 22: 77-83.

_____, _____ and N. P. Bellchikova. 1960. Biosynthesis of humic substances and their transformation during soil formation. Papers of Soviet Soil Scientists presented at the 7th International Congress in the U.S.A. Izd. Akad. Nauk. SSSR.

Kumada, K. and K. Aizawa. 1958. The infrared spectra of humic acids. Soil Plant Fd. 3: 152-159.

Lange, Willy. 1970. Blue-green algae and humic substances. Proc. 13th Conf. Great Lakes Res. 58-70.

Leclerc, E. and P. Beaujean. 1955a. Research relating to iron complexes. Bull. mens. Centre Belge et Document Eaux. No. 56: 170-176.

_____ and _____. 1955b. Research relative to iron contaminants, the complexing action of the Hautes-Fagnes organic substances. Centre Belge B'etude et de Documentation Des Eaux. June 1955. No. 56: 170.

_____ and T. Samuel. 1956. A study of the capacity of anion exchangers and their alteration. Centre Belge D'etude et de Documentation Des Eaux. No. 31:

Lee, G. Fred. 1973. Role of phosphorus in eutrophication and diffuse source control. Wat. Res. 7: (1-2): 111-128.

Lewin, Joyce and Ching-Hong Chen. 1971. Available Iron: A limiting factor for marine phytoplankton. Limnol. Ocean. 16: 670-675.

Lewis, T. E. and F. E. Broadbent. 1961. Soil organic matter-metal complexes Rt. and Nature and properties of exchange sites. Soil Sci. 91: 393.

Lindsay, W. L. 1972. Inorganic phase equilibria of micronutrients in soils. In: Micronutrients in agriculture. J. J. Mortvedt (ed.). Soil Science of America, Inc. Madison, Wisconsin. 666p.

Lindsay, W. L. and E. C. Moreno. 1960. Phosphate phase equilibria in soils. Soil Sci. Soc. Amer. Proc. 27: 177-182.

Loub, W., V. W. Kiermayer, A. Diskus and K. Hilbauer. 1954. Die algenzonievung in mooren des ostenurkisiken alpengebutes. Ost. Akad. Wess. Math. natur. 163: 447.

Mackenthum, K. M. 1968. The phosphorus problem. J. Amer. Water Works Assoc. 60: 1047-1054.

Manojlovic, S. 1965. Humic acid in the binding of phosphoric acid from soluble phosphates in the soil. Zemijiste Biljka. 14 (1): 25-35.

Martell, A. E. and M. Calvin. 1952. Chemistry of the metal chelate compounds. Soil Sci. R.R.

Martin, D. F., M. T. Doig and Richard H. Pierce, Jr. 1971. Distribution of naturally occurring chelators (humic acids) and selected trace metals in some west coast Florida streams, 1968-1969. Technical paper #12. Florida Dept. of Nat. Resources.

Menzel, D. W. and J. H. Ryther. 1961. Nutrients limiting the production of phytoplankton in the Sargasso Sea, with special reference to iron. Deep Sea Res. 7: 276-281.

Moreau, Richard B. 1972. The interaction of linear phosphate with soluble and particulate calcium and natural colored organic acids in the Thunder Bay, Alpena, Michigan. Ph.D. Thesis. Michigan State University. 242p.

Mortland, M. M. 1970. Clay-organic complexes and interactions. Adv. Agron. 22: 75.

Mun, A. I., R. A. Idrisova and A. B. Berkturov. 1966. Sorption of cobalt, nickel, copper and zinc by humic acids of lake deposits. Izu. Akad. Nauk. Kaz. SSR. Soil Khim. 16 (1): 3-9.

Murphy, D. and D. W. Moore. 1960. A possible structural basis of natural humic acid. Sci. Proc. Roy. Dublin Soc. Ser. Al. No. 40: 191-195.

Myers, J. 1947. Oxidative assimilation in relation to photosynthesis in Chlorella. J. Gen. Physiol. 30: 217-227.

Nissenbaum, Arie and I. R. Kaplan. 1972. Chemical and isotopic evidence for the in situ origin of marine humic substances. Limnol. Oceanog. 17: 570.

O'Brien, W. John. 1972. Limiting factors in phytoplankton algae: Their meaning and measurement. Science. 178: 616-617.

O'Kelly, J. C. 1968. Mineral nutrition of algae. Annu. Rev. Plant Physiol. 19: 89-112.

Oata, Yukito and Takahiko Tsudzuki. 1971. Resemblance of growth substances to metal chelators with respect to their actions on duckweed growth. Plant and Cell Physiol. 12: 619-631.

Oborn, E. T. 1960. A survey of pertinent biochemical literature. USGS Wtr. Supply Papers. No. 1459F.

Orlov, D. S. 1967. Spectrophotometric determination of humus in soils. Soviet Soil Sci. (English Trans.) 1278.

Otsuki, Akira and Takahisa Hanya. 1966. Fractional precipitation of humic acids from recent sediment by use of N,N-dimethylformamide and its infrared absorption spectra. Nature. 212 (5069), 1462-1463.

_____ and Robert G. Wetzel. 1973. Interaction of yellow organic acids with calcium carbonate in fresh water. Limnol. and Ocean. 18: 490-493.

Pearson, E. A., E. J. Middlebrooks, M. Tunzi, A. Aleti, P. H. McGauhey and G. A. Pohlick. 1969. Kinetic assessment of algal growth, Proc. of the eutrophication-biostimulation assessment workshop, E. J. Middlebrooks et al. (eds.).

Posner, A. M. 1963. Use of sephadex gel filtration with reference to humic acids. Importance of electrolyte in the determination of molecular weights by sephadex gel filtration, with special reference to humic acid. Nature. 198: 1161-1163.

Prakash, A. 1971. Terrigenous organic matter and coastal phytoplankton fertility, pp. 351-368. In: Fertility of the Sea. J. D. Costlow (ed.). V. II. Gordon and Breach.

Prakash, A. and M. A. Rashid. 1968. Influence of humic substances on the growth of marine phytoplankton dinoflagellates. (Gonyaulox) Limnol. Oceanog. 13: 598-606.

_____ and _____. 1969. The influence of humic substances on coastal phytoplankton productivity, pp. 431-438. In: Coastal lagoons, a symposium. Mem. Symp. Lagoons Costeras. A. Ayals Castanaces and F. B. Phleger (eds.) UNAM-UNESCO.

_____, _____, Anne Jensen and D. B. Subba Rao. 1973. Influence of humic substances on the growth of marine phytoplankton: Diatoms. Limnol. Ocean. 18: 516-524.

Prat, S. 1955. The effect of humus substances on algae. Folia Biol. 1. 321-326.

_____. 1968. Effect of humus on plant metabolism. Wiad. Bot. 12 (3): 165-175.

_____ and F. Pospisil. 1959. Humic acids with C^{14}. Biol. Plant. Acad. Sci. Bohemoslov. 1: 71-80.

_____, M. Smidova and A. I. Cinceroua. 1961. Penetration and effects of humic substances (Fractions) on plant cells. J. Intern. Congr. Biochem. Abstracts of Commun. 329. Moscow, 1961.

Prelog, V. 1964. Iron-containing compounds in microorganisms. In: Iron metabolism (F. Gross, ed.) Springer-Verlag, Berlin. pp. 73-83.

Provasoli, L. 1963. Organic regulation of phytoplankton fertility, pp. 165-219. In: The Sea, V. II. M. N. Hill (ed.). Interscience, New York.

_____, J. J. A. McLachlin and M. R. Droop. 1957. The development of artificial media for marine algae. Arch. Fur Microbiologie. 25: 392-428.

_____, and I. J. Pintner. 1959. Ecological implications of in vitro nutritional requirements of algal flagellates. Anal. N. Y. Acad. Sci. 56: 839-851.

Rashid, M. A. 1971. Role of humic acids of marine origin and their different molecular weight fractions in complexing di- and trivalent metals. Soil Sci. 111: 298-306.

Reilly, Peter J. 1972. Concurrent growth of bacteria and algae in a closed vessel. OWRR Project No. A-020-NEB.

Rice, J. K., D. E. Simon and R. C. Rice. 1962. Determination of naturally occurring organic acids in surface waters: Industrial Water and Wastes. 7: 75-78.

Rossatti, F. J. C. and H. Rossatti. 1961. The determination of stability constants and other equilibrium constants in solution. McGraw-Hill, New York.

Round, F. E. 1970. The biology of the algae. Edward Arnold Publishers, London. 269p.

Ruttner, Franz. 1972. Fundamentals of limnology. Univ. of Toronto Press, Toronto. 295p.

Sanders, George W. 1957. Interrelations of dissolved organic matter and phytoplankton. The Bot. Review. 23: 389-410.

Schelske, Claire L., F. F. Hoyser and E. J. Haerth. 1962. Responses of a marl lake to a chelated iron and fertilizer. Ecol. 43 (4): 646-653.

Schnitzer, M. 1971. Characterization of humic constituents by spectroscopy. In: Soil Biochemistry, Vol. II. (A. D. McLarin and J. Skyins, eds.). Marcel Dekker, Inc., New York.

_____ and S. U. Khan. 1972. Humic substances in the environment. Marcel Dekker, Inc., New York. 327p.

_____ and S. I. M. Skinner. 1963. Organo-metallic interactions between a number of metal ions and the organic matter of a podzol Bh horizon. Soil Sci. 96: 86-93.

_____ and _____. 1968a. Alkali versus acid extraction of soil organic matter. Soil Sci. 105: 392.

_____ and _____. 1968b. Gel filtration of fulvic acid, a soil humic compound, pp. 41-55. In: Isotopes and radiation in soil organic matter studies. IAEA.

Seelye, James G. 1971. A measurement of low level cesium isotope concentrations in a fresh water lake. M.S. Thesis. Michigan State University. 71p.

Shapiro, Joseph. 1957. Chemical and biological studies on the yellow organic acids of lake water. Limnol. Oceanog. 2: 161-179.

_____. 1961. Freezing-out, a safe technique for concentration of dilute solutions. Science. 133: 2063.

_____. 1966a. Iron available to algae: Preliminary report on a new approach to its estimation in lake water through the use of the ferrigram. In: Proceedings of an IBP Symposium Chemical Environment in the aquatic habitat, held in Amsterdam. October, 1966. 219-228.

_____. 1966b. Yellow organic acids of lake water: Differences in their composition and behavior. Proceedings of an IBP Symposium held in Amsterdam and Nieywerslyis. October, 1966. pp. 202-216.

Siegel, A. 1971. Meatal-organic interactions in the marine environment, pp. 265-295. In: Organic compounds in awuatic environment. D. Faust and J. V. Hunder (eds.). Marcel Decker.

Smidova, M. 1960. The influence of humate on the respiration of wheat. Acta. Agrolot. 9: 129.

Starr, Richard C. 1971. Culture collection of algae at Indiana University - additions to the collection. July 1966-July 1971. J. of Phycol. 7: 350-362.

Steelink, Cornelius. 1963. What is humic acid? J. Chem. Educ. 40: 379-384.

Stein, Janer R. (ed.). 1973. Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, Cambridge. 448p.

Sato, O. and K. Kumada. 1967. The chemical nature of the green fraction of P-type humic acid. Soil Sci. Plant Nutr. 13: 121.

Stevenson, F. J. and M. S. Ardakani. 1972. Organic matter reactions involving micronutrients in soil. In: micronutrients in agriculture. J. J. Mortvedt, P. M. Giordano and W. L. Lindsay (eds.). Proceedings of a symposium held at Muscle Shoals, Alabama, 1971. Co-sponsored by T.V.A. and S.S.S.A.

Taylor, F. J. 1950. Oxidative assimilation of glucose by Scenedesmus quadricauda. J. Exptl. Bot. 1: 301-321.

Theng, B. K. G., J. R. H. Wake and A. M. Posner. 1967. The humic acids extracted by various reagents from a soil. J. Soil Sci. 18: 349.

Tinsley, J. and A. Salem. 1961. Extraction of soil organic matter with aqueous solvents. Soils and Fert. 24: 81.

Toerien, D. F., O. H. Huang, J. Radimsky, E. A. Pearson and J. Scheifig. 1971. Final report provisional aglal assay procedures. Water Quality Office, Environmental Protection Agency. Project # 160100QB. 211p.

Van Beneden, G. 1959. Study of the elimination of iron hard water and the humic materials. Centre Belge D'etude et de Documentation Des Eaux. No. 43: 1959/1. p. 47.

Wallace, Arthur. 1962. A decade of synthetic chelating agents in inorganic plant nutrition. Edwards Bros., Inc. Ann Arbor. 195p.

Waris, H. 1953. The significanœ for algae of chelating substances in the nutrient solutions. Physiol. Plant. 6: 538-543.

Wershaw, R. L., S. J. Heller and D. J. Pinckney. 1970. Measurement of the molecular size of a sodium humate fraction. Advances in X-Ray analysis, Vol. 13. U. S. Geol. Survey. Denver, Colorado.

Wetzel, Robert G. 1967. Dissolved organic compounds and their utilization in two marl lakes. Hidrol. Kozlony. 47: 298-303.

Wheaton, R. M. and A. H. Seamster. 1966. A basic reference on ion exchange. Encyclopedia of chemical technology, 2nd ed. 11: 871-899.

Wilson, W. B. and A. Collier. 1955. Preliminary notes on the culturing of Gymnodinium brevis Davis. Science. 121: 394-395.

Wright, R. R. and M. R. Collings. 1964. Application of fluorescent tracing techniques to hydrologic studies. Amer. Water Works Ass. J. 56(6): 748-754.

Yentsch, C. S. and C. A. Reichert. 1962. The interrelationship between water soluble yellow substances and chloroplastic pigments in marine algae. Bolar. Marina. 3 (3/4): 64-74.

Zajic, J. E. and Chiu. 1970. Heterotrophic culture of algae. Properties and products of algae: Proceedings of the Symposium on the culture of algae sponsored by the division of microbial chemistry and technology of the American Chemical Society. (J. E. Zajic, Ed.) New York, Sept. 7-12, 1969. Plenum Press, New York. 153p.