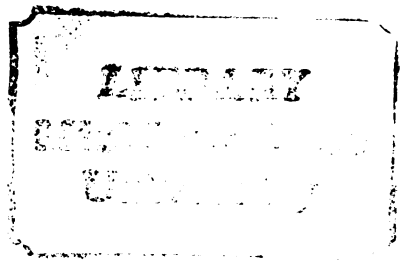

This is to certify that the

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A COMPARISON OF TWO ALGAL BIOASSAY PROCEDURES AND THEIR APPLICATION IN ASSESSING THE EFFECTS OF CUPRIC ION AND P-CRESOL ON ALGAL GROWTH KINETICS

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Michael John Toohill

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Major professor

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A COMPARISON OF TWO ALGAL BIOASSAY PROCEDURES AND THEIR APPLICATION IN ASSESSING THE EFFECTS OF CUPRIC ION AND P-CRESOL ON ALGAL GROWTH KINETICS

By

Michael John Toohill

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ABSTRACT

A COMPARISON OF TWO ALGAL BIOASSAY PROCEDURES AND THEIR APPLICATION IN ASSESSING THE EFFECTS OF CUPRIC ION AND P-CRESOL ON ALGAL GROWTH KINETICS

By

Michael John Toohill

The purpose of this study was to evaluate the effectiveness of two types of static algal bioassays in assessing toxicant effects on algal growth. Batch cultures of Chlorella vulgaris Beijerinck were assayed for changes in growth kinetics caused by cupric ion and p-cresol.

Cupric ion was found to be acutely toxic to Chlorella vulgaris cultures at a concentration of 0.15 mg/l. A concentration of 0.05 mg Cu^{2+}/l was found to have little effect on the algal cultures. Cupric ion levels of 0.075, 0.1, and 0.125 mg/l had increasing effects on algal growth. The sublethal effect was a graded decrease in photosynthesis with increasing cupric ion levels, prolonging the lag phase of growth and decreasing the maximum specific growth rate. This caused a prolongation of the growth cycle of the Chlorella vulgaris cultures. The maximum standing crop level was not affected.

The organic toxicant, p-cresol, was found to be rapidly degraded under nonaxenic algal culture conditions. Four possible causes of loss from the media were tested (photolysis, volatilization, algal sorbtion/degradation, bacterial

degradation) and the results indicated that bacterial degradation of the compound was the major cause of p-cresol loss.

A comparison of the two bioassay methods used showed that the pH method (Young and King, 1973) was more sensitive in assessing the toxic effects of cupric ion than was the Algal Assay Procedure (Miller et al, 1978). In the p-cresol test, the pH method was adversely affected by the breakdown of the p-cresol. The pH method was found to be a more time-efficient procedure.

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INTRODUCTION

In recent years much research has been carried out on the effects of pollutants in the environment. In general, however, the bulk of this effort has been focused on their effects on higher organisms. All too often the primary producers have been neglected or, at best, considered secondarily. While the importance of primary producers is stressed in the classrooms and in the literature, toxicological research generally centers on the heterotrophic life forms in natural systems. A more concerted effort needs to be established in the area of defining toxicant effects on the primary producers in the aquatic ecosystem.

As with researchers in any area of investigation, aquatic biologists have developed certain techniques useful in the determination of the effects of chemicals on organisms. Bioassays have been utilized for some time in these tests as technical tools. In this study, where the actions of two toxicants on Chlorella vulgaris Beijerinck were assessed, two of these bioassay methods were utilized. The first of these is the Algal Assay Procedure--Bottle Test (AAP-BT), currently listed by the United States Environmental Protection Agency as the accepted algal bioassay procedure. This method was outlined by the U.S. Environmental Protection

Agency (1971) and further defined by Miller et al (1978). In this procedure, an algal seed is introduced into a growth medium containing nutrients, and the variable under investigation and algal growth kinetics are studied for the effects of the variable. Quantification of the effects is via the change in cell counts (expressed as cells/ml) over time. The medium contains sufficient amounts of algal macro- and micronutrients to maintain the culture throughout the test period. Temperature and light conditions are held constant, and the pH and CO_2 supply are maintained by bubbling air through the medium, or by other means (Miller et al, 1978). The theory behind this procedure is that if the variable under study has an inhibitory effect on the algal culture, it will be exhibited by a reduced growth of the culture, as compared to controls. Conversely, any substance that augments the growth of the algae will exhibit an increase in cell counts as compared to the controls. While this method does give information on the effect of the variable under study, it gives no information on the interaction of the variable with the pH, inorganic carbon supply, and other growth factors in the system (Young and King, 1973). Also the method of quantification (cell counts) tends to be tedious and may introduce a large source of error into the results (Forsythe, 1973).

Many researchers have used the change in pH caused by algal uptake of CO_2 in a closed system as a measure of production (Verduin, 1951, Beyers and Odum, 1959; Beyers, 1963; Beyers et al, 1963; Young and King, 1973). The procedure of

Young and King (1973) will be followed in this study. As with the AAP-BT, this method (hereinafter called the pH method) supplies all of the nutrients needed by the algae but provides a limited source of inorganic carbon. The basis of the method is that if the amount of inorganic carbon available to the algae is known (the carbonate-bicarbonate alkalinity of the system) and the carbon present in the initial algal seed is known, the total amount of carbon in the closed system at any point in time is known. When, during growth, the algae fix some of the free CO_2 of the medium into algal biomass, the change in the free CO_2 concentration in the system may be measured as a change in the pH of the system. This effect may be seen in the first and second dissociations of carbonic acid (H_2CO_3) in water:

$$H_2CO_3\ (=CO_2 \cdot H_2O) \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+ \qquad (1)$$

or as it relates to the algal uptake of CO_2 (adapted from Wetzel, 1975);

$$2HCO^{3-} \longrightarrow CO_2 + CO_3^{2-} + HOH \qquad (2)$$

The rise in pH as the algae "pull" on the free CO_2 of the system also may be predicted with the following equation:

$$K_2 = \frac{(H^+)(CO_3^{2-})}{(HCO_3^-)} \qquad (3)$$

where K_2 is the temperature dependent second dissociation constant of carbonic acid. Rearranging this equation yields:

$$(H^+) = \frac{K_2\ (HCO_3^-)}{(CO_3^{2-})} \qquad (4)$$

and by taking the logarithm of both sides of the equation:

$$pH = pK_2 - \log \frac{(HCO_3^-)}{(CO_3^{2-})} \qquad (5)$$

As the CO_2 is removed from the system by the algae, the CO_3^{2-} concentration rises, the HCO_3^- concentration drops, and consequently the pH rises. In this system, a variable which has a deleterious effect on algal growth will express itself in a decreased pH rise as compared to the controls. Conversely, any variable which stimulates growth will cause a faster rise in pH of the cultures than is seen in the controls. A basic assumption in this method is that an increase in the pH of the system reflects an increase in algal biomass. This assumption was tested by Young and King (1973) and they found a correlation (r^2) of 0.986 between the calculated biomass estimates from the pH method and direct measurements of organic carbon.

This pH method has two advantages over the AAP-BT. First, the change in pH is more easily monitored than the change in the quantity of cells. Secondly, interactions between the variable under investigation and inorganic carbon (or other nutrient) availability may be seen in the pH method, but not in the AAP-BT.

The purposes of this study were twofold. The first was to examine the two bioassay methods discussed above and attempt to assess the relative sensitivity of each pertaining to changes in algal growth kinetics. By doing this, comparisons between the two methods and their relative sensitivities were made. Secondly, an attempt was made to assess the

inhibitory effects of cupric ion and p-cresol on Chlorella vulgaris. The choice of these two toxicants is discussed in the following paragraphs.

LITERATURE REVIEW

Cupric Ion

Copper is a trace nutrient in plant and animal metabolism and is found in many naturally occurring compounds (Lehniger, 1970). In plants, copper is an important constituent of cytochrome c oxidase, which catalyzes energy transformations during electron transport (Lehniger, 1970). Chloroplasts contain the blue copper protein plastocyanin, which may be the direct electron donor to P700+ in Photosystem I (Weier et al, 1974).

The form of copper of interest in this study is cupric ion (Cu^{2+}). This has been determined to be the active toxic form of the metal (den Dooren De Jong, 1965; Sunda and Guillard, 1976). Cupric ion is the active ingredient in one of the first known fungicides, Bordeaux mixture, which is a mixture of lime, water, and copper sulfate (Bullard, 1972). Copper sulfate also has been extensively used as an algicide, having been applied to lakes for that purpose as early as 1918 (Bullard, 1972). Besides its input into the environment as an algicide, cupric ion is found as a significant constituent in metal plating, pickling, and rayon manufacture wastes (Klein, 1962) as well as in mine tailings.

While much of the copper which is mined is not lost to the environment, the indiscriminant use of copper sulfate has

caused problems. In cases like Lake Monona, Wisconsin, where over two million pounds of copper sulfate had been added over a 26-year period for the control of algae (MacKenthun and Cooley, 1952) or the San Francisco water storage reservoirs where, in the period 1912 to 1962, 800,000 pounds of copper sulfate were added annually (Palmer, 1964), the problem of copper persisting in the aquatic environment is acute. Symmes (1975) reported that of all the copper added to a lake, only 2 percent left the system; 2 percent was in the water and the rest had accumulated in the sediments and biota. Wagner and Bohl's (1978) study showed that upon the addition of copper sulfate or cutrine to the aquatic system, the copper content of the fish in a pond was raised twofold, but the content of the plants and sediments was raised 18 to 80 fold.

Cutrine (copper triethanolamine complex) was chosen as the form of copper to be used in this study because of its present use as an algicide and because of the reactions of copper in the aqueous environment. Under slightly acidic (pH = 6) conditions, cupric ion precipitates as a hydroxide (Britton, 1927). Under alkaline conditions, cupric ion forms soluble and insoluble carbonate complexes (Stiff, 1971a and 1971b). Depending on the pH and alkalinity of the system, these forms include $CuCO_3$, $Cu(CO_3)_2^{2-}$ and $CuCO^3(OH)_2^{2-}$ (Stiff, 1971a). The solubility of these complexes is pH dependent and Stiff (1971a) gives equilibrium constants for these complexes. Cutrine was chosen because

this complex is less susceptible to precipitation as a carbonate, oxide, or sulfide than other copper compounds, and thus is more likely to remain actively toxic. Stiff (1971b) states that copper complexes with amino acids, polypepotides, and humic substances are the most soluble forms of copper.

Much work has been done on assessing the toxicity of cupric ion towards aquatic life. Copper has been found to be toxic to fish in concentrations ranging from 0.05 ppm (Sprague, 1963) to 1.2 ppm (Todd, 1970), depending on the species tested and the test conditions. As previously stated, copper, applied in various forms, has been used for some time as an algicide. Young and Lisk (1972) reported complete inhibition of growth in Anabaena variablis by 0.30 ppm cupric ion and stated that green algae appear to be less sensitive to copper than are blue-green algae. This finding was supported by Kallqvist and Meadows (1978) when they reported that cupric ion applied at a concentration of 24 ppb prevented a blue-green algae bloom, but that the green algae developed normally. They also found that photosynthetic production of a natural assemblage was reduced to 80 percent of the control with the addition of 0.10 ppm cupric ion and to 50 percent of the control with 0.15 to 0.20 ppm cupric ion. They further report that the addition of 0.05 ppm cupric ion reduced the growth rate of the algae to 40 percent of the control within a few days (Kallqvist and Meadows, 1978). Bartlett et al (1974) found that 0.05 ppm cupric ion initiated growth inhibition in Selenastrum

capricornutum and that 0.09 ppm cupric ion was completely inhibitory towards growth. McBrien and Hassal (1967) found that a concentration of 1uM $CuSO_4$ (0.255 ppm $CuSO_4$) caused an increase in the lag phase of Chlorella culture growth and resulted in decreased cell counts after four days. They noted that a concentration of 2uM $CuSO_4$ (0.51ppm $CuSO_4$) completely inhibited culture growth. Sunda and Guillard (1976) found that growth rate inhibition and copper toxicity are related to cupric ion activity and not to the total copper concentration.

Various modes of action of cupric ion toxicity have been reported. Several researchers (McBrien and Hassal, 1967; Cedeno-Maldonado and Swader, 1976) reported that cupric ion reacts with protein sulfhydryl groups and in this way inhibits algal growth. Cedeno-Maldonado et al (1972) also reported that cupric ion inhibits electron transport in chloroplasts and, in this way, cuts down on the photosynthetic production. Gross et al (1970) noted that at a concentration ratio of 1.27ug $Cu^{2+}/10^6$ Chlorella cells, there was a significant drop in cell chlorophyll content. Furthermore, McBrien and Hassal (1965) found that cupric ion caused a release of potassium from Chlorella cells in amounts exceeding the number of equivalents of cupric ion. This indicates an increase in cell permeability.

In the course of this study, the exact mode of action of copper toxicity will not be assessed. Because of the extensive amounts of research done on the element and because of

its known algicidal properties, it is useful as a test of the sensitivities of the two algal assay procedures chosen.

p-Cresol

p-Cresol (4,methyl-phenol or 4,hydroxy-toluene) is a phenolic compound with the chemical formula C_7H_8O. p-Cresol is a weak acid with a pK_a of 10.2 at 25°C. It melts at 34.8°C (Chemical Rubber Co., 1974). p-Cresol has a solubility in water of 1.8 mg/ml at 25°C (Lang, 1973) and has a low vapor pressure (Chemical Rubber Co., 1974). It is a constituent of creosote, which is widely used as a wood preservative and for a variety of other uses (U.S. Environmental Protection Agency, 1978). This phenolic compound is also a significant by-product of coal gasification, coal liquification, and petroleum refining processes (U.S. Environmental Protection Agency, 1978; Boling, personal communication). With the increased production of these fuel products, water contamination with p-cresol is a distinct possibility. Campbell et al (1979) have shown very clearly how underground coal gasification projects can lead to groundwater contamination with p-cresol. They also proved that there may be a delayed release of this chemical due to the disruption of water movements in the area surrounding these coal gasification projects.

In the aqueous environment, p-cresol has been shown to react in several ways. p-Cresol may be chemically oxidized

to yield Pummerer's ketone, 2,2'-dihydroxy-4,4'-dimethyl-biphenol and the trimer of this compound (U.S. Environmental Protection Agency, 1978). Furthermore, p-cresol may undergo photolysis to yield a variety of organic compounds (Joschek and Miller, 1966). Both of these processes occur at a very slow rate (U.S. Environmental Protection Agency, 1978). The most significant degradation pathway of p-cresol in the aquatic environment is via biodegradation (U.S. Environmental Protection Agency, 1978). This is considered virtually the only breakdown pathway in eutrophic systems and photolysis becomes a significant factor only under oligotrophic conditions. Adsorption of p-cresol onto suspended particles and into the sediments is considered negligible (U.S. Environmental Protection Agency, 1978). These relationships can be seen in Table 1. Several genera of bacteria have been found to be biodegrade p-cresol. These include Escherichia coli (Landa et al, 1953), Aerobacter aerogenes (Phillips and Hinschelwood, 1953), Pseudomonas sp. (Dagley and Patel, 1957; Claus, 1964; Bayly et al, 1966), and Achromobacter sp. (Claus, 1964). Bunch and Chambers (1967) found that greater than 99.5 percent of the p-cresol initially in their samples was degraded in seven days in their biodegradability tests. A representative breakdown pathway for p-cresol in Pseudomonas is:

p-cresol → p-hydroxymethylphenol → p-hydroxybenzaldehyde → p-hydroxybenzoic acid → protocatechnic acid → ring cleavage (U.S. Environmental Protection Agency, 1978).

Table 1. Predictions based on a nine-compartment environmental exposure model for p-cresol (U.S. Environmental Protection Agency, 1978)

Environment	Half life (hr)	Solution (g/ml)	Suspended Solids (g/g)	Sediments (g/g)	Percent sorbed
River	0.55	0.980	9.80	9.65	0.1
Pond	12.0	0.017	0.17	0.17	0.3
Eutrophic Lake	12.0	0.037	0.37	0.10	0.05
Oligotrophic Lake	2400	0.0221	2.20	0.94	0.05

p-Cresol may indeed be biodegraded in less than one hour (Boling, personal communication); however, little work has been done on this chemical in relation to its effect on aquatic life.

LD_{50}'s ranging from 5 to 15 ppm p-cresol have been reported for fish and invertebrates (McKee and Wolf, 1963). Boling and Cooper (1980) found a significant shift in the nocturnal drift patterns of invertebrates upon the addition of 8 ppm p-cresol to their experimental stream. They also noted that bluegills exhibited abnormal behavior when exposed to 2.5 ppm p-cresol and a cessation of feeding when exposed to 8 ppm p-cresol. No literature was found pertaining to the effects of this chemical on algal growth.

Chlorella vulgaris

The experimental algae, Chlorella vulgaris Beijerinck, is a planktonic, unicellular green algae with large cup-shaped chloroplasts (Trainor, 1978). Its planktonic character makes it suitable for a batch culture testing procedure, minimizing the loss of algae cells due to adhesion to the walls of the test vessel. Its unicellular habit makes it easy to enumerate, and its large cup-shaped chloroplast is an easily identifiable characteristic of this genus (Trainor, 1978). Chlorella is fairly ubiquitous in nature (Smith, 1933) and divides about as rapidly as any green plant, making it ideal for phycological studies (Trainor, 1978). It is a relatively small alga (4 to 5u), necessitating the use of a

high-power, high-resolution microscope (Smith, 1933). Its nearly perfect spherical shape makes it an ideal specimen for enumeration by electronic particle counters. This was one of the primary reasons why Chlorella vulgaris was chosen for this study. However, mechanical problems with the available particle counter precluded its use in this study.

ALGAL CULTURE METHODS

One of the aims of this study was to assess the effectiveness of two algal bioassay procedures in detecting the effects of toxicants on algal culture growth. The two methods chosen, the Alagal Assay Procedure--Bottle Test (Miller et al, 1978) and the method of Young and King (1973), differ in several respects. In this study, an attempt was made to adhere as closely as possible to the standards put forth by the United States Environmental Protection Agency (Miller et al, 1978) for algal bioassays. The following sections briefly describe these standards and any modifications which were made in order to make the two methods more comparable. For brevity, the modification of the Algal Assay Procedure--Bottle Test used in this study is referred to as the AAP. The method of Young and King (1973) is referred to as the pH method.

Physical Conditions

An assay platform, similar to that described by Miller et al (1978), was constructed and divided into 64 equally sized and spaced sections, each of sufficient size to accommodate one culture vessel. Miller et al (1978) recommend that the algal cultures be grown under 400 $\pm$10 percent

footcandles of constant fluorescent illumination. The lighting for this study was supplied by eight 48-inch-long General Electric fluorescent plant-grow light bulbs mounted on a scaffolding above the assay platform. Light intensity measurements (footcandles) were taken prior to and following the conclusion of each experiment with a Model 756 Weston Illumination Meter equipped with a quartz filter Number 2403. Those spaces falling in the 360 to 440 footcandle range were used in the test. The Photosynthetically Active Radiation (PAR) also was measured because recent literature suggests that this is a more realistic measurement of the light available to plants for photosynthesis (Trainor, 1978). A LiCor Integrating Photometer, Model Li 18813, equipped with a Li 1925B underwater sensor was used for this purpose. Figures showing the variation of both footcandles and PAR across the assay platform surface are presented in the text.

Glassware was cleaned with a laboratory soap solution, rinsed with distilled water, and acid washed with chromic acid. Following these steps, the glassware was soaked in a 30 percent hydrochloric acid solution for six hours and rinsed with distilled water. These steps were taken to minimize contamination of the Chlorella vulgaris cultures.

Miller et al (1978) recommend that the pH of the cultures be maintained by continuous mixing on a shaker table at 100 rpm. Efforts were made to fulfill this requirement; however, the heat generated by the shaker table raised the temperature of the cultures to 32°C, far above the

recommended temperature of 25°C. An alternative method of pH control was devised.

Line air was delivered through a filter and water saturator (glass wool in a 250 ml flask containing approximately 100 ml of distilled water) and a series of brass aquarium gang valves to the culture vessels. The valves allowed regulation of the airflow to each flask. A pasteur pipette inserted through a Number 9 two-hole rubber stopper delivered the air to the bottom of the culture vessel. A second pasteur pipette, inserted tip up and capped with a foam plug, provided a pressure release while minimizing culture evaporation and contamination. This closure was used for all AAP culture vessels and for the stock culture vessels.

The closure for the pH method test vessels was a modification of those described by Young and King (1973). A Number 9 two-hole rubber stopper was fitted with a Number 12 serum cap and a pressure-release apparatus. The pressure release consisted of a piece of tygon tubing inserted through the open hole of the stopper. The opposite end of the tubing was placed underwater in a beaker. This apparatus allowed for the release of evolved oxygen from the cultures while minimizing atmospheric recarbonation of the medium.

Algal Culture Medium

The components of the algal culture medium are listed in Table 2. This medium is a modification of that developed by Kevern (personal communication) and is higher in nutrient

Table 2. Composition of synthetic algal nutrient medium. The components listed are given as final concentrations in milligrams per liter.

MACRONUTRIENTS

Compound	Concentration (mg/l)
$NaHCO_3$	168.0
KNO_3	114.0
$CaCl_2 \cdot 2H_2O$	13.2
$MgSO_4 \cdot 7H_2O$	20.0
KH_2PO_4	3.0

MICRONUTRIENTS

Compound	Concentration (mg/l)
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$(NH_4)_6Mo_7O_{24}$	0.18
$CuSO_4$	0.05
$CoCl_2 \cdot 6H_2O$	0.40
$FeCl_3 \cdot 6H_2O$	1.20
Na_2EDTA	3.00

concentrations than the medium recommended by Miller et al (1978). The intent of raising the nutrient concentrations was to ensure that all components were in nonlimiting quantities. The exception to this is carbon in the case of the pH method, but not in the AAP where the medium is constantly recarbonated. Fitzgerald (1975) states that whereas the maximum yield of an algal culture depends on the concentration of nutrients in the medium, the rate of growth of cultures in different media remains the same up to the point of nutrient deficiency. The medium chosen for these experiments should have alleviated any problems of nutrient deficiency which would have complicated the analyses.

The medium was prepared with reagent grade chemicals and distilled-deionized water on a regular basis. Macronutrients were added directly to 18 l of distilled-deionized water in a 5-gallon glass carboy. This mixture was aerated for 48 hours to ensure carbonation of the medium. The micronutrients, with the exceptions of $FeCl_3 \cdot 6H_2O$ and Na_2EDTA, were combined in a stock solution at 1,000 times the final medium concentration. The $FeCl_3 \cdot 6H_2O$ and Na_2EDTA were combined in the same manner. Eighteen ml of each stock were added to the aerated macronutrient mixture.

Experimental Algae

The experimental algae, Chlorella vulgaris Beijerinck, were obtained from the University of Texas Algal Collection.

Upon receipt, the culture was transferred into freshly prepared nutrient medium. Weekly inoculations of fresh medium with 1 ml of 6- to 8-day-old culture kept the algae in an actively growing condition.

AAP Sampling, Enumeration, and Calculation Methods

The USEPA (1971) lists various methods of estimating the biomass of algal cultures. Although gravimetric methods may be preferable, these researchers state that indirect measurements of biomass, such as cell counts, normally will be required because of the difficulty in making accurate gravimetric measurements at low cell densities. Therefore, the preferred method is indirect enumeration via an electronic particle counter. This was the method chosen for this study; however, as previously noted, an alternative method had to be found. This other method, found to be reliable by Miller et al (1978), is direct microscopic enumeration. The direct counting method chosen was that of McNabb (1960). A twenty-five-milliliter sample was withdrawn from each culture with a pipette. Portions of this sample were then filtered at 8 cm pressure through a 0.45u Millipore filter. The filters were air dried for 1 to 3 hours prior to mounting them on glass microscope slides with Type A, microscope objective immersion oil. This process had the effect of making the filters transparent while preserving the algae in their natural form.

After enumeration, the raw cell counts were converted to biomass estimates (cells/ml) by the following formula:

$$\text{cells/ml} = \left(\frac{NC}{F}\right)\ (MF)\ \left(\frac{NF}{V}\right) \qquad (6)$$

where NC is the number of cells counted, F is the number of fields counted, NF is the number of filters counted, V is the volume of sample filtered (ml), and MF is the magnification factor. This factor is determined by the equation

$$MF = \frac{\text{effective filtering area}}{\left(\frac{\text{microscope field width}}{2}\right)^2} \qquad (7)$$

Using these cell number estimates, the specific growth rate, u, for the cultures can be determined by the equation

$$u = \frac{N/\Delta t}{n} \qquad (8)$$

where N is the change in cells/ml over the time increment Δt and n is the average number of cells/ml during this time increment.

The USEPA (1971) states that the maximum specific growth rate (u_{max}) for an individual flask is the largest u obtained at any time during incubation. Since this is an observed value, as opposed to a calculated u_{max}, this parameter was referred to as the maximum u observed (observed u_{max}) in this study. As discussed in the following section, the pH method allows for the calculation of u_{max} rather than taking the observed u_{max}. To avoid confusion, the distinction between these two parameters is noted.

The recorded biomass estimates (cell counts), u values, and observed u_{max} values for each treatment were compared in

order to detect changes in culture condition due to the toxicant being studied.

pH Method Sampling, Measurement, and Calculations

The basis for the estimation of algal growth response in the pH method is the change of culture pH over time. The starting point for the calculations in this method is the initial carbonate-bicarbonate alkalinity (a) of the medium, the initial pH of the medium, and the organic carbon content of the algal seed inoculum. The alkalinity of the medium was measured prior to the start of a test by the potentiometric method (American Public Health Assoc., 1965). The potentiometric method yields the total alkalinity of the system. To determine the noncarbonate portion of the alkalinity, a replicate sample of the medium was acidified to pH 3 with 1N H_2SO_4 and sparged with nitrogen gas for 5 minutes to remove CO_2. The CO_2-free sample was back titrated to the initial pH of the medium with 0.02N NaOH to determine the noncarbonate alkalinity. Subtraction of the noncarbonate alkalinity from the total alkalinity yields the alkalinity due to the carbonate-bicarbonate buffering system (a).

The total organic carbon content of the algal seed inoculum was determined with a Beckman Model 915A carbon analyzer. Replicate samples of the cultures were acidified to pH 3 with 1N HCl and sparged with nitrogen gas for 5 minutes to rid the samples of inorganic carbon. Fifty ul samples of the cultures were injected into the 900°C. oven of

the carbon analyzer. The results were compared to standard curves of known organic carbon concentrations prepared by analysis of potassium acid phtlalate standards.

Samples from the pH method test vessels were taken with 25-ml syringes fitted with 18-gauge hypodermic needles. The pressure release tubes were sealed with a cork stopper prior to sampling. The contents of the test vessels were mixed by swirling and inverting the vessels for sampling. The sampling procedure, penetration of the septum of the serum cap with the hypodermic needle, and withdrawal of the sample, created a slight vacuum in the test vessel. This was equalized by injecting 25 cc of nitrogen gas into the test vessel with a hypodermic needle. The samples were then injected into a 50 ml beaker. This was done by placing the tip of the needle at the bottom corner of the beaker and slowly injecting the sample into the beaker so as to minimize recarbonation. The pH of the sample was measured with a Beckman Zeromatic pH meter equipped with an Orion Research Needle Tip combination electrode. Prior to each use, this instrument was calibrated with standard buffer solutions for the culture pH anticipated. In the first few days of growth, buffer solutions in the range of pH 7 to 10 were used. After the culture exceeded pH 10, the meter was calibrated with buffer solutions in the pH range 9 to 11 in an attempt to minimize interferences usually encountered in solutions at high pH (American Public Health Assoc.,

1965). The accuracy of the pH meter and electrode was determined to be approximately 0.1 pH unit.

Young and King (1973) recommend taking the pH of the sample in a nitrogen gas-filled apparatus, so as to avoid recarbonation of the sample. Testing of their method versus the one mentioned above revealed no appreciable difference in pH between replicate samples. Even at high pH, the recarbonation of the sample occurred at a slow enough rate as to allow accurate determination of the sample pH.

Using the pH measurements, initial medium alkalinity (a) and the organic carbon content of the algal inoculum, the calculation method of Young and King (1973) was used to estimate the growth kinetics of the cultures. The pH measurements were transformed into values of carbon fixed by the algae by the equation

$$M_t = CO_2 = (CO_{2t_0} - CO_{2t}) \qquad (9)$$

where M_t is the change in biomass (mM organic carbon/l) over the time increment t and CO_2 is determined by the equation

$$CO_2 = a\left(\frac{H^2/K_1 + H + K_2}{H + 2K_2}\right) \qquad (10)$$

where CO_2 is the total molar sum of the inorganic carbon species in the medium (mM/l); a is the carbonate-bicarbonate alkalinity of the medium (meq/l) which has been corrected for the hydroxide ion concentration of the medium; H is the hydrogen ion activity as determined by a pH meter; and K_1 and K_2 are the first and second dissociation constants of

carbonic acid. Values of K_1 and K_2 are temperature-dependent and, for the purposes of this study (temperature = 27°C.), the values used were $K_1 = 4.3 \times 10^{-7}$ and $K_2 = 4.9 \times 10^{-11}$.

Likewise, the free carbon dioxide concentration of the medium was determined from the pH and initial alkalinity of the medium by the equation

$$CO_{2f} = a\left(\frac{H^2}{K_1 (H + 2K_2)}\right) \tag{11}$$

where CO_{2f} is the free carbon dioxide concentration of the medium (mM/l).

The specific growth rate, u, is calculated over the time interval t by the equation

$$u = \frac{M/t}{\bar{m}} \tag{12}$$

where M is the change in biomass (mM carbon fixed by the algae/l) over the time increment t, and $\bar{m}$ is the average standing crop biomass (mM C/l) during the time increment.

Models predicting u as a function of the concentration of a limiting substrate have been used for some time. One such model is the Monod Equation (after Lehniger, 1970);

$$u = u_{max} \frac{S}{K_s + S} \tag{13}$$

where S is the concentration of a substrate, u_{max} is the maximum specific growth rate, and K_s is the saturation constant (substrate concentration at which $u = 1/2\ u_{max}$. Equation (13) is also known as the Michaelis-Menten Equation (Lehniger, 1970).

If an assumption is made that there exists a lower threshold concentration of the substrate in question; that is, a concentration below which the algae cannot assimilate the substrate, equation (13) can be shown as:

$$u = u_{max} \left(\frac{S - Sq}{(K_s - Sq) + (S - Sq)} \right) \qquad (14)$$

where Sq is the lower threshold concentration of the substrate. Therefore, when S = Sq, u = 0.

In the pH method, where CO_{2f} is the limiting substrate, equation (14) can be shown as:

$$u = u_{max} \left(\frac{CO_{2fo} - K_{CO_{2fo}}}{(K_{CO_{2fo}} - CO_{2fq}) + (CO_{2fo} - CO_{2fq})} \right) \qquad (15)$$

where CO_{2fo} is the observed CO_{2f} concentration of the medium and CO_{2fq} is the lower threshold concentration of CO_{2f}.

The substrate concentration represented by $CO_{2fo} - CO_{2fq}$ is the actual concentration of CO_{2f} in the medium available to the algae for photosynthisis (CO_{2fA}).

To obtain CO_{2fA}, one must find CO_{2fq}. Two methods of obtaining CO_{2fq} were assessed. The first is to estimate CO_{2fq} by observation. That is, when during the course of the experiment the calculated u for a replicate = 0, the CO_{2fq} concentration at that point can be taken as CO_{2fq}. Averaging the CO_{2fq} values for the replicates then gives an estimate of CO_{2fq} for that treatment.

A second method is to estimate CO_{2fq} graphically from the experimental data. Since growth curves for algae are logrithmic in nature (up to the stationary growth phase), one

should expect that in a closed system that nutrient uptake also would be logrithmic. Since CO_{2f} is the limiting nutrient, a "mirror image" of CO_{2f} uptake vs growth (biomass) is anticipated. Therefore, plots of u vs CO_{2fo} were used to estimate the CO_{2f} concentration at which u = 0, the CO_{2fq} value. These curves are of the form

$$u = a + b\ (\ln CO_{2fo}) \tag{16}$$

The second method of estimating CO_{2fq} was chosen for use in this study because it utilizes all of the data from the active growth phases of each replicate, as opposed to one value per replicate. Although this method led to some extrapolation of the data, the CO_{2fq} estimates yielded by this method were several orders of magnitude below the CO_{2f} values obtained and did not influence the determination of u_{max} or $K_{CO_{2f}}$.

The value for CO_{2fq} obtained by this method was then subtracted from CO_{2fo} to yield CO_{2fA} (the actual CO_{2f} concentration available to the algae for photosynthesis at time t). Rearranging equation (15) and substituting CO_{2fA} for $CO_{2fo} - CO_{2fq}$ yields

$$1/u = 1/u_{max} + \frac{K_{CO_{2fo}} - CO_{2fq}}{u_{max}} (1/CO_{2fA}) \tag{17}$$

which is a linear regression equation of the form Y = a + bX. This transformation is known as the Lineweaver-Burk Equation (Lehniger, 1970).

Curves of the linear transform 1/u vs $1/CO_{2fA}$ were therefore used to determine the kinetic constants u_{max} and $K_{CO_{2fo}} - CO_{2fo}$. The latter kinetic constant, as determined by the curve-fitting procedure, is the corrected CO_{2f} concentration where u equals $1/2u_{max}$, or $K_{CO_{2fA}}$.

The specific growth rates and corresponding CO_{2fA} concentrations which occurred during the active growth phase of the cultures were used in this curve-fitting process.

Attempts were made to use the Monod Equation as a mathematical model in this study. The three kinetic constants, derived from algal cultures exposed to various toxicant concentrations, were examined for changes due to the toxicant. When either CO_{2fq}, $K_{CO_{2f}}$, or u_{max} varied as a function of the toxicant concentration, they were substituted into the Monod equation as follows:

$$u = f_1(Z) \left(\frac{CO_{2f} - f_3(Z)}{[f_2(Z) - f_3(Z)] + [(CO_{2f} - f_3(Z)]} \right) \qquad (18)$$

where $f_1(Z)$ equals u_{max} as a function of the toxicant concentration, $f_2(Z)$ equals $K_{CO_{2f}}$ as a function of the toxicant concentration, and $f_3(Z)$ equals CO_{2fq} as a function of the toxicant concentration. Such models would have been useful in predicting the sublethal effects of a toxicant on the specific growth rates of Chlorella vulgaris cultures at various toxicant and medium CO_{2f} concentrations. However, as discussed in Experiments 4 and 5, the growth of the algal cultures did not follow the Michaelis-Menton growth kinetics

equations closely enough to warrant the extrapolation of the data in further modeling.

Statistical Analyses and Computer Software

The statistical methods employed varied with the setup of the experiments. Complete randomization was used wherever possible. Because of the volume of the data, analyses of variance, t-tests, multiple-range procedures, and the other statistics used were calculated using the Statistical Package for the Social Sciences (SPSS) computer software package (Nie et al, 1975). Assay platform light level contour maps were produced by using the Surface II Graphics Software package (Sampson, 1975) and a CalComp Plotter. Calculation of the algal growth kinetics was performed by using a Fortran routine designed by the experimenter. All computations were performed by the Control Data Corporation Model 170 Cyber Series 750 computer maintained by the Michigan State University Computer Laboratory.

PRELIMINARY EXPERIMENTS

The aim of this series of tests was to locate and attempt to minimize various sources of error in the experimental methods. The first two experiments dealt with sources of error encountered in the enumeration procedure of the AAP. The third experiment was a trial run of the two algal bioassay methods chosen for use in this study.

EXPERIMENT 1

Cell Counting Procedure Error

Purpose

In this experiment, an attempt was made to locate and eliminate sources of error in the enumeration procedure of the AAP.

Procedure

Three 5 ml replicate samples were withdrawn from a stock culture of Chlorella vulgaris. Three 0.5 ml subsamples of each sample were filtered and fixed on glass microscope slides. Twenty microscope fields were counted for each subsample at 1,000X. A nested analysis of variance (Sokal and Rohlf, 1969) was used to test the significance of the variance components in the model

$$Y_{ijk} = u + S_i + F_{ij} + C_{ijk} \qquad (19)$$

where Y_{ijk} is the observed sample mean; u is the true population mean; S_i is the effect due to sampling; F_{ij} is the effect of subsamples within samples; and C_{ijk} is the effect of cell counts within subsamples.

Results

The results of the cell counting procedure are tabulated in the Appendix (Table A1). The results of the analysis of

variance indicate that the variability due to sampling is not significant (Table 3). The low level of significance due to the variability involved in subsampling indicates variability due to the filtering procedure as well as subsampling. Since some clumping of the algal culture sampled was noted, and since pasteur pipettes were used in the sampling procedure, it was felt that the error due to subsampling makes up the majority of the variability in this term.

Conclusions

The majority of the variation in the enumeration procedure was due to the cell counting. The sampling effect was not significant. Since some variation was due to subsampling, future samples were directly enumerated. Also, volumetric glassware was used.

The clumping of the algal cells appeared to be a function of culture age. This was determined by microscopic examination of cultures at different stages of growth. This effect was minimized by thoroughly mixing the culture prior to sampling and mixing the samples prior to filtration. As indicated in the analysis, the maximum effort should have been placed in counting rather than in sampling. Therefore, in the following experiments one sample was withdrawn from each culture flask at each sampling time and analyzed.

Table 3. Nested analysis of variance studying the variability in the sampling, subsampling, and counting procedures used for the AAP.

Source	df	SS	MS	F
Total	179	3,115.4		
Sample	2	38.9	19.5	0.577 ns
Subsample	6	203.0	33.8	2.012 *
Count	171	2,873.5	16.8	

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (Not significant)

EXPERIMENT 2

The Effects of Culture Concentration of the Cell Counting Procedure

Purpose

In Experiment 1, all samples were of the same volume and were enumerated at the same magnification. This experiment was designed to test the effects of the use of various sample volumes and microscope magnifications on the enumeration procedure. This gave an indication of the responsiveness of the modified McNabb (1960) method to differing biomass levels.

Procedure

A sample of 8-day-old Chlorella vulgaris culture was taken and various dilutions made (Table 4). These subsamples were filtered, fixed on slides, and enumerated. The results were analyzed by linear regression.

Results

One would have expected a linear relationship between the expected and observed cell numbers, if the volume of the sample filtered and/or the microscope magnification used made no difference in the enumeration procedure used here. The high degree of correlation found between these variables ($r^2 = 0.992$, $n = 6$, log transformation) indicates that this

Table 4. Experimental setup and results from a test of the modified McNabb (1960) method of enumerating algal cells. Shown for cell count and cells/ml are the mean ±1 standard deviation for ten replicates.

Dilution factor	Filtered volume (ml)	Microscope magnification	Cell count	Cells/ml
1	2.0	1,000	TNC	--
1	0.5	400	159 ±53	2.9×10^6 $\pm 9.8 \times 10^5$
5	5.0	1,000	TNC	--
5	1.0	1,000	16 ±3	9.3×10^5 $\pm 1.7 \times 10^5$
10	1.0	1,000	6 ±2	3.5×10^5 $\pm 1.2 \times 10^5$
50	20.0	1,000	33 ±7	9.6×10^4 $\pm 2.0 \times 10^4$
100	20.0	1,000	9 ±7	2.6×10^4 $\pm 2.0 \times 10^4$
500	20.0	400	14 ±4	6.4×10^3 $\pm 1.8 \times 10^3$
1,000	20.0	400	6 ±3	2.8×10^3 $\pm 1.4 \times 10^3$

TNC = Too numerous to count.

was the case for the range tested in this experiment (Figure 1). It was noted that counting the Chlorella vulgaris cells at low magnification (400X) was difficult because of their small size. Increasing the sample size at low-culture densities eliminated the need for enumeration at this magnification.

Conclusions

From the results of Experiments 1 and 2, it was concluded that the modified McNabb (1960) method of enumerating algal cells was an acceptable alternative to indirect enumeration by an electronic particle counter. The use of direct enumeration for the estimation of algal culture biomass is preferred by Miller et al (1978) over other methods such as absorbance and chlorophyll-a fluorescence. By varying the filtered volume and microscope magnification, one can accurately estimate the algal culture density over a broad range (10^3 to 10^6 + cells/ml).

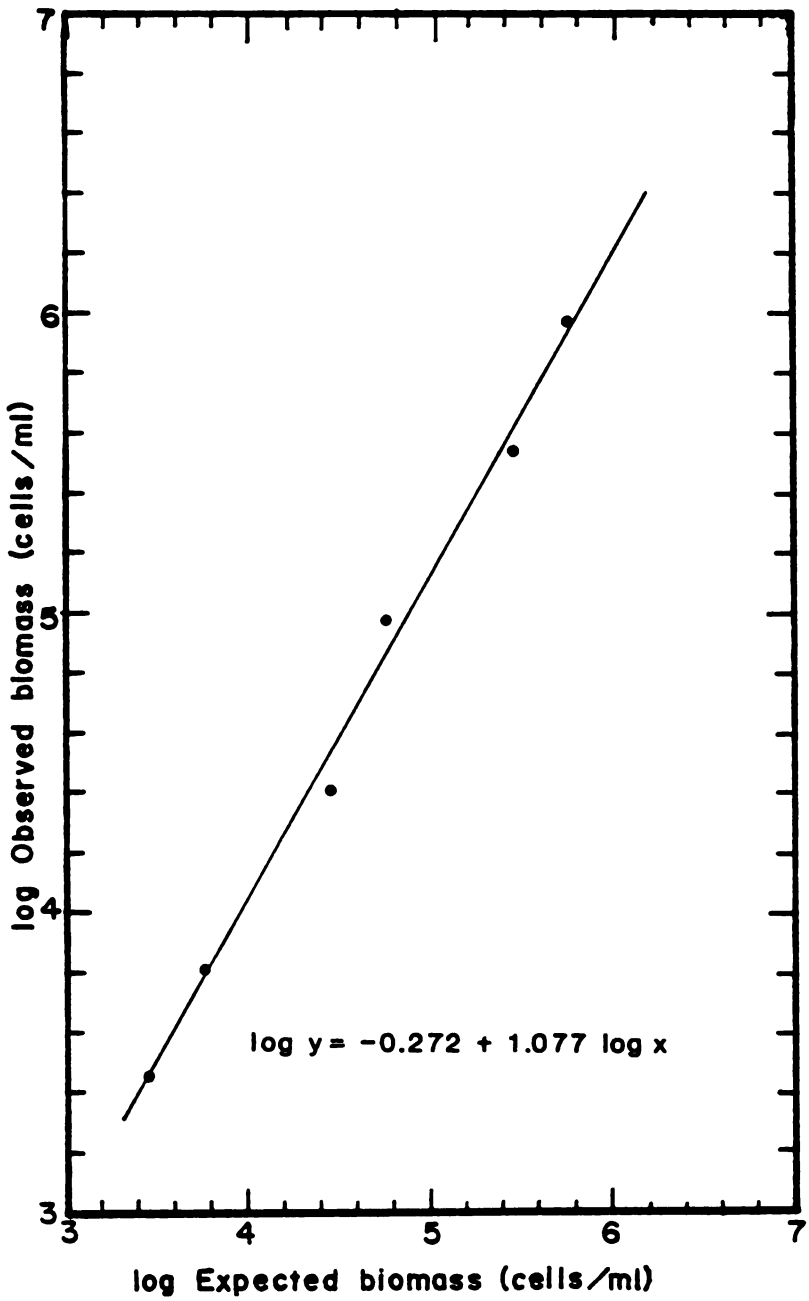


Figure 1. The relationship between the expected and observed biomass estimates using the modified McNabb (1960) method of algal cell enumeration.

EXPERIMENT 3

Trial Run of the AAP and pH Methods

Purpose

This test was designed to familiarize the experimenter with the two algal bioassay methods chosen for use in this study. The purpose also was to isolate and correct procedural difficulties in these algal assay techniques.

Procedure

Ten culture vessels were each half filled with 500 ml of fresh nutrient medium (a = 1.77 meq CO_3^{2-}--HCO_3^- alkalinity/l). Five of these vessels were randomly assigned to the pH method and five to the AAP. Measurements of light reaching the assay platform were taken and 25 of the 64 spaces on the assay platform were within the acceptable range of 400 $\pm$40 footcandles of illumination (Figure 2). The test vessels were randomly assigned to 10 of these 25 spaces. Figure 3 shows the positions of the test vessels in relation to the photosynthetically active radiation (PAR) falling on the platform.

Each flask was inoculated with 0.5 ml of 6-day-old *Chlorella vulgaris* stock culture, giving a final concentration of 5,300 cells/ml. The organic carbon content of the stock culture was 146 mg/l, as determined by carbon analysis.

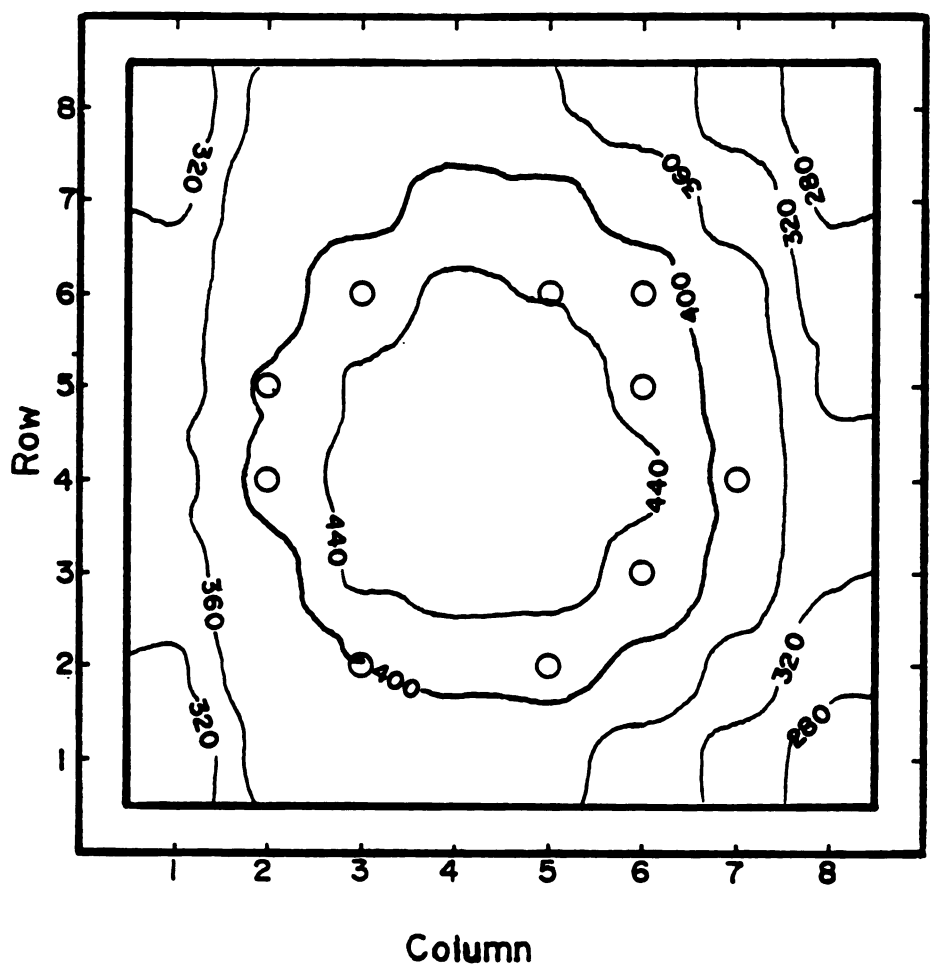


Figure 2. Contour map of the light intensity (footcandles) falling on the assay platform in Experiment 3. The position of each test vessel is indicated by a circle.

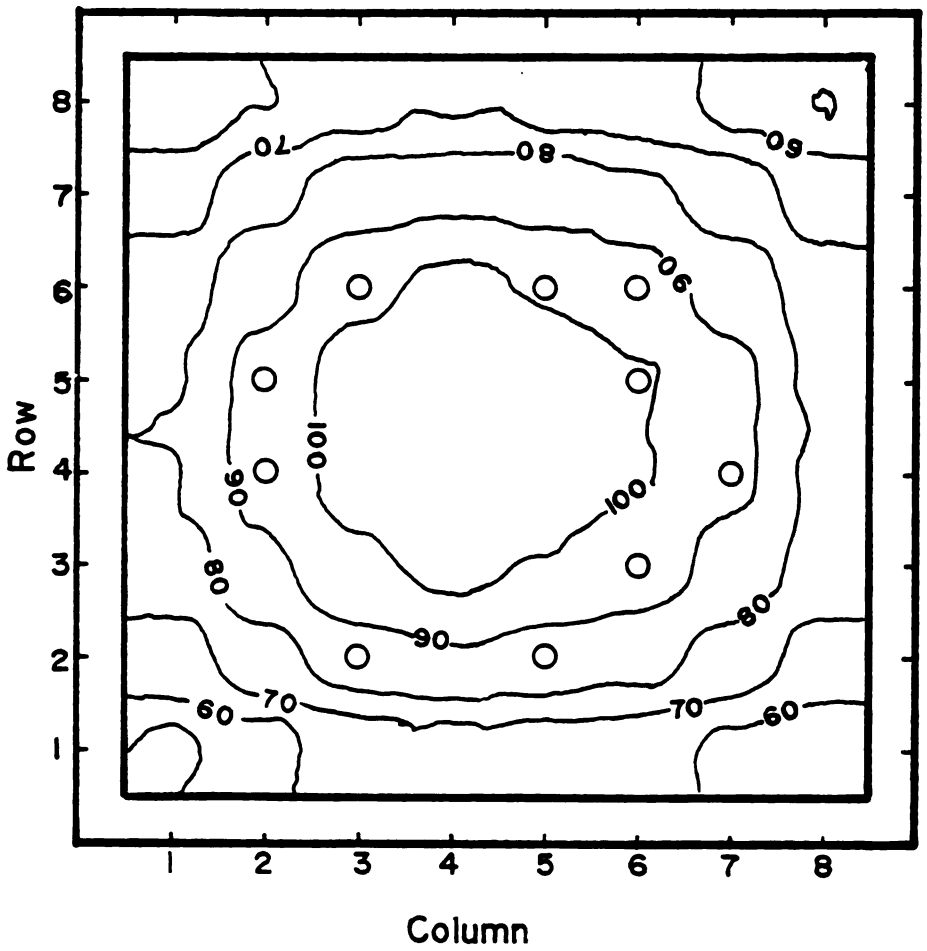


Figure 3. Contour map of the photosynthetically active radiation (uE/m^2/sec) falling on the assay platform in Experiment 3. The position of each test vessel is indicated by a circle.

This equates to an experimental culture content of 0.0122 mM organic C/l. Daily samples of the AAP cultures were taken and the number of cells/ml counted. Samples of the pH method cultures were taken on a daily basis and the pH determined. The pH of the AAP cultures was also monitored to ensure proper recarbonation of the medium by the pH control apparatus. The test was run at 27°C.

The length of the test period was determined by examining growth curves plotted as the test proceeded. The test continued until the stationary growth phase had been reached.

Results

The constant aeration of the medium kept the pH of the AAP cultures at a moderate level. The sharp rise in pH between days 4 and 5 (Figure 4) was the result of a break in the air delivery system. The return of the pH to a moderate level between days 5 and 6 illustrates the effectiveness of the pH control method used. The results of AAP culture pH control from Experiments 4 and 5 are also shown in Figure 4. In all three experiments, the pH control apparatus kept the pH of the AAP cultures within the guidelines of Miller et al (1978).

The daily cell counts of the AAP cultures are tabulated in the Appendix (Table A2). These cell counts were used to produce the growth curves in Figure 5. The five AAP cultures remained in the lag phase of growth for approximately one day and reached their maximum standing crop biomass levels by day 5, at which time a lack of light due to shading effects

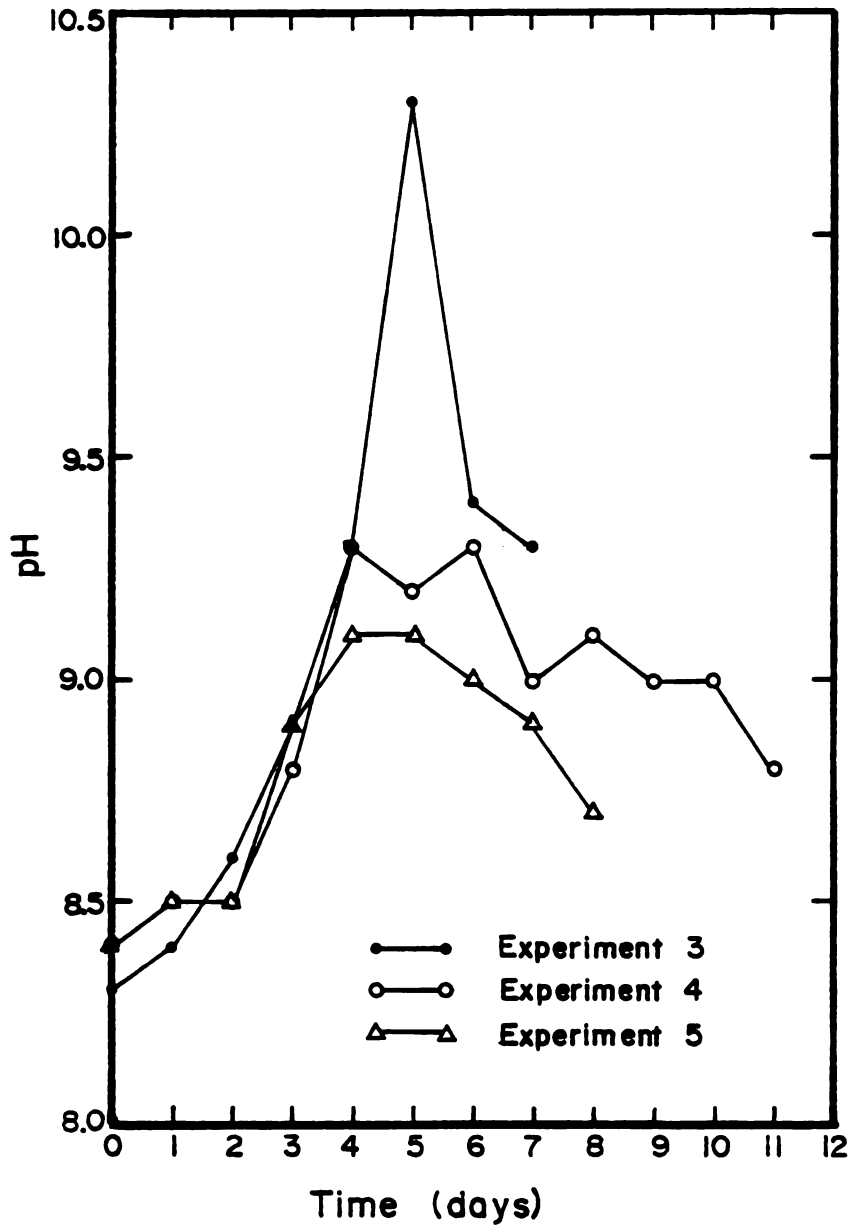


Figure 4. pH fluctuation of AAP cultures of Chlorella vulgaris from Experiments 3, 4 and 5.

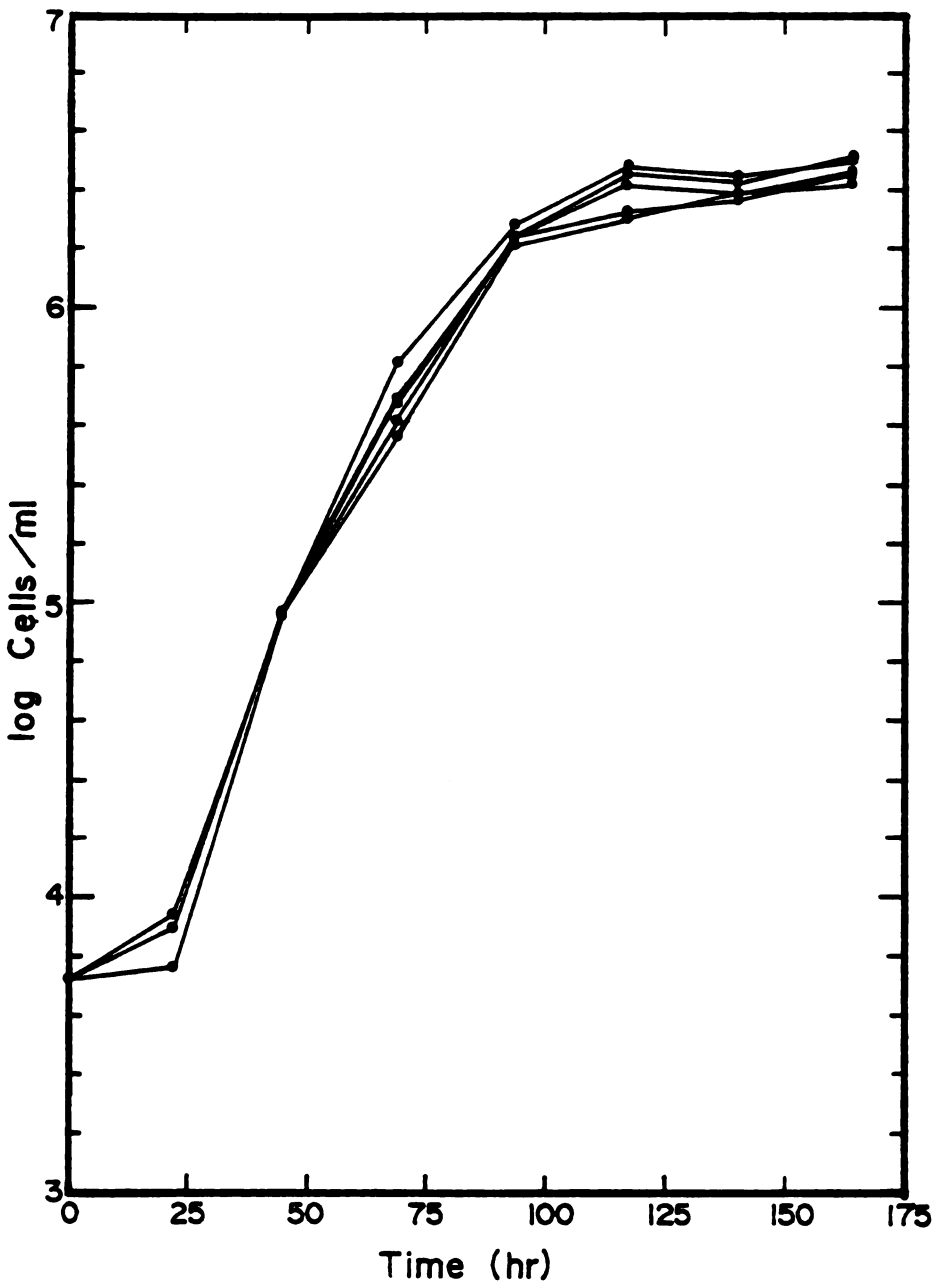


Figure 5. AAP growth curves for Chlorella vulgaris cultures in Experiment 3.

within the cultures may have become the limiting growth factor.

The statistical analysis of the data (Table 5) indicates that although the growth curves are fairly uniform, at several samplings there existed a significant difference between the biomass estimates of the cultures. It also was noted that the biomass order of the cultures changed on a daily basis (Table 5). These results indicate a nonuniformity of growth in the various cultures, independent of initial algal inoculum. The variability of the growth rates of the five replicates is shown in Table 6. All five replicates reached their respective maximum specific growth rates (observed u_{max}) in the interval between sampling times 21 and 44.5 hours. This observation is in accordance with the findings of Forsythe (1973) and Miller et al (1978) who stated that the observed u_{max} is generally reached in the first few days of culture growth. The mean observed u_{max} for the five replicates was 0.072 $hour^{-1}$.

The daily pH measurements of the pH method test cultures are tabulated in the Appendix (Table A3). The biomass curves for the pH method cultures (Figure 6) also are fairly uniform. As shown in Table A3, the pH measurements for all five replicates vary only by a few tenths of a pH unit throughout the experiment. Given the inherent variability of the pH meter ($\pm$0.1 pH unit), there was a high degree of replicability with the pH method in this experiment. The means and standard deviations of the transformed data are given in Table 5.

Table 5. The statistical analysis of Experiment 3. The one-way analysis of variance F test and coefficient of variability are listed for each sampling period for the AAP and pH method, respectively. For the AAP, the replicates are listed in ascending order of biomass. Underlining indicates no significant difference within the underlined group as judged by a Student-Newman-Keuls multiple comparison test (P = 0.95). For the pH method, the mean of 5 replicates ±1 standard deviation is shown.

Time (hr)	AAP F value	AAP replicate	pH method (biomass)
21.0	7.08 ***	3 1 4 2 5	0.0241 ±0.0090
44.5	1.51 ns	3 4 1 5 2	0.1274 ±0.0325
68.5	21.17 ***	1 4 2 5 3	0.5447 ±0.1373
93.5	0.72 ns	1 2 5 4 3	1.6127 ±0.0744
116.5	3.39 **	1 5 2 4 3	1.7636 ±0.0439
140.5	2.58 **	1 5 2 4 3	1.7636 ±0.0439
164.0	2.27 *	5 1 2 3 4	1.7636 ±0.0439

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

Table 6. Specific growth rates (u) of Chlorella vulgaris cultures in Experiment 3, as calculated by the AAP. Maximum specific growth rates (u_{max}) are underlined. Units of u are 1/hour.

Interval	Replicates 1	2	3	4	5
1	0.0182	0.0191	0.0047	0.0186	0.0235
2	<u>0.0717</u>	<u>0.0719</u>	<u>0.0747</u>	<u>0.0714</u>	<u>0.0704</u>
3	0.0503	0.0563	0.0635	0.0541	0.0570
4	0.0511	0.0452	0.0392	0.0495	0.0452
5	0.0093	0.0187	0.0195	0.0204	0.0092
6*	0.0065	-0.0029	-0.0023	-0.0024	0.0059
7	0.0078	0.0071	0.0046	0.0087	0.0036

*Individual replicates exhibited a decline in biomass. The group as a whole had an average u = 0.0006 $hour^{-1}$.

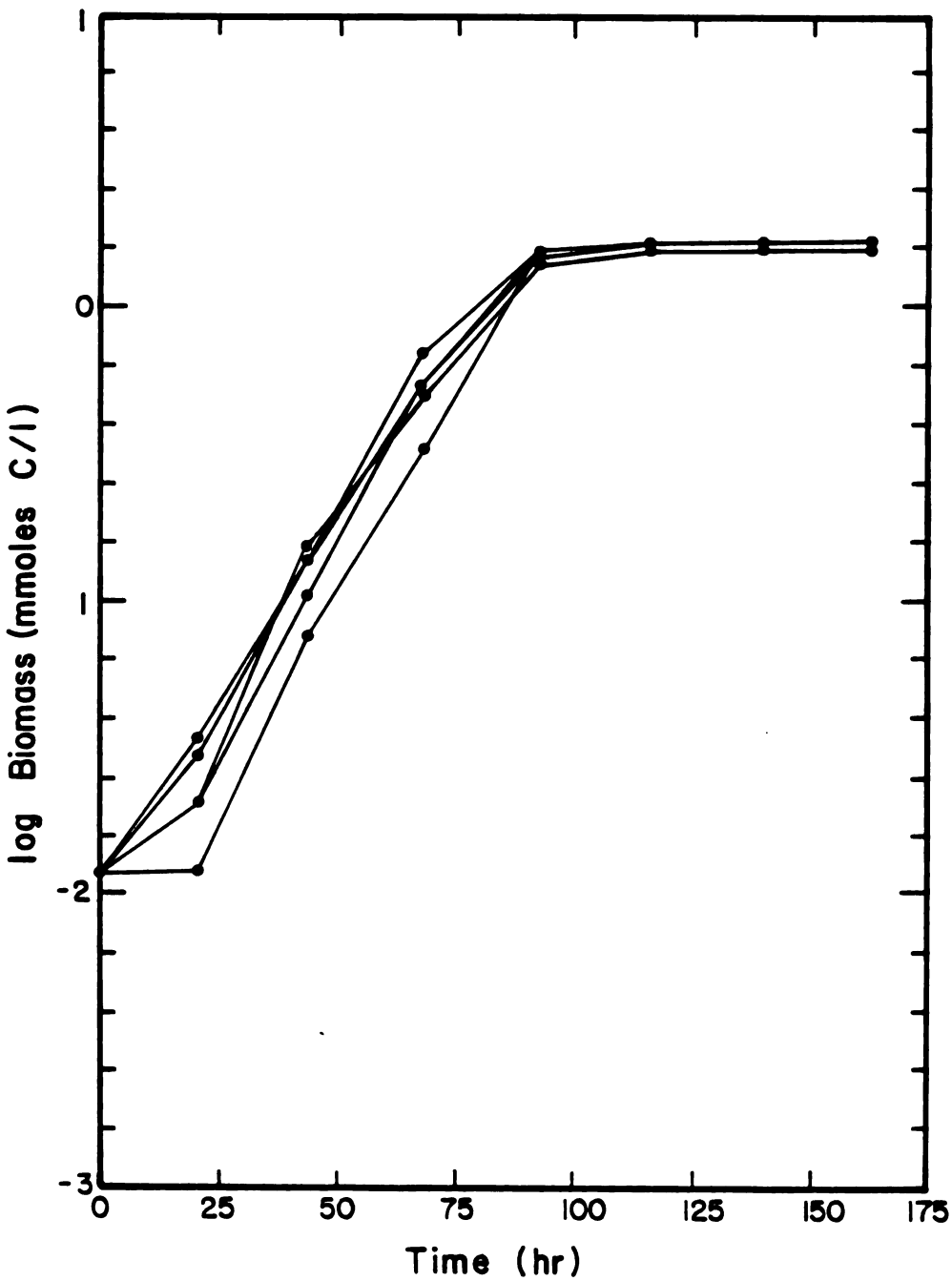


Figure 6. pH method growth curves for Chlorella vulgaris cultures in Experiment 3.

To estimate the photosynthetic CO_2 minima, the specific growth rates from the active phase of growth were plotted versus the corresponding average CO_{2fo} concentrations of the medium (Figure 7). The data were fit with a natural logarithmic regression curve and gave a CO_{2fq} estimate of $9x10^{-5}$ uM CO_2/l (r^2 = 0.970, n = 18). After correcting CO_{2fo} for CO_{2fq}, a double reciprocal plot of 1/u vs $1/CO_{2fA}$ was produced in order to estimate u_{max} and $K_{CO_{2f}}$ (Figure 8). A least-squares regression analysis of Figure 8 (r^2 =0.867, n =15) yielded $K_{CO_{2f}}$ estimate of 0.0283 uM CO_{2f}/l and a u_{max} estimate of 0.0547 $hour^{-1}$ for the combined replicates.

Conclusions

The pH of the AAP cultures was maintained within the guidelines of Miller et al (1978) by constant aeration. This was the case for Experiments 4 and 5 as well as this experiment.

The estimates of u_{max} given by the two bioassay methods varied greatly. This may have been a function of the setups of the two methods and/or the variables used to estimate culture changes (cell counts versus carbon uptake). Because of these differences, the u_{max} estimates from the two methods could not be compared statistically.

The significant variations between replicates indicated a need for greater replication. However, because of the light-level restrictions and the time consumed in sampling

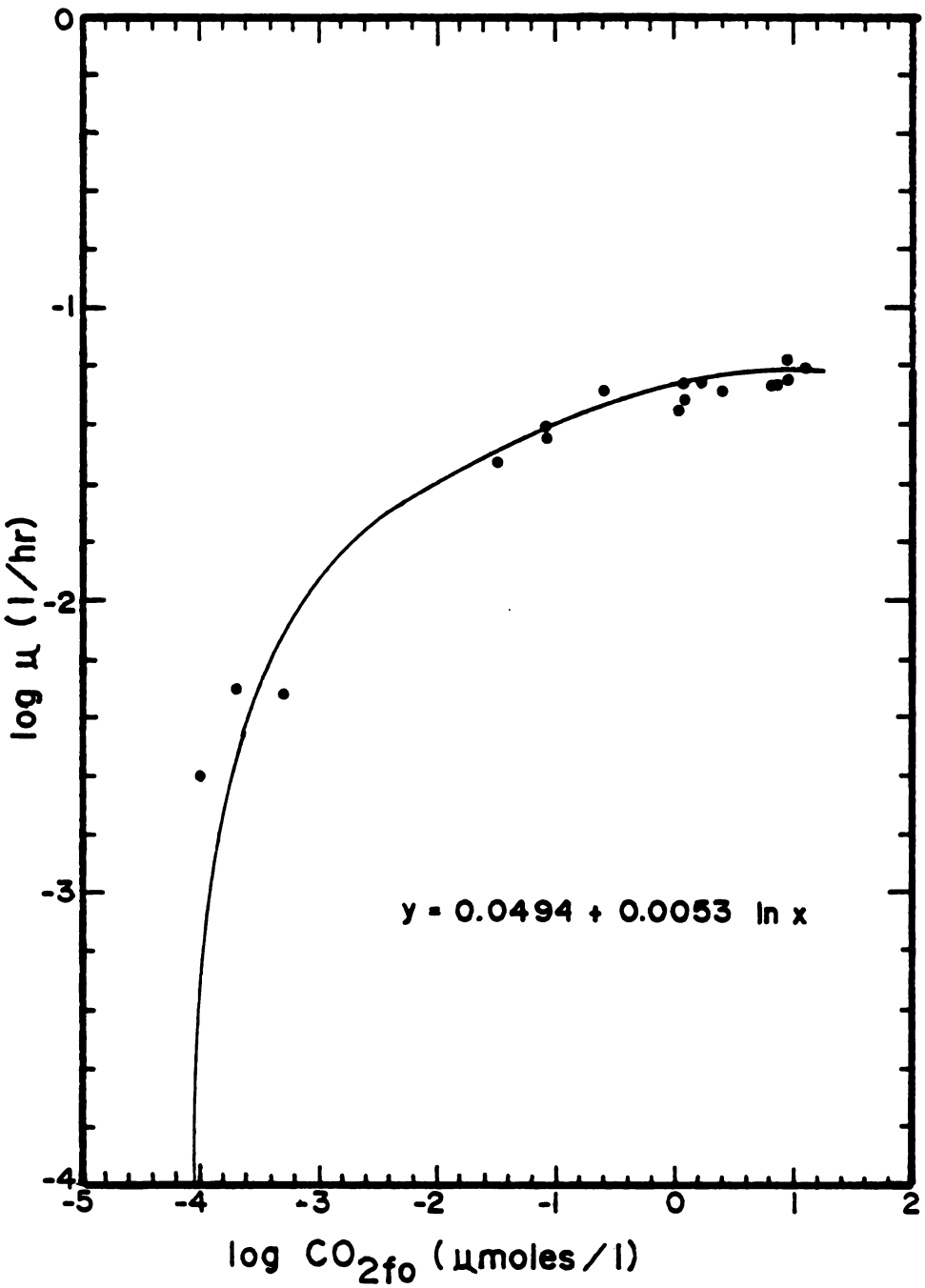


Figure 7. Specific growth rates of cultures of Chlorella vulgaris in Experiment 3 as a function of the corresponding free CO_2 concentrations of the medium.

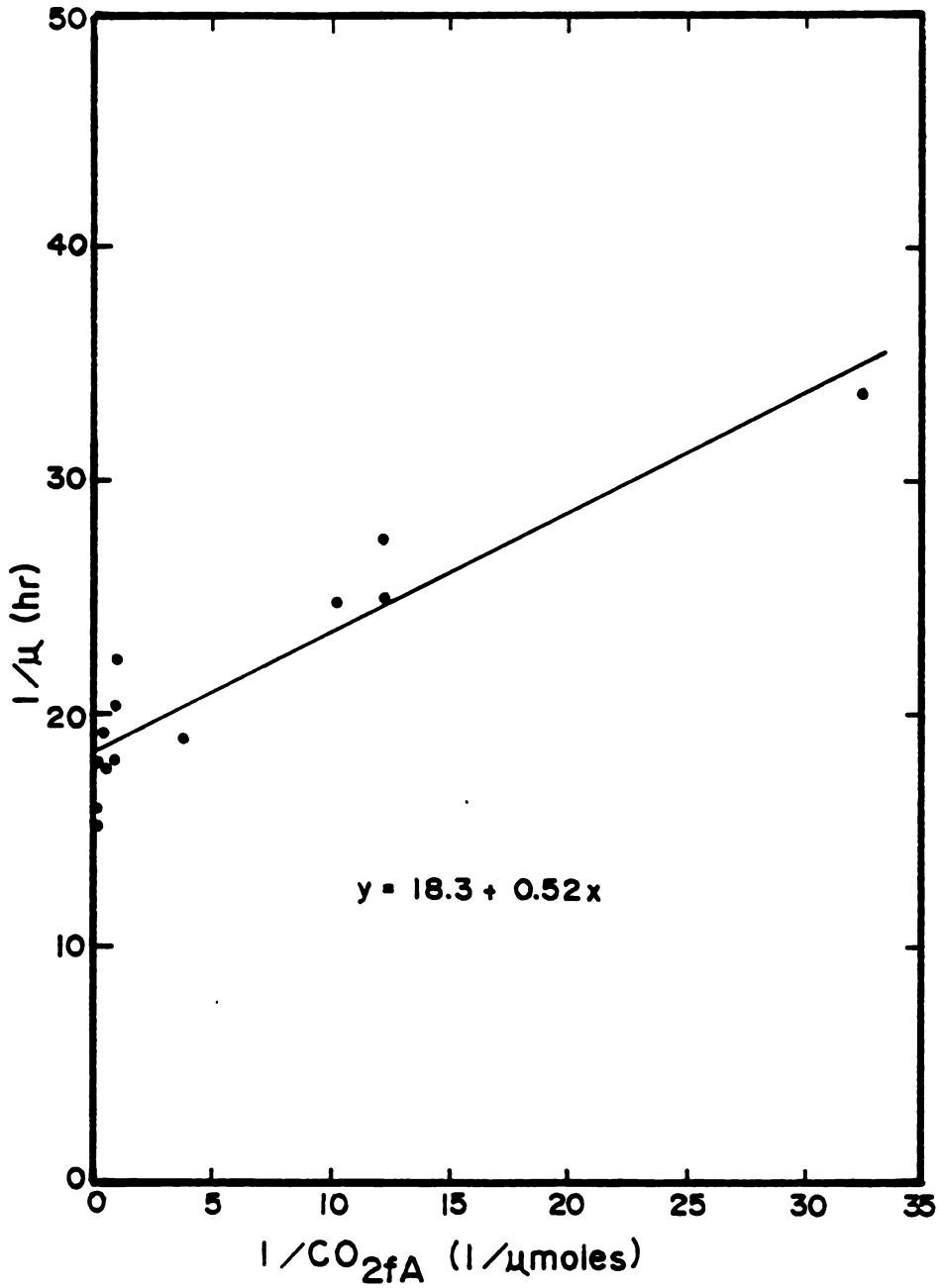


Figure 8. Reciprocal plot of the specific growth rates of Chlorella vulgaris cultures in Experiment 3 versus the corresponding free CO_2 concentrations of the medium.

and enumeration, this was not possible in this study. Therefore, a more detailed analysis of variance, separating the error due to replication from that due to the treatment effects, was used in subsequent experiments.

TOXICOLOGY EXPERIMENTS

The following experiments were applications of the AAP and pH method algal bioassay techniques in assessing the effects of two toxicants on algal growth kinetics. The first two experiments dealt with the effects of cupric ion. The third experiment in this series was designed to assess the effects of p-cresol. The fourth experiment was a test of the degradation of p-cresol under algal bioassay conditions.

EXPERIMENT 4

The Effects of Cupric Ion on *Chlorella vulgaris* Culture Growth

Purpose

This experiment was designed to test the effects of three concentrations of cupric ion on the growth kinetics of *Chlorella vulgaris* cultures. It was intended to show how a known algicide exhibits its inhibitory effect on algal growth in the two bioassay methods chosen for use in this study.

Procedure

Twenty-four test vessels were each half filled with 500 ml of fresh nutrient medium (a = 2.038 meq CO_{32-}--HCO_3^- alkalinity/l). The average light intensity measurements (Figure 9) indicate that only 20 of the 64 spaces of the assay platform were in the acceptable range of 400 $\pm$40 footcandles of illumination during the test. However, at the beginning of the test, 24 of the 64 spaces were within this range, and the amount of bulb decay observed was not anticipated prior to beginning the experiment. The 24 test vessels were randomly assigned to these spaces. The positions of the test vessels in relation to the photosynthetically active radiation falling on the platform are shown in Figure 10.

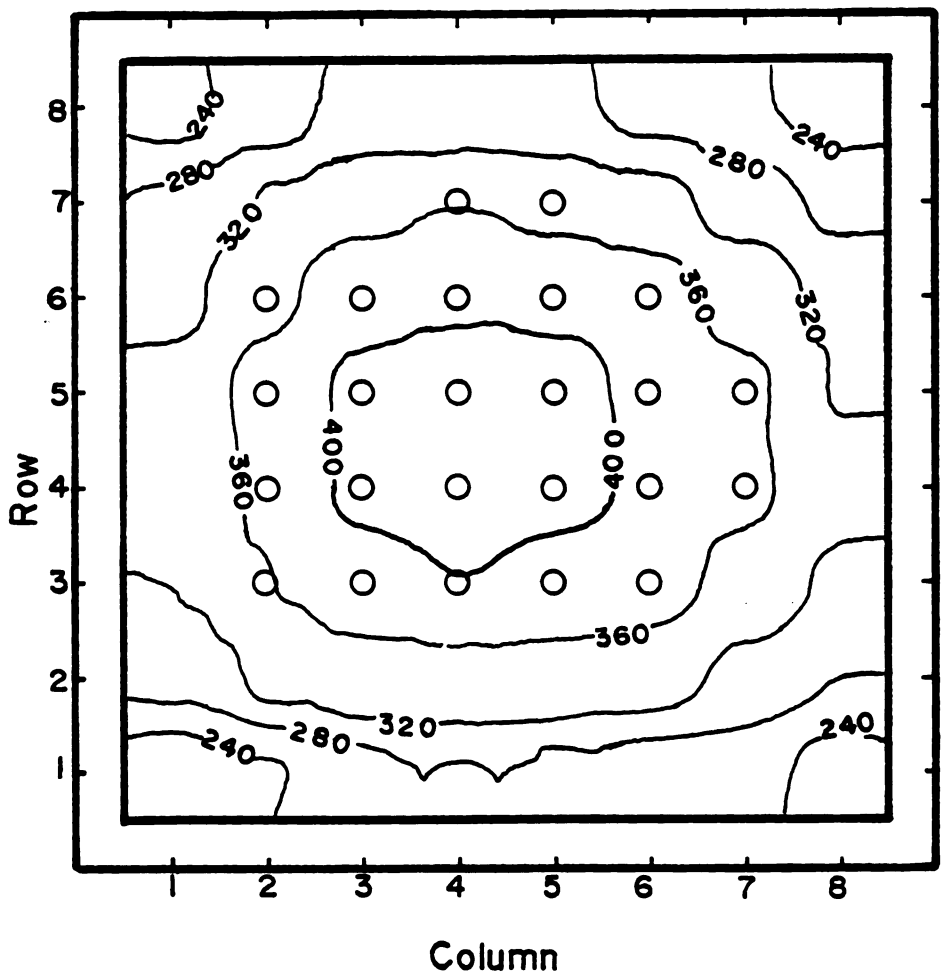


Figure 9. Contour map of the light intensity (footcandles) falling on the assay platform in Experiment 4. The position of each test vessel is indicated by a circle.

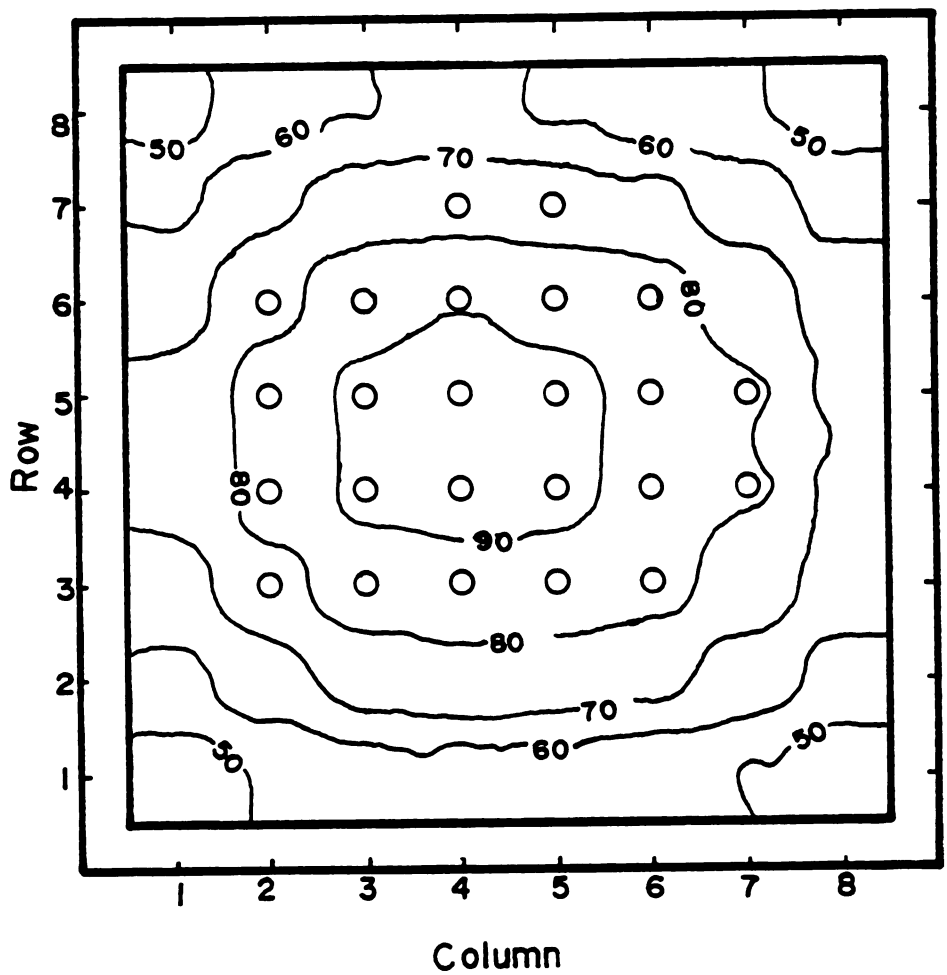


Figure 10. Contour map of the photsynthetically active radiation ($uE/m^2/sec$) falling on the assay platform in Experiment 4. The position of each test vessel is indicated by a circle.

Twelve test vessels were randomly assigned to the AAP and twelve to the pH method. Within each method, three flasks were assigned to each of the three treatment levels of cupric ion. The remaining three vessels in each method were used as controls.

One milliliter of cutrine (91.071 g Cu^{2+}/l) was added to 999 ml of distilled deionized water to give a stock solution concentration of 91.071 mg Cu^{2+}/l. This stock solution was added directly to the test vessels within each method to give three replicates each of 0.05, 0.10, and 0.15 mg Cu^{2+}/l.

Each test vessel was inoculated with 0.5 ml of 7-day-old Chlorella vulgaris stock culture to give a final concentration of 3,400 cells/ml. Carbon analysis of the stock culture yielded a test culture concentration of 0.01133 mM organic carbon/l.

Daily samples of the AAP cultures were taken and the number of cells/ml computed. Samples of the pH method cultures were taken daily and the pH determined. The test was run at 27°C.

The daily cell counts (cells/ml) from the AAP were tested for significant differences between treatments by a nested analysis of variance (Sokal and Rohlf, 1969) and Student-Newman-Keuls multiple comparison procedure (Steel and Torrie, 1960). The biomass estimates from the pH method (mM C fixed by the algae/l) were tested by an analysis of variance and Student-Newman-Keuls multiple comparison procedure (Steel and Torrie, 1960).

Results

The results of the daily cell counts of the AAP cultures are tabulated in the Appendix (Table A4). The resulting growth curves (Figure 11) indicate a graded response to the four levels of cupric ion tested, with the 0.15 mg Cu^{2+}/l treatment cultures exhibiting acute toxicity. All four treatments were judged significantly different from one another at various sampling times (Table 7). The three cupric ion treatments were judged significantly different from the control until day 6 (104.5 hr), when both the control and the 0.05 mg Cu^{2+}/l treatment had almost reached their maximum standing crop biomass levels (Figure 11). The 0.10 mg Cu^{2+}/l treatment remained significantly different from the control until day 10 (236 hr) when it too had almost reached the stationary phase of growth (Figure 11). The 0.15 mg Cu^{2+}/l treatment cultures remained in the lag phase of growth for several days and eventually entered the death phase (Figure 11).

The specific growth rates calculated from these biomass estimates are shown in Table 8. The control, 0.05 and 0.10 mg Cu^{2+}/l, treatments reached their maximum specific growth rates in interval 4 (between the 68 and 91.5 hr samplings). The specific growth rates indicate that the control cultures apparently reached the observed u_{max} at an earlier time than did the 0.05 mg Cu^{2+}/l treatment cultures, leading to the conclusion that the control may have had a higher u_{max} than observed. The observed u_{max} of the 0.10 mg Cu^{2+}/l treatment

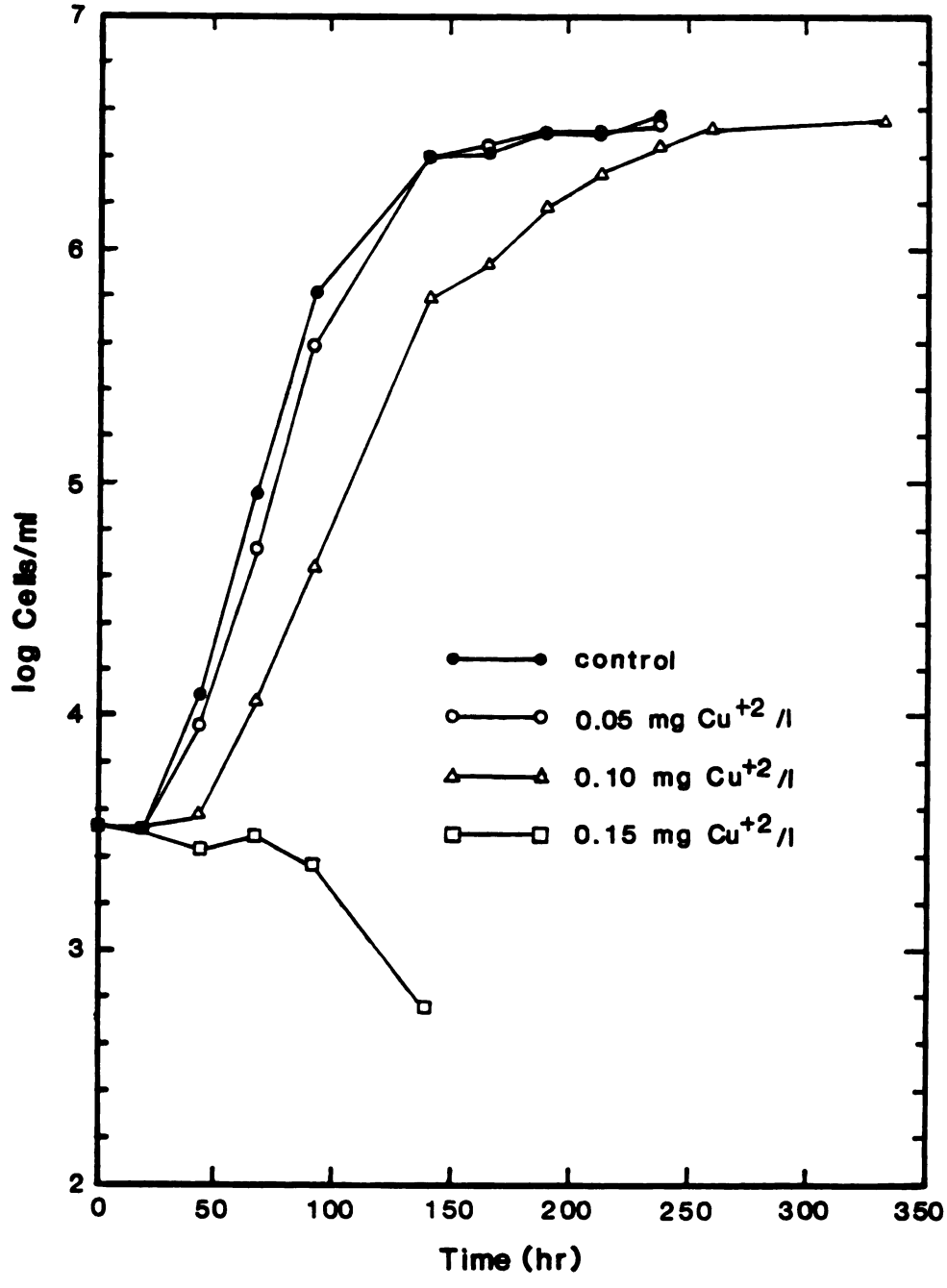


Figure 11. AAP growth curves for Chlorella vulgaris cultures in Experiment 4. Each point represents the mean of three replicates.

Table 7. Statistical analysis of the AAP results from Experiment 4. F test values and associated significance levels from a nested analysis of variance are listed for each sampling time. The treatments are listed in ascending order of biomass (cells/ml) with those means judged not significantly different using a Student-Newman-Keuls multiple comparison procedure (P = 0.95) grouped by underlining.

Time (hr)	F value	Treatment (mg Cu^{2+}/l)
20.0	0.35 ns	<u>0.15 0.05 0.00 0.10</u>
44.0	82.16 ***	0.15 0.10 0.05 0.00
68.0	122.00 ***	0.15 0.10 0.05 0.00
91.5	129.20 ***	0.15 0.10 0.05 0.00
140.5	21.85 ***	0.15 0.10 <u>0.05 0.00</u> #
164.0	6.01 **	0.10 <u>0.00 0.05</u>
188.5	4.93 *	0.10 <u>0.00 0.05</u>
212.0	2.57 +	0.10 <u>0.00 0.05</u>
236.0	2.55 +	<u>0.10 0.05 0.00</u>

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

The 0.15 mg Cu^{2+}/l treatment was terminated after this sampling.

Table 8. Specific growth rates (u) of Chlorella vulgaris cultures in Experiment 4, as determined by the AAP. Maximum specific growth rates are underlined.

	Treatment (mg Cu^{2+}/l)			
Interval	Control	0.05	0.10	0.15*
1	-0.0015	-0.0030	0.0000	-0.0030
2	0.0481	0.0407	0.0035	-0.0071
3	0.0636	0.0585	0.0428	0.0057
4	<u>0.0644</u>	<u>0.0648</u>	<u>0.0496</u>	-0.0126
5	0.0238	0.0297	0.0352	-0.0248
6	0.0010	0.0044	0.0160	
7	0.0081	0.0069	0.0218	
8	-0.0007	-0.0011	0.0137	
9	0.0074	0.0024	0.0117	
10			0.0079	
11			0.0009	

*The u values obtained from the 0.15 mg Cu^{2+}/l treatment cultures may be due to sampling error and are shown for discussion purposes only.

was much lower than the observed u_{max} of either the control's or the 0.05 mg Cu^{2+}/l treatment cultures.

The differences between the u values of the control and 0.05 mg Cu^{2+}/l treatment cultures appears to have been minimal (Table 8). The u values obtained for the 0.15 mg Cu^{2+}/l treatment cultures were probably due to sampling error. For the purpose of discussion, the 0.15 mg Cu^{2+}/l treatment was assumed to be acutely toxic. Because of these factors, models predicting u_{max} as a function of (Cu^{2+}) were not derived from the AAP data in this experiment.

The rise in the u values of the 0.10 mg Cu^{2+}/l treatment cultures late in the test (intervals 5 to 9) and the final maximum standing crop biomass level reached by these cultures (Figure 11) seemed to indicate an ability of the algae to overcome the sublethal effects of cupric ion. This could have been the result of binding of cupric ion in older cells and a subsequent decrease in cupric ion concentration. Conversely, the cells could have excreted a binding agent into the medium. Other possibilities include microbial degradation of the chelating agent in cutrine and the subsequent precipitation of cupric ion as an inorganic salt, or precipitation caused by physical/chemical changes of the medium. These hypotheses were not tested.

Several observations were made upon microscopic examination of culture samples. In the cultures receiving cupric ion treatment, the individual Chlorella vulgaris cells were

noticably chlorotic. This was especially prevalent in the 0.10 and 0.15 mg Cu^{2+}/l treatments and particularly noticeable in the first few days of culture growth. The second observation was that the cells of the 0.15 mg Cu^{2+}/l treatment were noticably smaller than the cells of the other three treatments. These observations are similar to those noted by Cedeno-Maldonado et al (1972) and McBrien and Hassal (1965), and may indicate a decrease in the chlorophyll content of the cells.

The daily pH measurements from the pH method cultures are tabulated in the Appendix (Table A5). The growth curves for the four cupric ion treatment algal cultures (Figure 12) show a graded response to the treatments. An analysis of variance and corresponding multiple range test revealed that the biomass estimates of all three cupric ion treatments were significantly different from the control during most of the control cultures' active growth phase (Table 9). In the case of the 0.05 mg Cu^{2+}/l treatment cultures this difference was probably caused by an increase of the lag phases of growth. After these cultures entered the active growth phase, their growth curve parallels that of the controls. The 0.10 mg Cu^{2+}/l treatment cultures' growth was more drawn out than either the control or 0.05 mg Cu^{2+}/l treatment cultures, lasting over 350 hours. Sampling of the 0.10 mg Cu^{2+}/l treatment cultures was stopped at 355 hours because there was insufficient culture volume remaining for representative sampling. The 0.15 mg Cu^{2+}/l treatment cultures

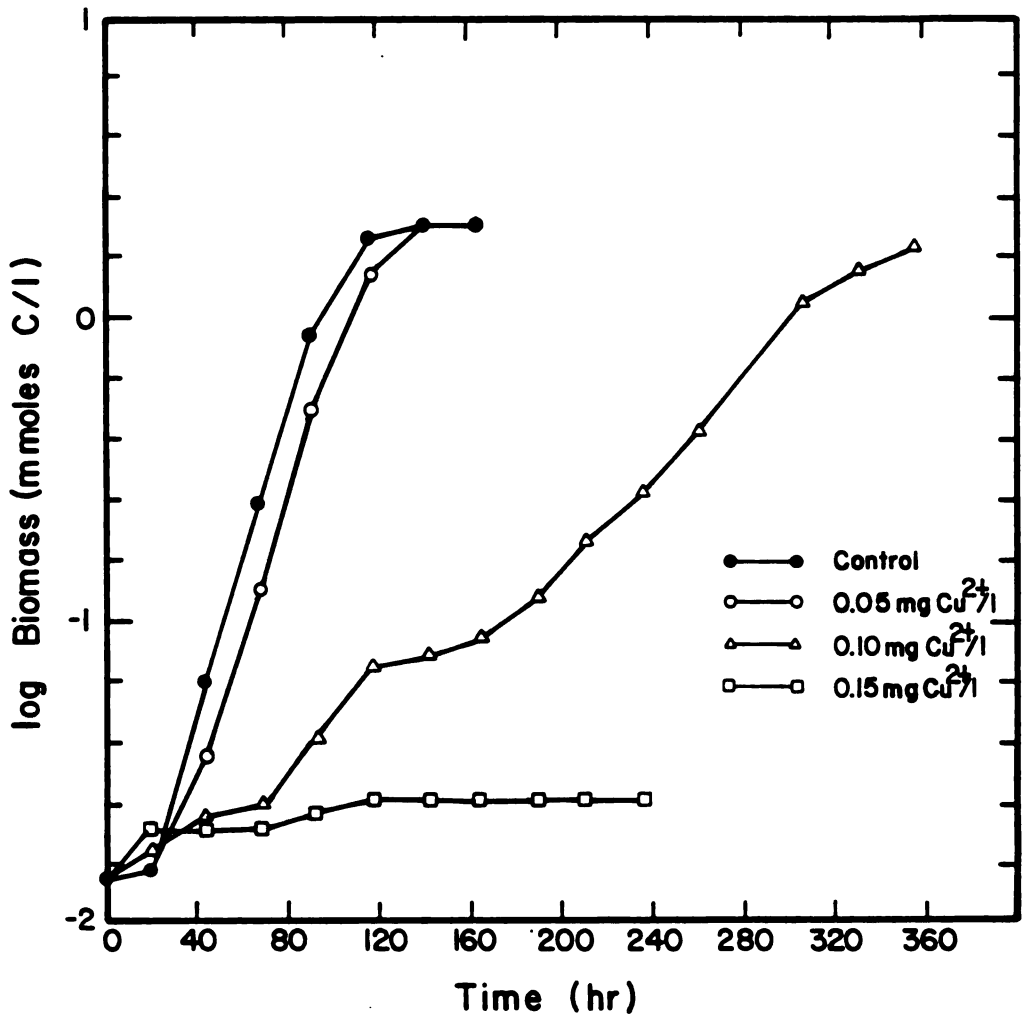


Figure 12. pH method growth curves for Chlorella vulgaris cultures in Experiment 4. Each point represents the mean of three replicates.

Table 9. Statistical analysis of the pH method results from Experiment 4. F test values and associated significance levels from an analysis of variance are listed for each sampling time. The treatments are listed in ascending order of biomass (mM C fixed by the algae/l), with those means judged not significantly different using a Student-Newman-Keuls multiple comparison procedure (P = 0.95) grouped by underlining.

Time (hr)	F value	Treatment (mg Cu^{2+}/l)
20.0	1.36 ns	<u>0.00 0.05 0.10 0.15</u>
44.0	35.43 ***	<u>0.15 0.10</u> 0.05 0.00
68.0	169.07 ***	<u>0.15 0.10</u> 0.05 0.00
91.5	47.98 ***	<u>0.15 0.10</u> 0.05 0.00
117.0	53.50 ***	<u>0.15 0.10</u> <u>0.05 0.00</u>
140.5	145.15 ***	0.15 0.10 <u>0.05 0.00</u> #

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

The 0.15 and 0.10 mg Cu^{2+}/l treatment cultures remained significantly different from one another, as judged by t-tests (P = 0.99), for the remainder of the former's growth cycle.

never entered the active growth phase, indicating acute toxicity of cupric ion towards these cultures.

The photosynthetic CO_2 minima was estimated from curves of u versus CO_{2fo}. The curves for the three treatments in which growth occurred (the control, 0.05, and 0.10 mg Cu^{2+}/l) are shown separately in Figures 13, 14, and 15 for clarity. The resulting CO_{2fq} values from these plots are shown in Table 10. A linear regression analysis of the CO_{2fq} concentration estimate versus the toxicant concentration indicated that the two variables were not linearly correlated ($r^2 = 0.047$, $n = 3$).

Double reciprocal plots of $1/u$ vs $1/CO_{2fA}$ were used to estimate u_{max} and $K_{CO_{fA}}$. Figures 16, 17, and 18 are the plots for the three treatment cultures which exhibited an active growth phase. The resulting $K_{CO_{2fA}}$ and u_{max} values from these Lineweaver-Burk plots are given in Table 10. The u_{max} estimates from the pH method were lower than the observed u_{max} values from the AAP, as was the case in the previous experiment. However, as can be seen in Figures 16, 17, and 18, the Michaelis-Menton growth kinetics line drawn through the data does not appear to fit the data as well as might be indicated by the derived regression coefficients (Table 10). To test the applicability of Michaelis-Menton kenetics to this experiment, two other transformations of the Monod model were used. The first is known as the Eadie-Hofstee plot (Lehniger, 1970) and takes the form

$$u = u_{max} - K_{CO_{2fA}} (u/CO_{2fA}) \qquad (20)$$

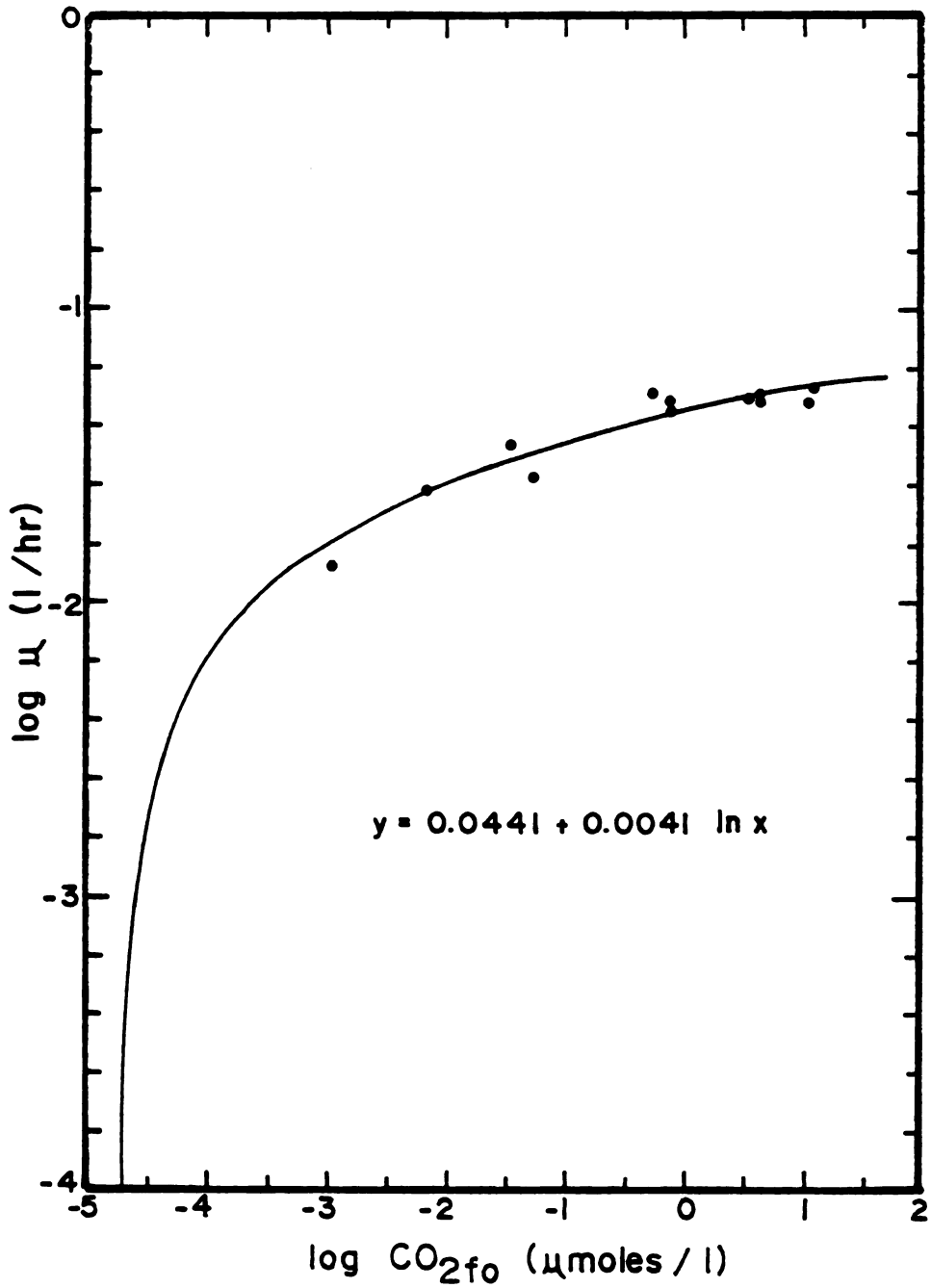


Figure 13. Specific growth rates of the control Chlorella vulgaris cultures in Experiment 4 as a function of the CO_{2fo} concentration of the medium.

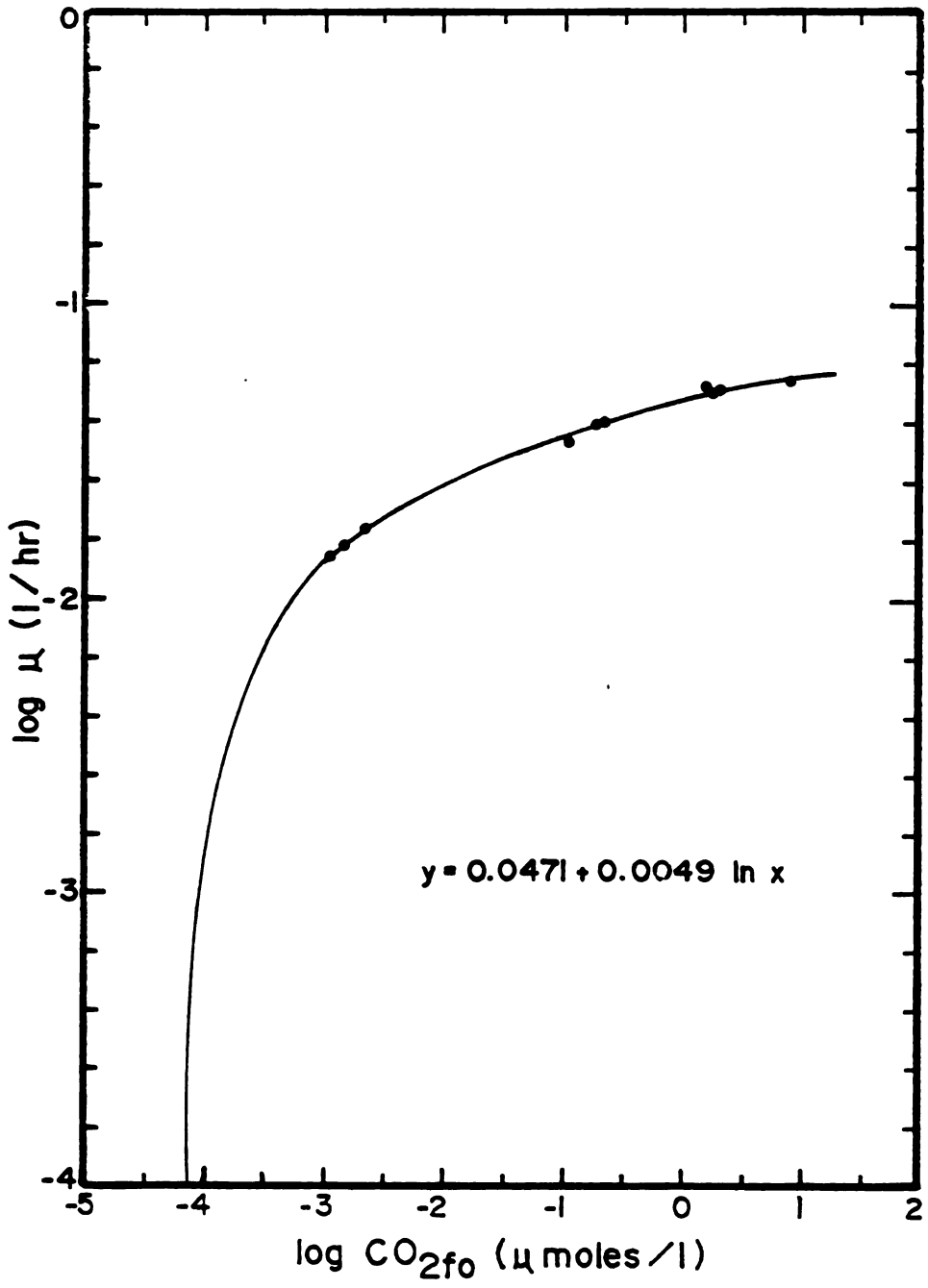


Figure 14. Specific growth rates of the 0.05 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 4 as a function of the CO_{2fo} concentration of the medium.

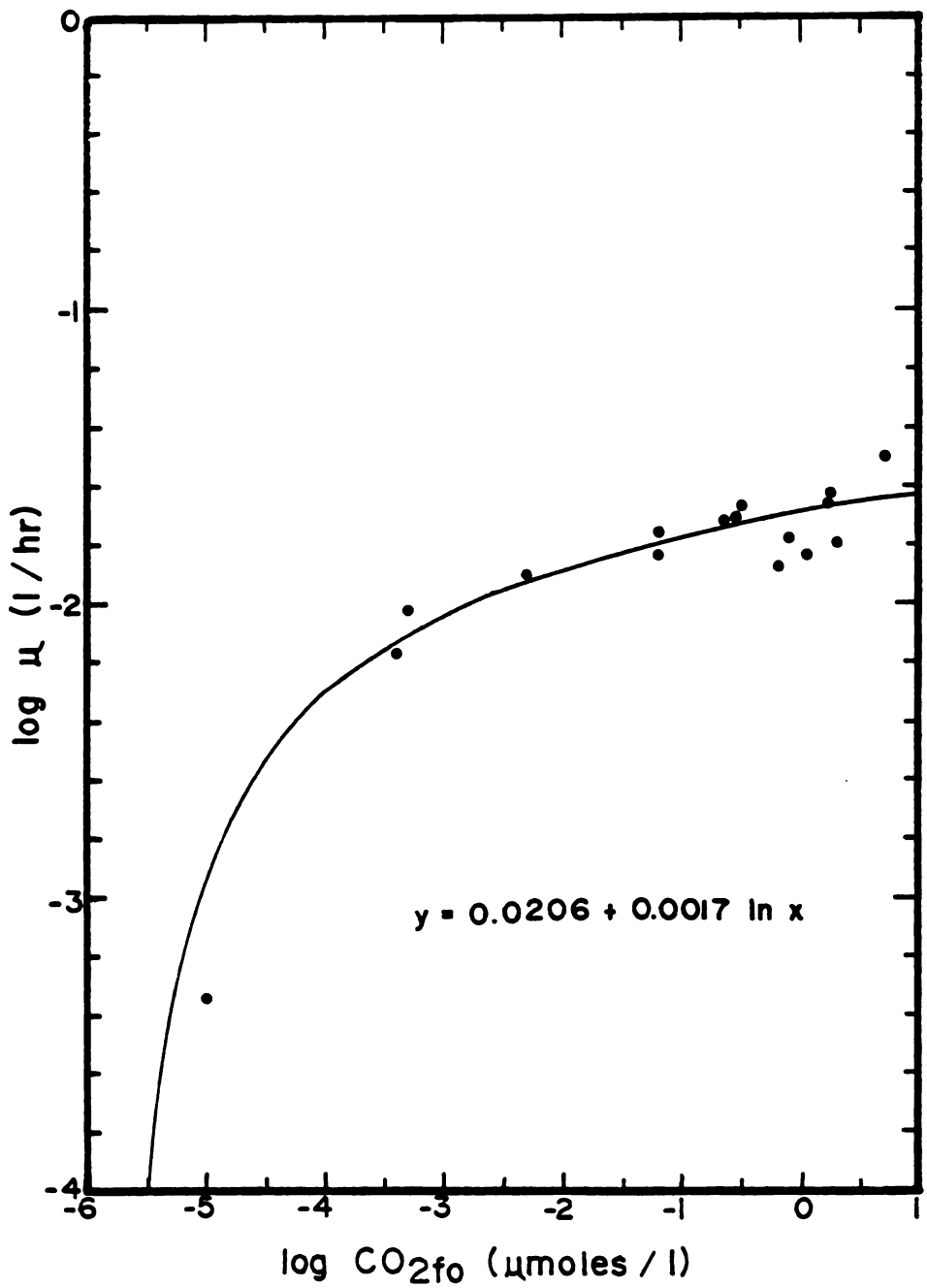


Figure 15. Specific growth rates of the 0.10 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 4 as a function of the CO_{2fo} concentration of the medium.

Table 10. Kinetic constants for the Chlorella vulgaris cultures in Experiment 4, as determined by the pH method.

Treatment (mg Cu^{2+}/l)	CO_{2fq} (uM CO_2/l)	r^2 for CO_{2fq}	K_CO_{2fA} (uM CO_2/l)	u_{max} (hr^{-1})	r^2 for $K_{CO_{2fA}}$ and u_{max}
Control[a]	$2x10^{-5}$	0.888	$2.51x10^{-3}$	0.0429	0.858
Control[b]	--	--	$3.07x10^{-3}$	0.0458	0.618
Control[c]	--	--	$7.18x10^{-2}$	0.0514	0.996
0.05	$7x10^{-5}$	0.991	$2.52x10^{-3}$	0.0437	0.950
0.10[a]	$5x10^{-6}$	0.721	$5.77x10^{-4}$	0.0182	0.795
0.10[b]	--	--	$6.29x10^{-4}$	0.0193	0.395
0.10[c]	--	--	$3.25x10^{-1}$	0.0290	0.861

[a]Kinetic constants derived from double reciprocal plots of $^1/u$ vs $^1/CO_{2fA}$ (Lineweaver-Burk equation).

[b]Kinetic constants derived from u/CO_{2fA} vs u plots (Eadie-Hofstee plot).

[c]Kinetic constants derived from CO_{2fA}/u vs CO_{2fA} plots.

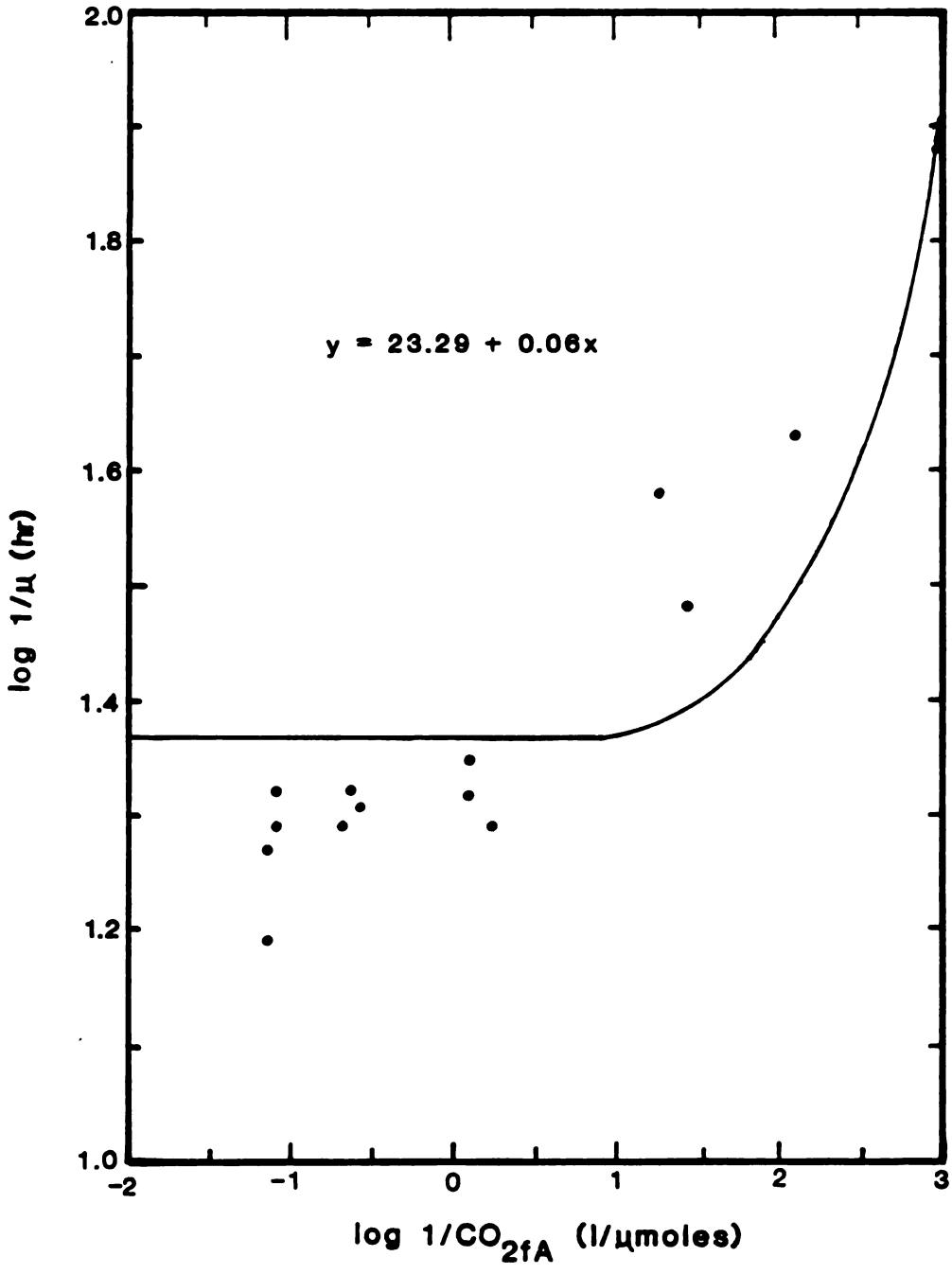


Figure 16. Reciprocal plot of the specific growth rates of the control Chlorella vulgaris cultures in Experiment 4 as a function of the CO_{2fA} concentration of the medium.

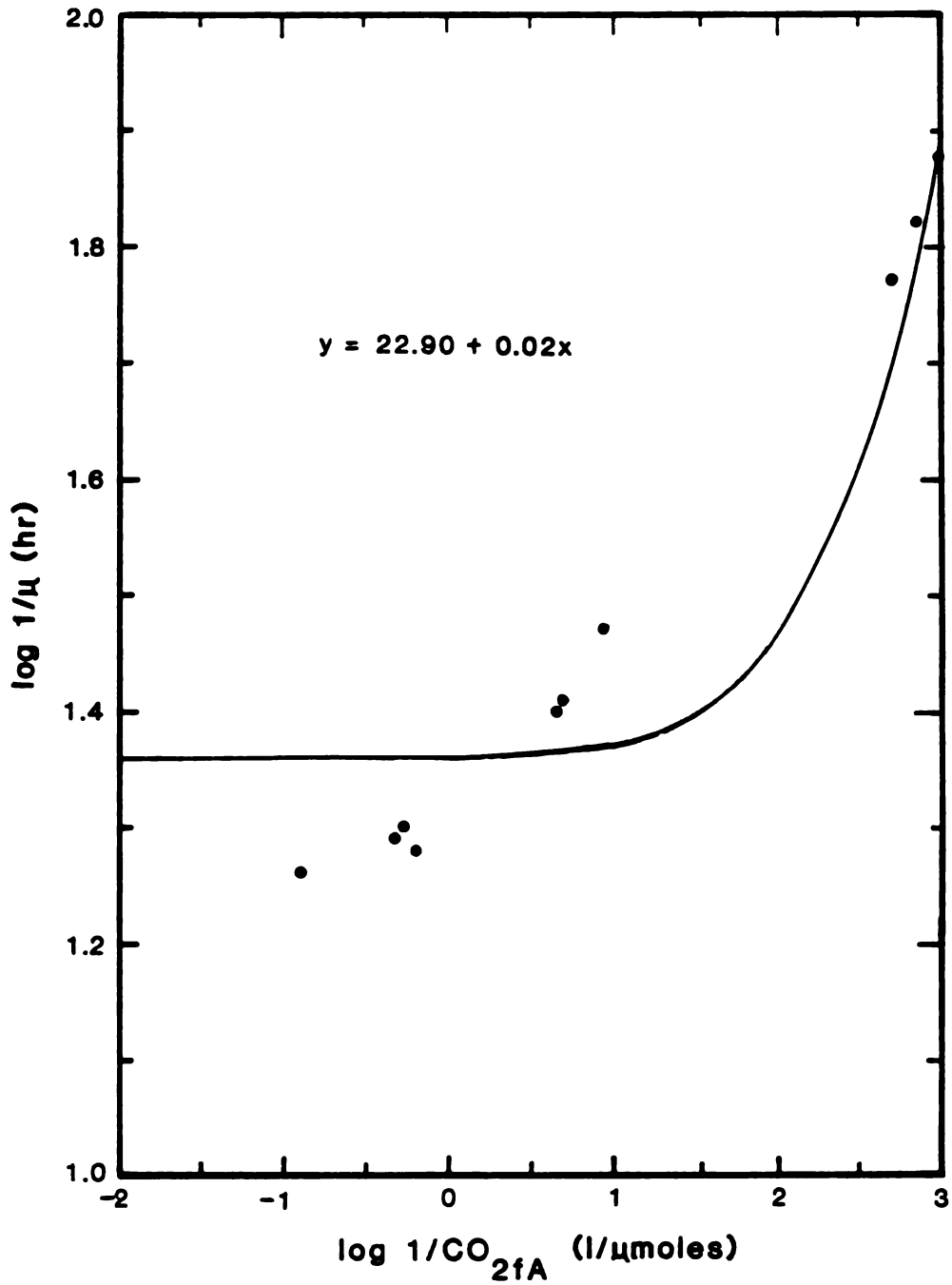


Figure 17. Reciprocal plot of the specific growth rates of the 0.05 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 4 as a function of the CO_{2fA} concentration of the medium.

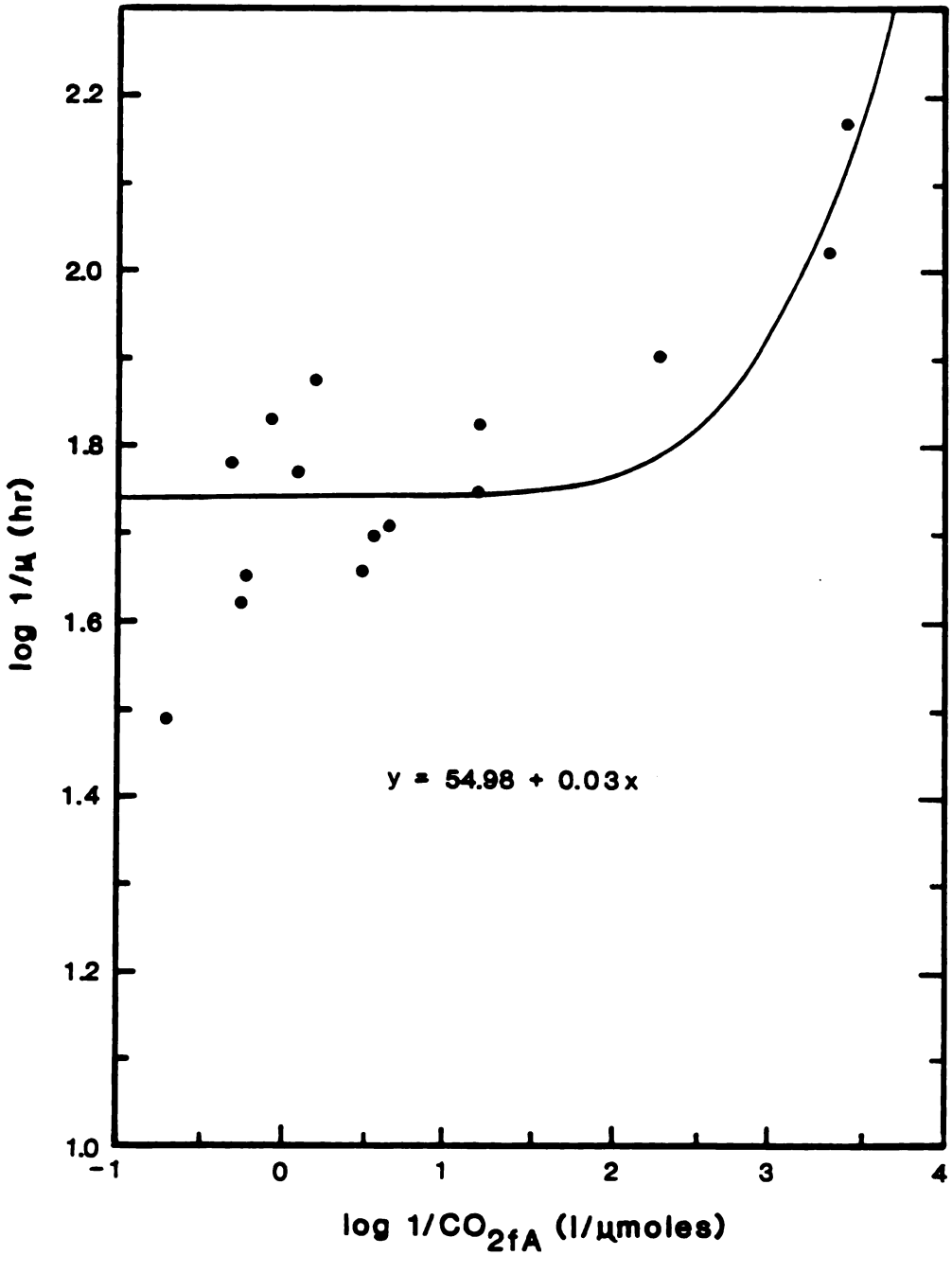


Figure 18. Reciprocal plot of the specific growth rates of the 0.10 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 4 as a function of the CO_{2fA} concentration of the medium.

The second rearrangement of the Monod model takes the form

$$CO_{2fA}/u = K_{CO_{2fA}}/u_{max} + 1/u_{max} (CO_{2fA}) \quad (21)$$

Plots of u/CO_{2fA} vs u and CO_{2fA} vs CO_{2fA}/u of the control and the 0.10 mg Cu^{2+}/l treatments are shown in Figures 19, 20, 21, and 22. The resulting u_{max} and $K_{CO_{2fA}}$ values derived from these plots are given in Table 10. The results of the Eadie-Hofstee plots (Figures 19 and 20) indicate that both the u_{max} and $K_{CO_{2fA}}$ estimates for the control and 0.10 mg Cu^{2+}/l treatment cultures are of the same order of magnitude as predicted by the Lineweaver-Burk plots (Figures 16 and 18). However, the correlation coefficients for these plots are significantly lower than those for the Lineweaver-Burk plots. The Eadie-Hofstee plots magnified the departures from linearity which were not evident in the r^2 values of the Lineweaver-Burk plots. The plots from the third transformation, CO_{2fA} vs CO_{2fA}/u, are shown in Figures 21 and 22. The kinetic constants derived from these figures were significantly higher than those derived by the previous transformations (Table 10) although the u_{max} values were still lower than those obtained from the AAP method.

Apparently, a higher order interaction exists in the treatment cultures, requiring a more complex model than the Monod model. Other interactions with nutrients, pH, light, algal by-products, or other factors may have been involved. The cause(s) of departure from Michaelis-Menton kinetics were

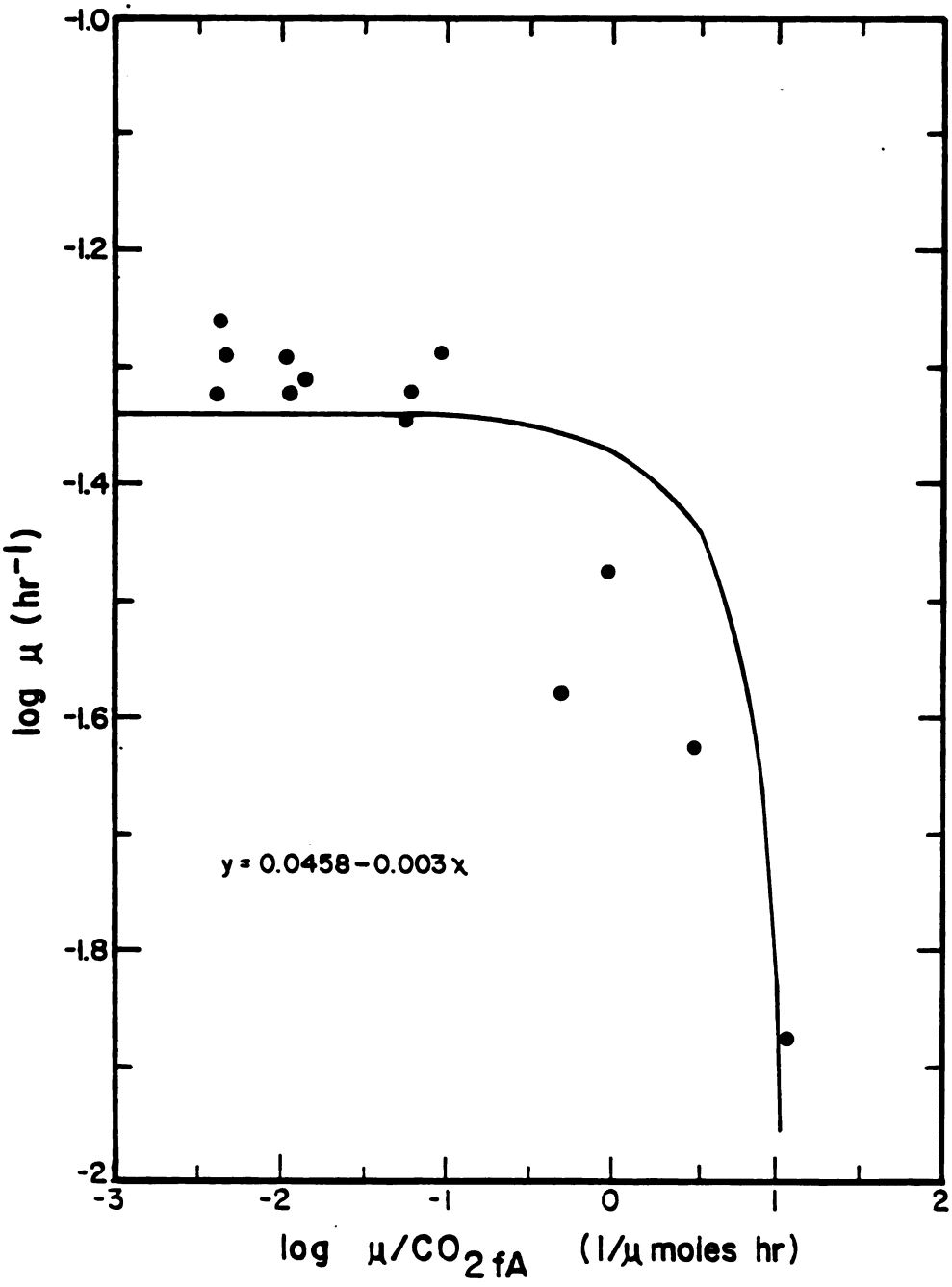


Figure 19. Eadie-Hofstee plot of the control Chlorella vulgaris cultures in Experiment 4.

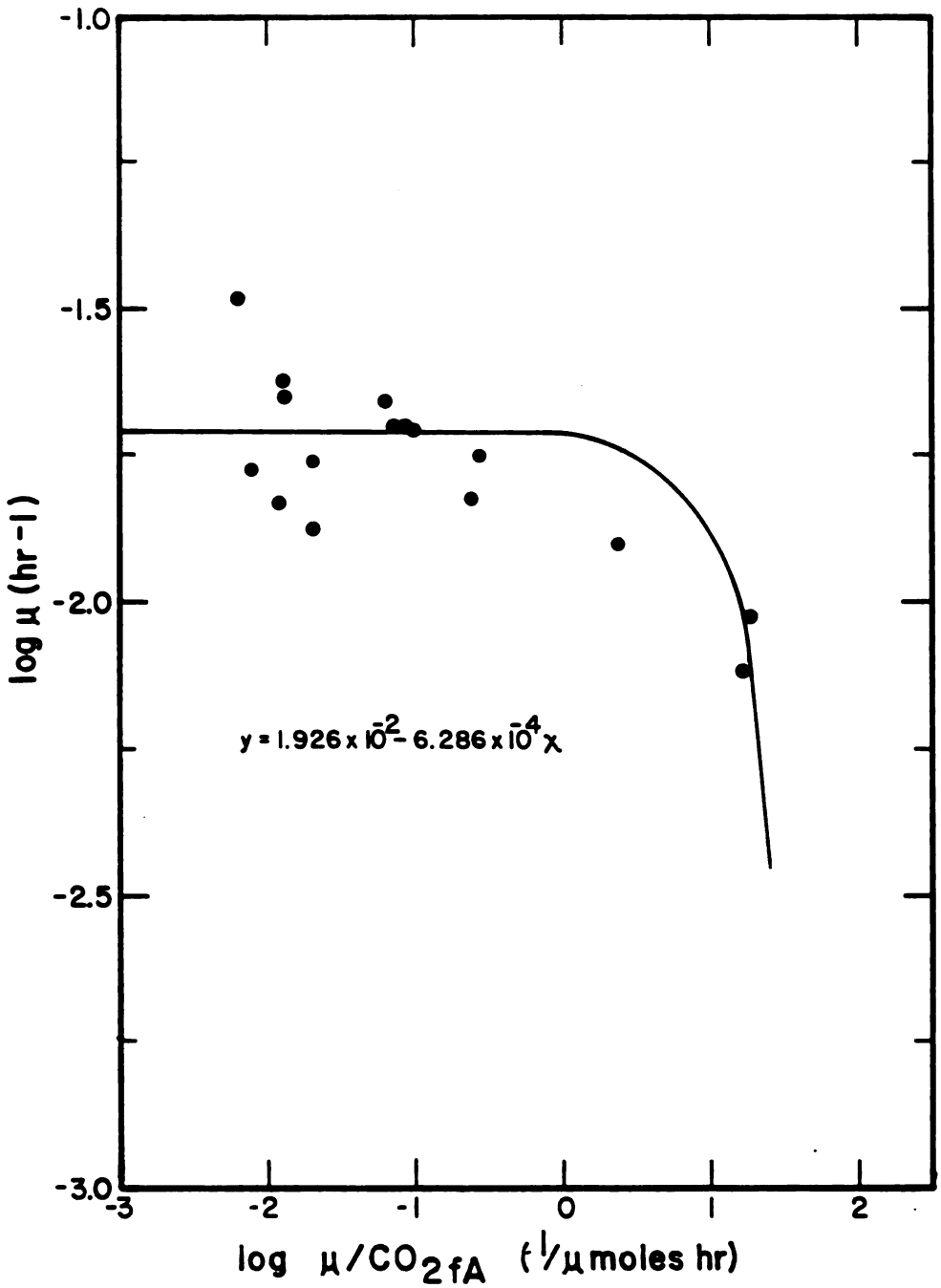


Figure 20. Eadie-Hofstee plot of the 0.10 mg Cu^{2+}/l treatment Chorella vulgaris cultures in Experiment 4.

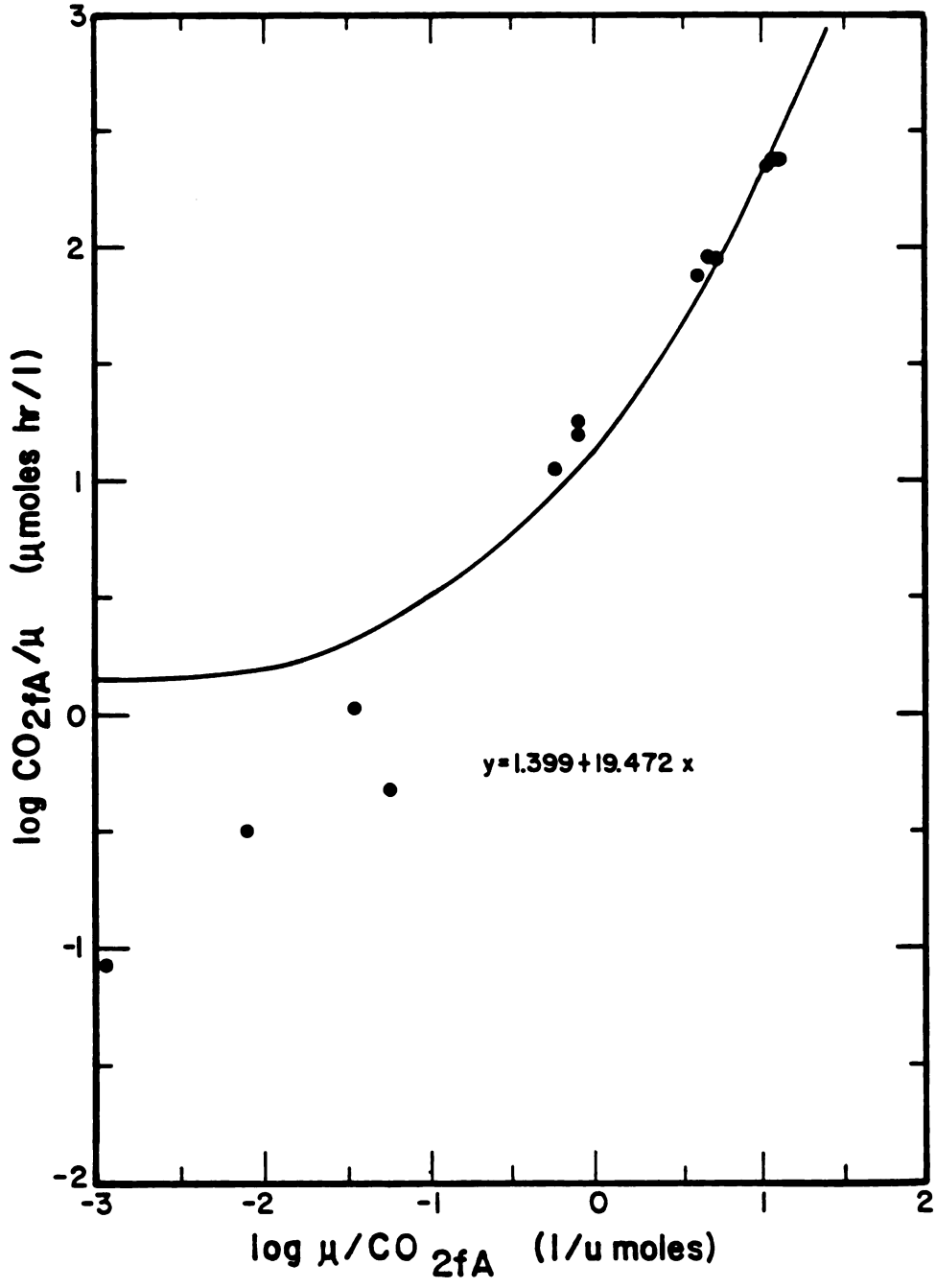


Figure 21. CO_{2fA}/u vs. CO_{2fA} transformation of the control Chlorella vulgaris cultures in Experiment 4.

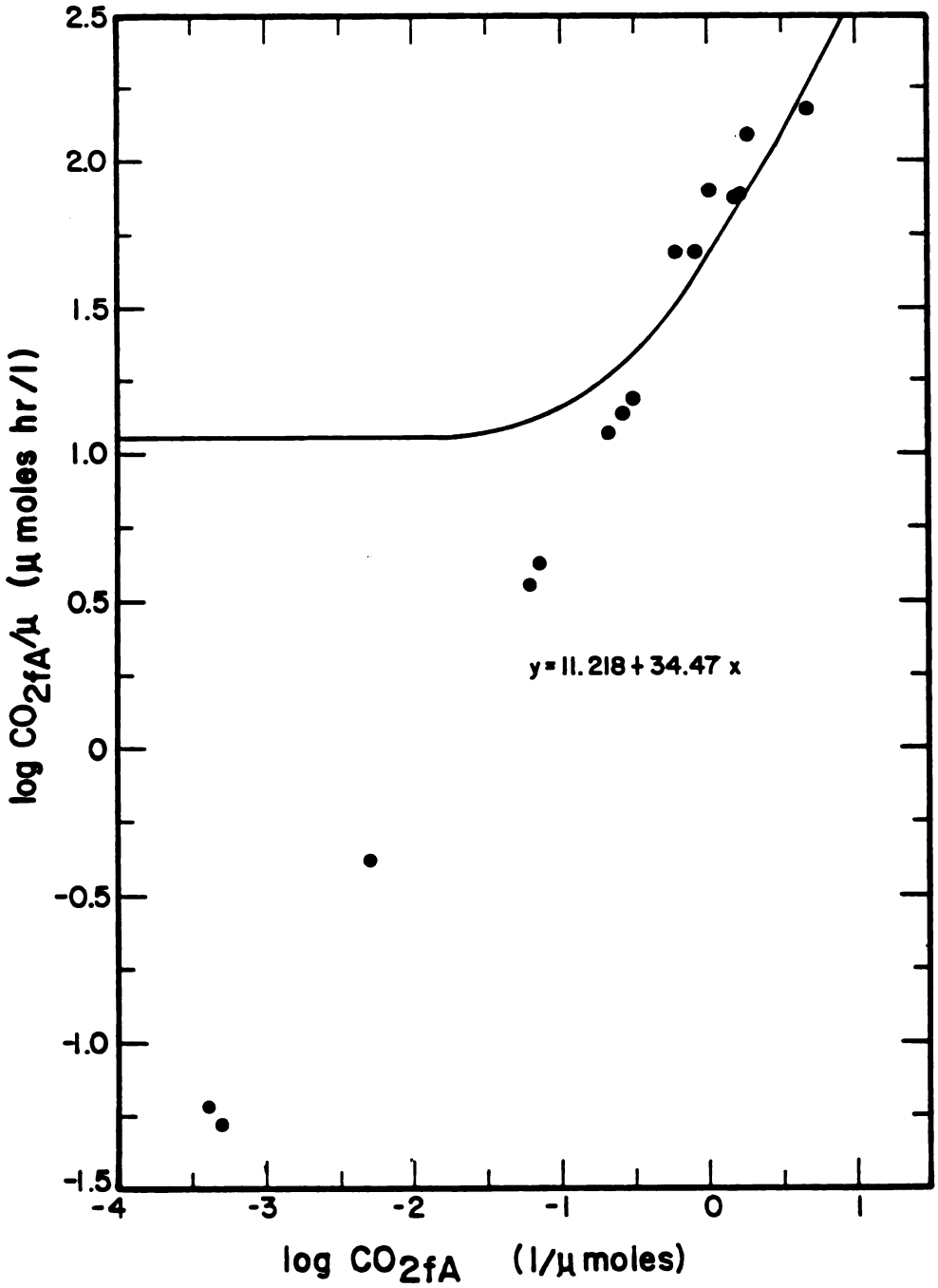


Figure 22. CO_{2fA}/u vs. CO_{2fA} tranformation of the 0.10 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 4.

not investigated. Because of the deviation from the Monod model, models predicting u as a function of Cu^{2+} were not derived.

Conclusions

The results of the AAP and pH method indicated that cupric ion inhibits algal growth by prolonging the lag phase of growth. This finding, and the fact that the treatment cultures were chlorotic during this phase, supports the findings of Cedeno-Maldonado et al (1972). These researchers reported that cupric ion inhibits electron transport in chloroplasts. The findings of Gross et al (1970), who reported that the chlorophyll content of cells exposed to cupric ion drops significantly, are supported by the results of this experiment. The smaller size of the Chlorella vulgaris cells exposed to the 0.15 mg Cu^{2+}/l treatment may have been due to an increase in cell permeability. Cupric ion reportedly has this effect on algal cells (McBrien and Hassal, 1965).

In this study, the 0.15 mg Cu^{2+}/l treatment was found to be acutely toxic to Chlorella vulgaris cultures. This is lower than the toxic level reported by McBrien and Hassal (1965) for Chlorella. The 0.10 mg Cu^{2+}/l treatment decreased the maximum specific growth rate and the saturation constant for CO_{2fA} of the algal cultures. The lag phase of growth of these cultures was prolonged; however, the cultures appeared to be able to recover their photosynthetic abilities, although at a diminished level. The 0.05 mg Cu^{2+}/l treatment

had only a slight effect on culture growth. Other concentrations of cupric ion between 0.05 and 0.15 mg/l needed to be assayed for their chronic effects on algal growth.

Both the AAP and pH method showed similar relationships between the four treatments. At this juncture, neither method appeared to be superior in its ability to assess the toxic effects of cupric ion on algal growth. The biggest advantage of the pH method over the AAP was the ease of sampling and measurement. Further study into the toxic effects of cupric ion was needed in order to test these two methods more completely.

EXPERIMENT 5

Further Study into the Effects of Cupric Ion on *Chlorella vulgaris* Culture Growth

Purpose

This test was a repeat of Experiment 4, testing several additional levels of cupric ion. The purpose was to assess the effectiveness of the two algal bioassay methods in detecting the chronic effects of cupric ion on *Chlorella vulgaris* culture growth.

Procedure

Measurements of light reaching the assay platform were taken before and after the experiment. The resulting contour map of light intensity is shown in Figure 23. Of the 64 spaces on the assay platform, 30 of these were within the acceptable range of 400 $\pm$40 footcandles of illumination, because of an adjustment made to the light support apparatus after the previous experiment.

Twenty-four test vessels were each half filled with 500 ml of fresh nutrient medium (a = 1.811 meq CO_3^{2-}--HCO_3^- alkalinity/l) and were randomly assigned to 24 of the 30 available spaces. The positions of these vessels in relation to the photosynthetically active radiation falling on the platform are shown in Figure 24.

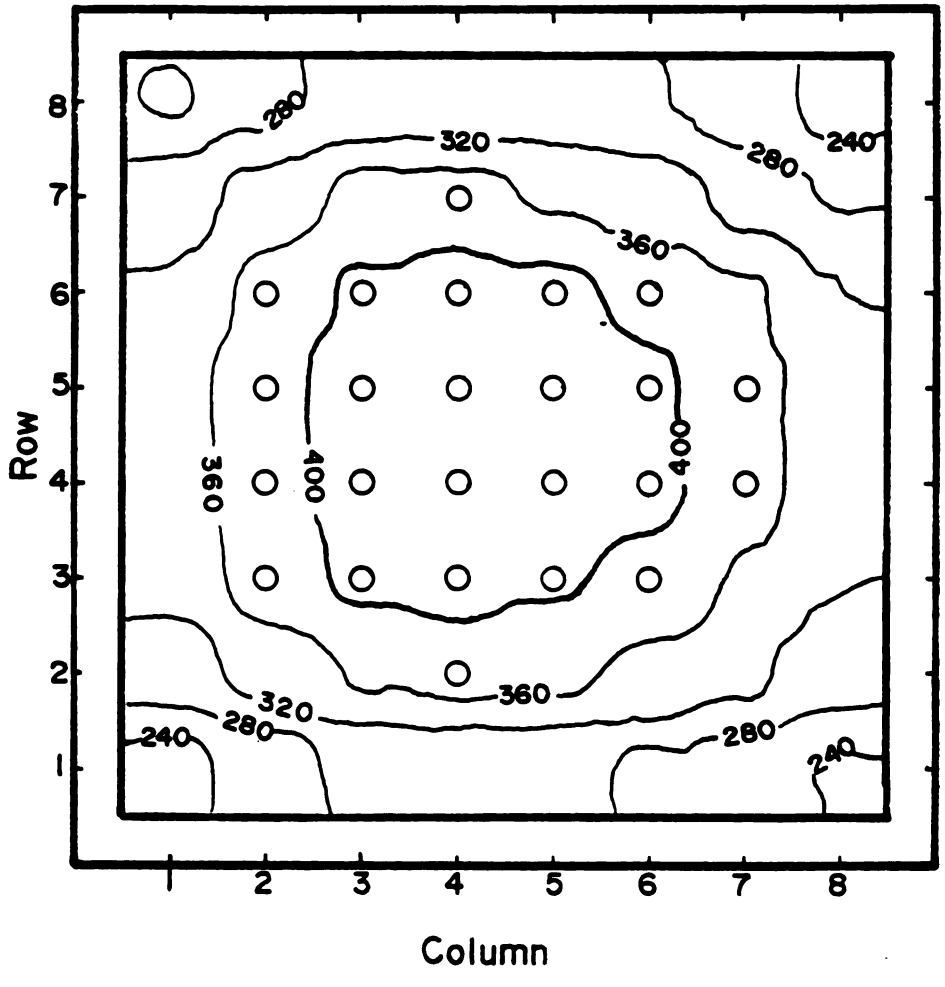


Figure 23. Contour map of the light intensity (footcandles) falling on the assay platform in Experiment 5. The position of each test vessel is indicated by a circle.

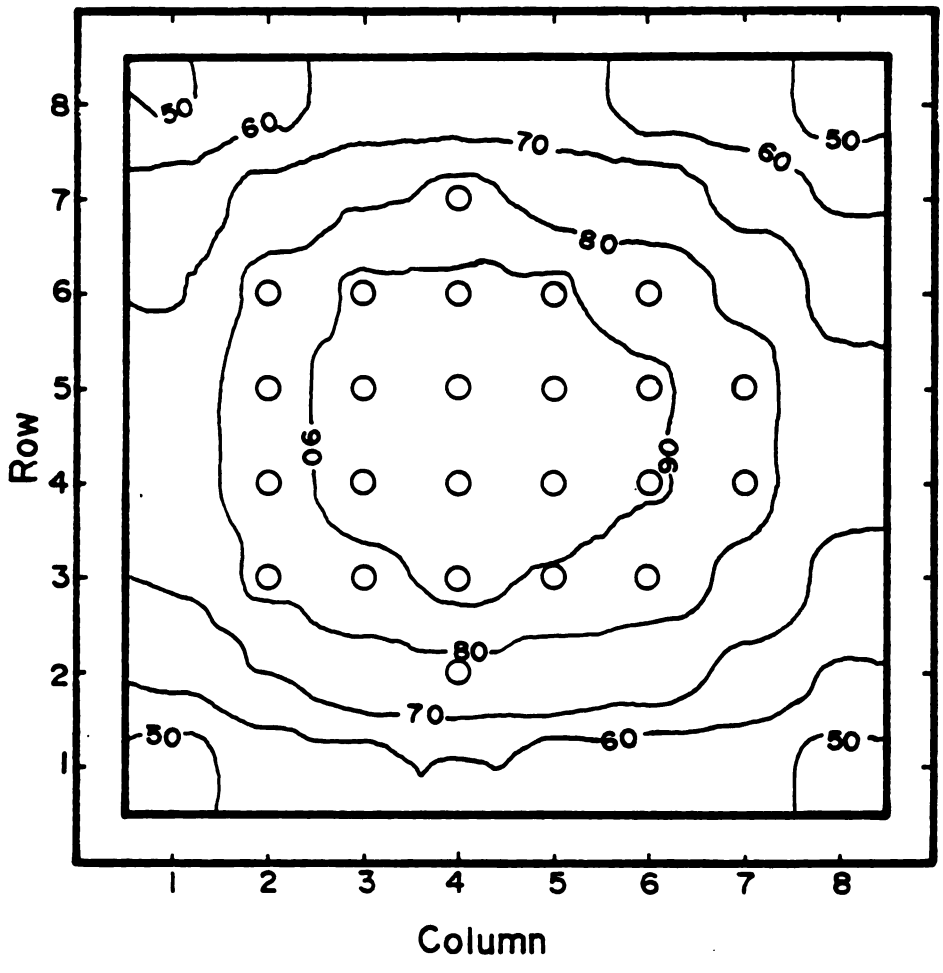


Figure 24. Contour map of the photosynthetically active radiation (uE/m^2/sec) falling on the assay platform in Experiment 5. The position of each test vessel is indicated by a circle.

Four levels of cupric ion were tested. A cutrine stock solution (91.071 mg Cu^{2+}/l) was added directly to the medium in the test vessels to give 6 replicates each of 0.075, 0.10, and 0.125 mg Cu^{2+}/l. The remaining 6 test vessels were used as controls. Three replicates of each treatment were randomly assigned to the AAP and 3 to the pH method.

Each flask was inoculated with 0.5 ml of 5-day-old Chlorella vulgaris stock culture to give a final concentration of 3,300 cells/ml. These test cultures had an initial organic carbon content of 0.0092 mM C/l, as determined by analysis with a carbon analyzer.

Daily samples of the AAP cultures were taken and the number of cells/ml computed. Samples of the pH method cultures were taken daily and the pH determined. The size of the samples taken from the pH method cultures was decreased to 20 ml in an effort to increase the test length. The test was run at 27°C.

The daily biomass estimates from the AAP cultures (cells/ml) were tested for significant differences between treatments by a nested analysis of variance (Sokal and Rohlf, 1969) and Student-Newman-Keuls multiple comparison procedure (Steel and Torrie, 1960). The biomass estimates from the pH method cultures (mM C fixed by the algae/l were tested by an analysis of variance and Student-Newman-Keuls multiple comparison procedure (Steel and Torrie, 1960).

Results

The results of the daily cell counts of the AAP test cultures are tabulated in the Appendix (Table A6). The AAP growth curves for the four treatments show a graded response to cupric ion (Figure 25). The seeding of the test medium with a younger stock culture than previously used significantly decreased the lag phase of growth of the control cultures. The lag phase of the 0.10 mg Cu^{2+}/l treatment cultures, the only cupric ion treatment from the previous experiment that was repeated here also was decreased.

The results of the statistical analysis show a significant difference between the biomass estimates of the four treatments (Table 11). The treatment cultures remained significantly different throughout the active growth phase of the control cultures. The 0.075 mg Cu^{2+}/l treatment cultures were judged not significantly different from the controls after both had reached the maximum standing crop biomass level. Statistical testing between the 0.10 and 0.125 mg Cu^{2+}/l treatments showed that these cultures remained significantly different throughout the remainder of the test (Table 12).

Although the control and 0.075 mg Cu^{2+}/l treatment cultures were judged significantly different in terms of daily biomass estimates, the u values calculated for these cultures indicate that this difference may have been only slight (Table 13). The observed u_{max} of the control cultures was only slightly higher than that of the 0.075 mg Cu^{2+}/l

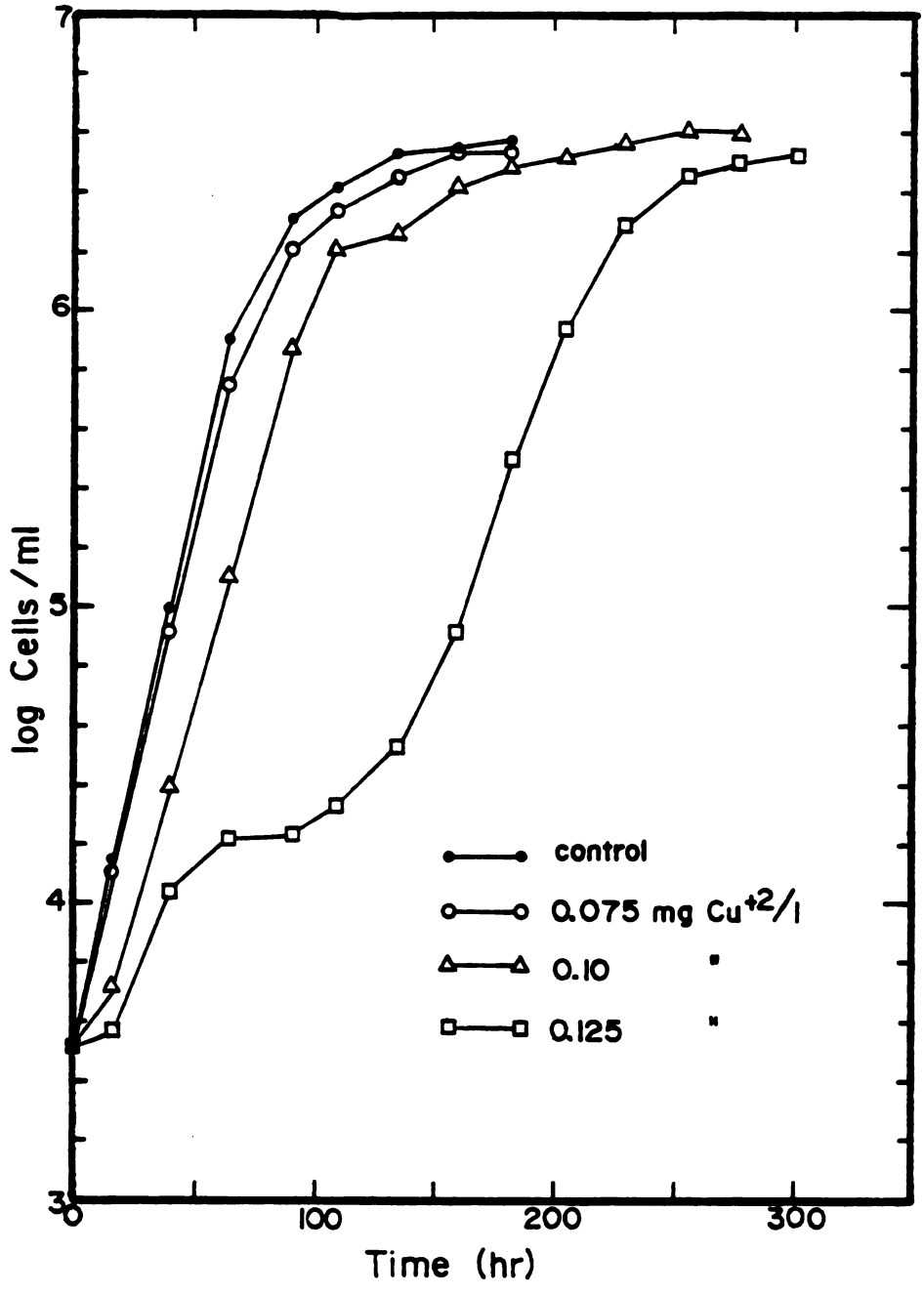


Figure 25. AAP growth curves for Chlorella vulgaris cultures in Experiment 5. Each point represents the mean of three replicates.

Table 11. Statistical analysis of the AAP results from the first eight sampling times of Experiment 5. F test values and associated significance levels from a nested analysis of variance are listed for each sampling time. The treatments are listed in ascending order of biomass (cells/ml) with those means judged not significantly different, using a Student-Newman-Keuls multiple comparison procedure (P = 0.95), grouped by underlining.

Time (hr)	F value	Treatment (mg Cu^{2+}/l)
16.0	46.61 ***	0.125 0.10 0.075 0.00
39.5	68.02 ***	0.125 0.10 0.075 0.00
64.5	94.98 ***	0.125 0.10 0.075 0.00
91.0	58.80 ***	0.125 0.10 0.075 0.00
109.5	88.11 ***	0.125 0.10 0.075 0.00
134.5	135.13 ***	0.125 0.10 0.075 0.00
159.5	51.24 ***	0.125 0.10 0.075 0.00
182.5	133.42 ***	0.125 0.10 0.075 0.00

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

Table 12. Statistical analysis of the AAP results from the last four sampling times of Experiment 5. Two tailed t-test values and associated significance levels are listed for each sampling time. The treatments are listed in ascending order of biomass (cells/ml).

Time (hr)	t value	Treatment (mg Cu^{2+}/l)
205.0	23.16 ***	0.125 0.10
229.0	13.25 ***	0.125 0.10
256.0	8.29 ***	0.125 0.10
277.0	4.21 ***	0.125 0.10

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

Table 13. Specific growth rates (u) of Chlorella vulgaris cultures in Experiment 5, as determined by the AAP. Maximum specific growth rates are underlined.

	Treatment (mg Cu^{2+}/l)			
Interval	Control	0.075	0.10	0.125
1	<u>0.0773</u>	<u>0.0744</u>	0.0268	0.0071
2	0.0641	0.0620	<u>0.0560</u>	0.0423
3	0.0623	0.0596	0.0536	0.0162
4	0.0333	0.0365	0.0536	0.0007
5	0.0129	0.0161	0.0407	0.0127
6	0.0104	0.0108	0.0044	0.0177
7	0.0020	0.0071	0.0144	0.0337
8	0.0025	0.0000	0.0069	<u>0.0508</u>
9			0.0035	0.0418
10			0.0045	0.0313
11			0.0032	0.0148
12			-0.0002	0.0054
13				0.0021

treatment cultures. Furthermore, the other u values for this treatment were only slightly lower than those recorded for the control cultures. This was the case up to the point where the control cultures' growth began to decrease.

The observed u_{max} for the 0.10 mg Cu^{2+}/l treatment cultures occurred at a later time and was lower than the observed u_{max} of the controls. The u values indicate that these cultures grew at a slower rate than did the control cultures. The 0.125 mg Cu^{2+}/l treatment had an unusual effect on Chlorella vulgaris culture growth. The growth of these cultures appeared to have reached a minimum by interval 4 (between sampling times 64.5 and 91.0 hours), but then the cultures began growing again. The observed u_{max} for these cultures appeared much later in the test and was the lowest u_{max} recorded for any of the treatments. The reason for this two-step growth curve is unclear.

Microscopic examination of the treatment cultures revealed that the Chlorella vulgaris cells in the cupric ion treatment cultures were lighter in color and smaller than their counterparts in the control cultures. This was particularly evident in the 0.10 and 0.125 mg Cu^{2+}/l treatment cultures. These conditions became less evident as the test proceeded.

The daily pH measurements of the pH method cultures are recorded in the Appendix (Table A7). As was the case with the growth curves produced with the AAP biomass estimates, the pH method growth curves exhibit a graded response of the

algae to the cupric ion treatments (Figure 26). Both the lag and active phases of growth became more drawn out as the cupric ion level was increased. The 0.125 mg Cu^{2+}/l treatment cultures did not exhibit the double growth curve anomoly that was noted in the AAP results. As in the previous experiment, one treatment (0.125 mg Cu^{2+}/l) had to be terminated prior to reaching the stationary phase of growth because of a lack of culture volume. These curves (Figure 26) and the AAP growth curves (Figure 25) indicate that at sublethal cupric ion concentrations, an increase of the cupric ion level does not affect the maximum standing crop biomass level reached by the Chlorella vulgaris cultures.

The results of the statistical analysis of the four treatments indicated that the biomass estimates from the treatments were significantly different from one another throughout the active growth phase of the control cultures. This difference became more pronounced as the cupric ion concentration was raised (Table 14). Following the temination of the control and 0.075 mg Cu^{2+}/l treatments, the 0.10 and 0.125 mg Cu^{2+}/l treatment cultures remained significantly different from one another for the remainder of the 0.10 mg Cu^{2+}/l treatment cultures' growth cycle (Table 15). Even after 300 hours of testing, the 0.125 mg Cu^{2+}/l treatment cultures continued to grow at a slow rate, showing the chronic effect of cupric ion.

The photosynthetic CO_2 minima for the four treatments was estimated by curves of u versus CO_{2fo}. These curves are

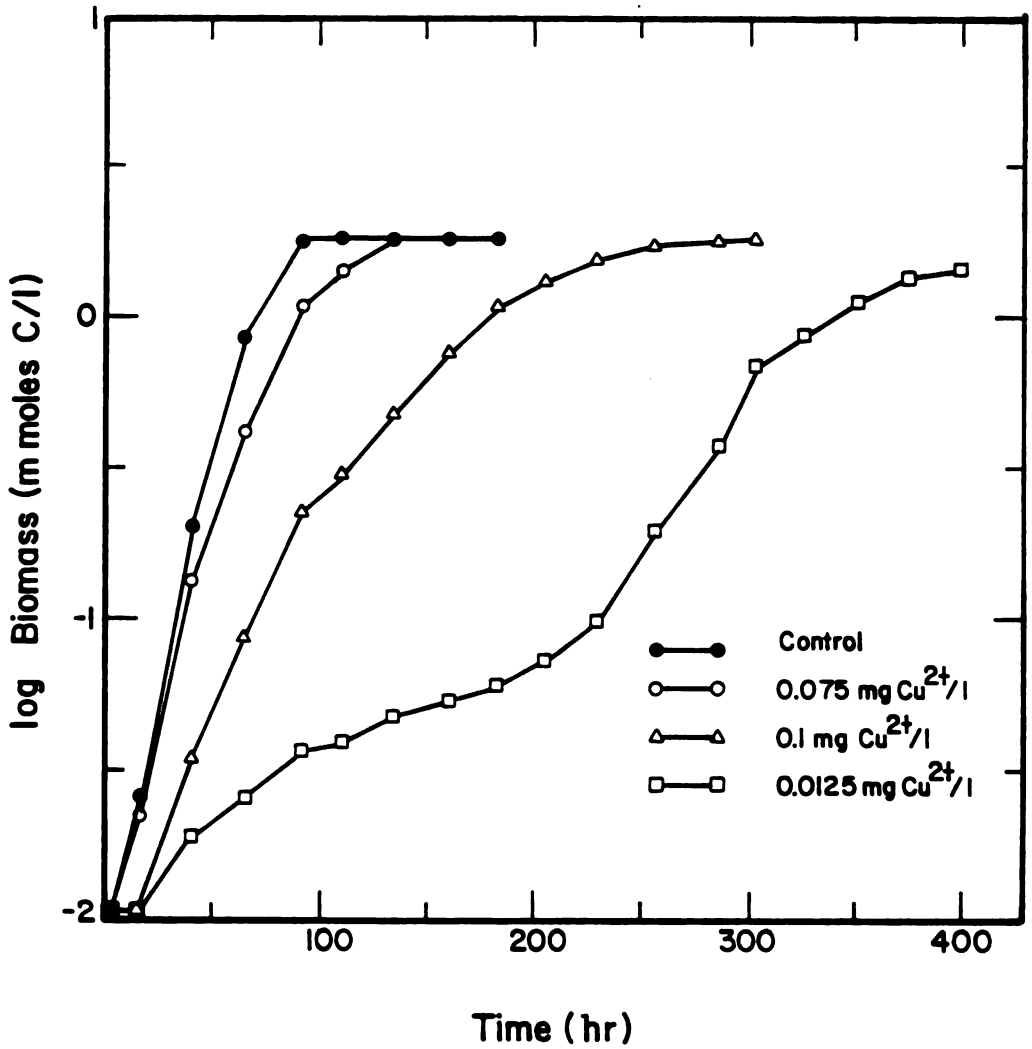


Figure 26. pH method growth curves for Chlorella vulgaris cultures in Experiment 5. Each point represents the mean of three replicates.

Table 14. Statistical analysis of the pH method results from the first six sampling times of Experiment 5. F test values and associated significance levels from an analysis of variance are listed for each sampling time. The treatments are listed in ascending order of biomass (mM C fixed by the algae/l) with those means judged not significantly different, using a Student-Newman-Keuls multiple comparison procedure (P = 0.95), grouped by underlining.

Time (hr)	F value	Treatment (mg Cu^{2+}/l)
16.0	31.61 ***	<u>0.10 0.125</u> <u>0.075 0.00</u>
39.5	747.81 ***	0.125 0.10 0.075 0.00
64.5	527.06 ***	0.125 0.10 0.075 0.00
91.0	655.46 ***	0.125 0.10 0.075 0.00
109.5	704.85 ***	0.125 0.10 0.075 0.00
134.5	379.58 ***	0.125 0.10 0.075 0.00

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

Table 15. Statistical analysis of the pH method results from the last seven sampling times of Experiment 5. Two tailed t-test values and associated significance levels are listed for each sampling time. The treatments are listed in ascending order of biomass (mM C fixed by the algae/l).

Time (hr)	t value	Treatment (mg Cu^{2+}/l)
159.5	5.04 **	0.125 0.10
182.5	6.95 **	0.125 0.10
205.0	9.07 **	0.125 0.10
229.0	12.02 ***	0.125 0.10
256.0	25.32 ***	0.125 0.10
277.0	13.76 ***	0.125 0.10
302.5	8.90 **	0.125 0.10

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

shown separately in Figures 27, 28, 29, and 30 for clarity. The resulting CO_{2fq} estimates from these plots are given in Table 16. The $K_{CO_{2fA}}$ and u_{max} values obtained from these plots are given in Table 16.

As shown in Figures 31 through 34, the growth of the algal cultures (with the possible exception of the control treatment cultures) did not adhere to classical Michaelis-Menton kinetics, as was also the case in Experiment 4. Therefore, this model does not adequately explain the interactions in the treatment cultures, and extrapolation of the results in estimating u_{max} or $K_{CO_{2fA}}$ as a function of the Cu^{2+} concentration was avoided.

Conclusions

As in the previous experiment, the results indicated that cupric ion prolongs the lag phase of growth. The results of this experiment clearly indicated that cupric ion does cause a decrease in the maximum specific growth rate and subsequently retards the growth of the Chlorella vulgaris cultures. Cupric ion, in sublethal concentrations, does not appear to affect maximum standing crop biomass levels that the cultures reach.

The results of both methods indicated a graded response of the algae to various cupric ion levels. However, this was more clearly shown by the pH method results than the AAP results. The kinetic constant u_{max} had a greater range of values in the pH method than in the AAP and the change in u_{max} due to cupric ion was more linear in the pH method.

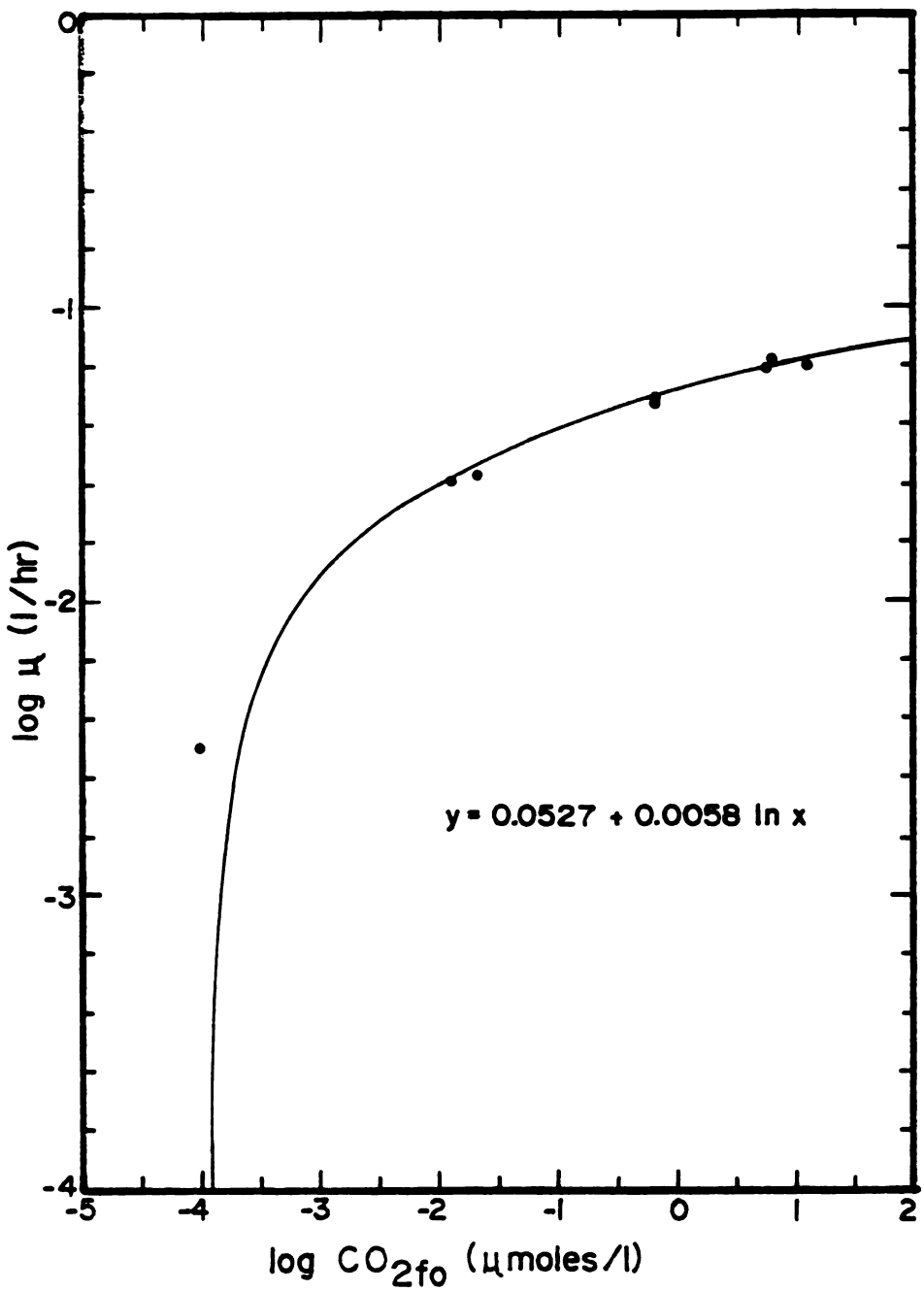


Figure 27. Specific growth rates of the control cultures of Chlorella vulgaris in Experiment 5 as a function of the CO_{2fo} concentration of the medium.

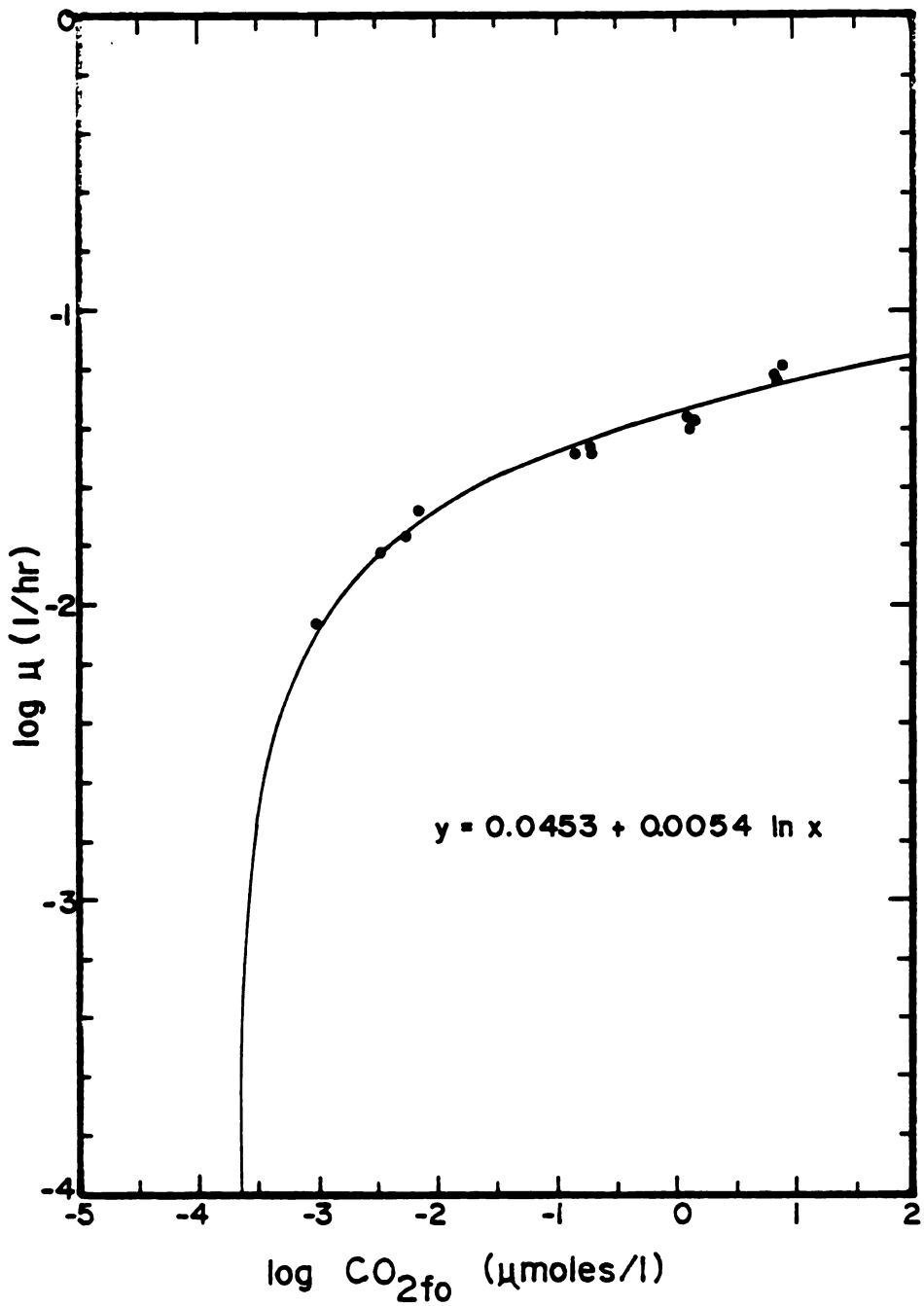


Figure 28. Specific growth rates of the 0.075 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 5 as a function of the CO_{2fo} concentration of the medium.

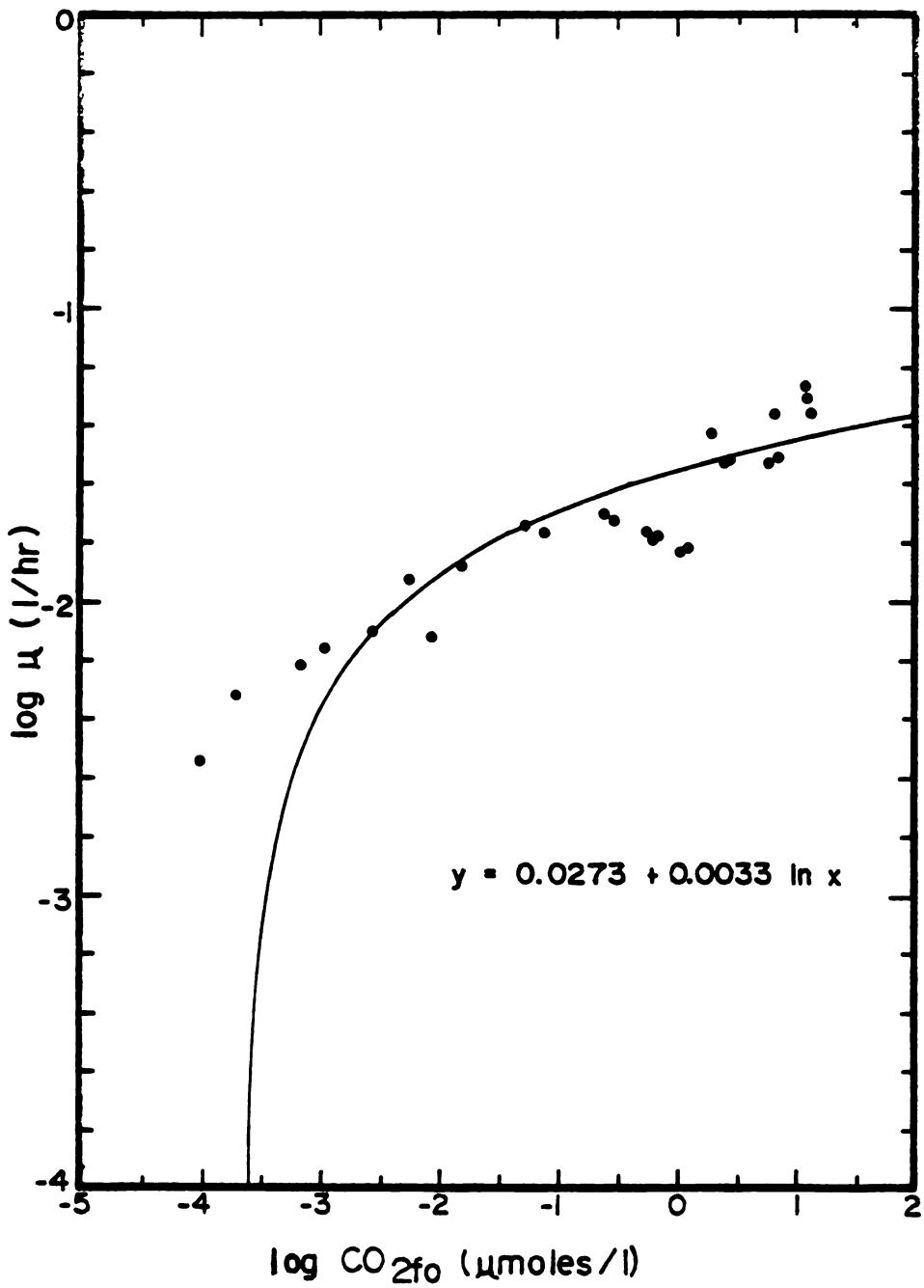


Figure 29. Specific growth rates of the 0.10 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiemnt 5 as a function of the CO_{2fo} concentration of the medium.

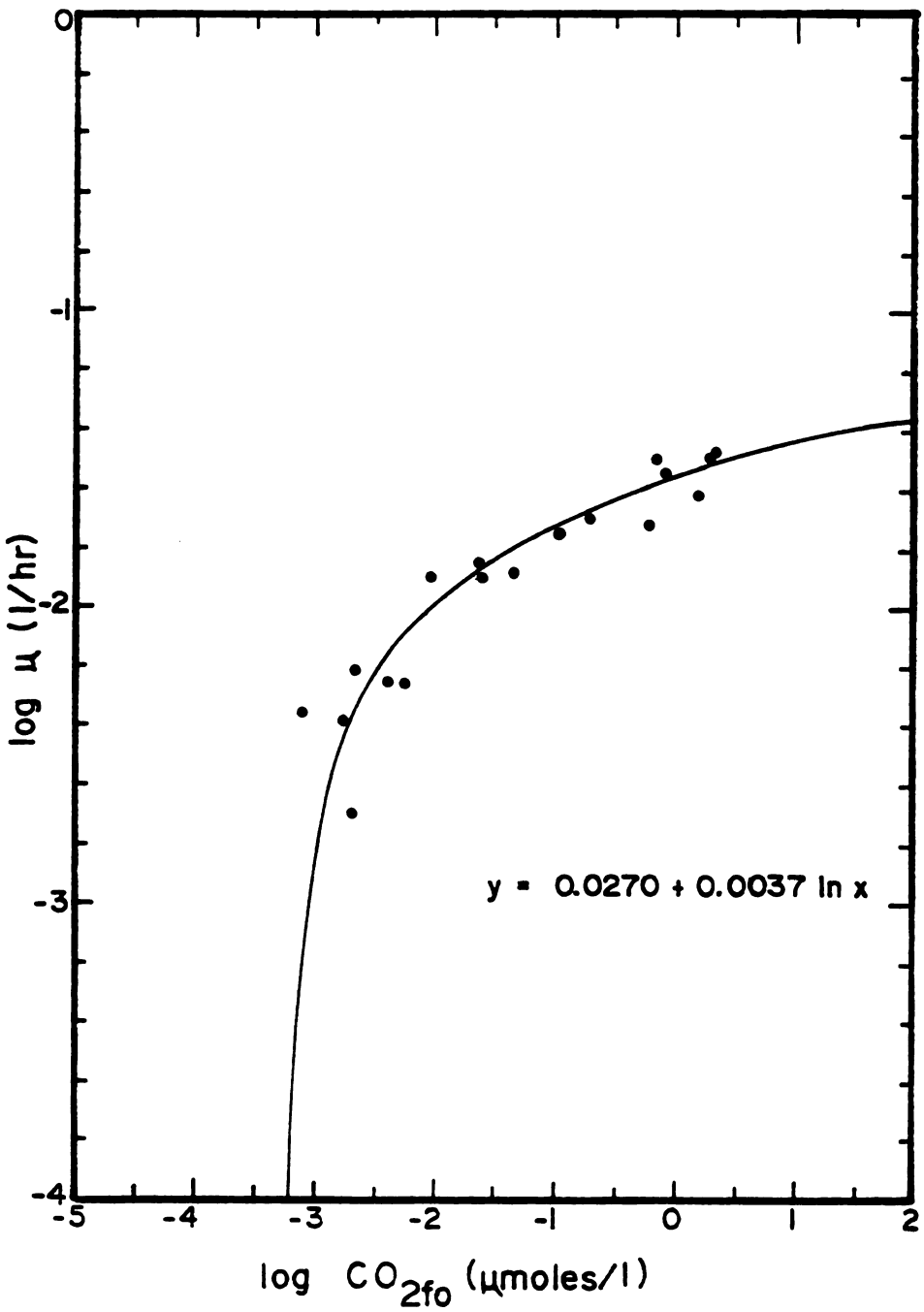


Figure 30. Specific growth rates of the 0.125 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 5 as a function of the CO_{2fo} concentration of the medium.

Table 16. Kinetic constants for the Chlorella vulgaris cultures in Experiment 5, as determined by the pH method.

Treatment (mg Cu^{2+}/l)	CO_{2fq} (uM CO_2/l)	r^2 for CO_{2fq}	$K_{CO_{2fA}}$ (uM CO_2/l)	u_{max} (hr^{-1})	r^2 for $K_{CO_{2fA}}$ and u_{max}
Control	1.2×10^{-4}	0.983	1.76×10^{-2}	0.0575	0.929
0.075	2.2×10^{-4}	0.923	6.56×10^{-3}	0.0411	0.841
0.10	2.5×10^{-4}	0.733	2.14×10^{-3}	0.0197	0.574
0.125	6.4×10^{-4}	0.911	4.17×10^{-3}	0.0180	0.790

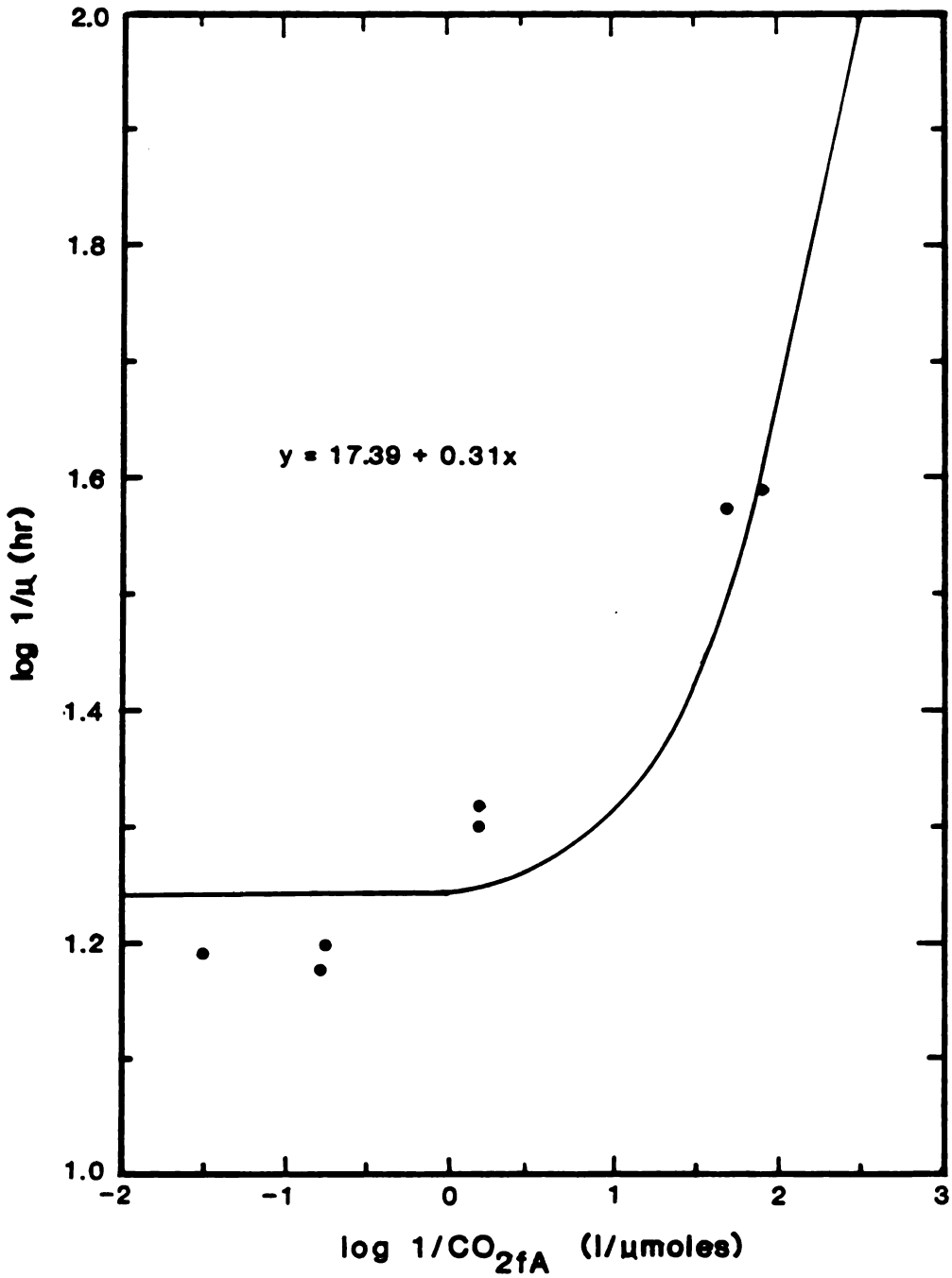


Figure 31. Reciprocal plot of the specific growth rates of the control Chlorella vulgaris cultures in Experiment 5 as a function of the CO_{2fA} concentration of the medium.

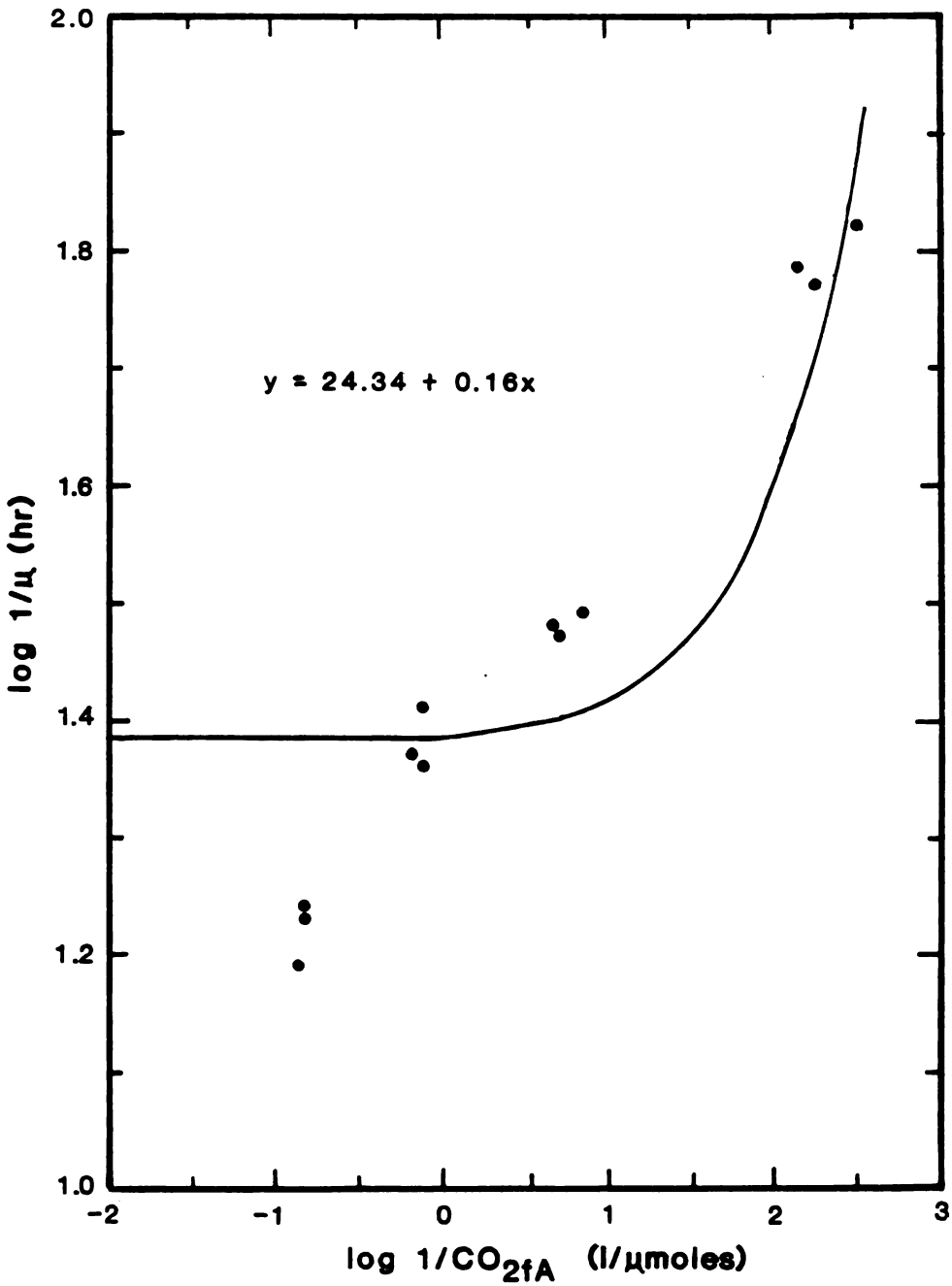


Figure 32. Reciprocal plot of the specific growth rates of the 0.075 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 5 as a function of the CO_{2fA} concentration of the medium.

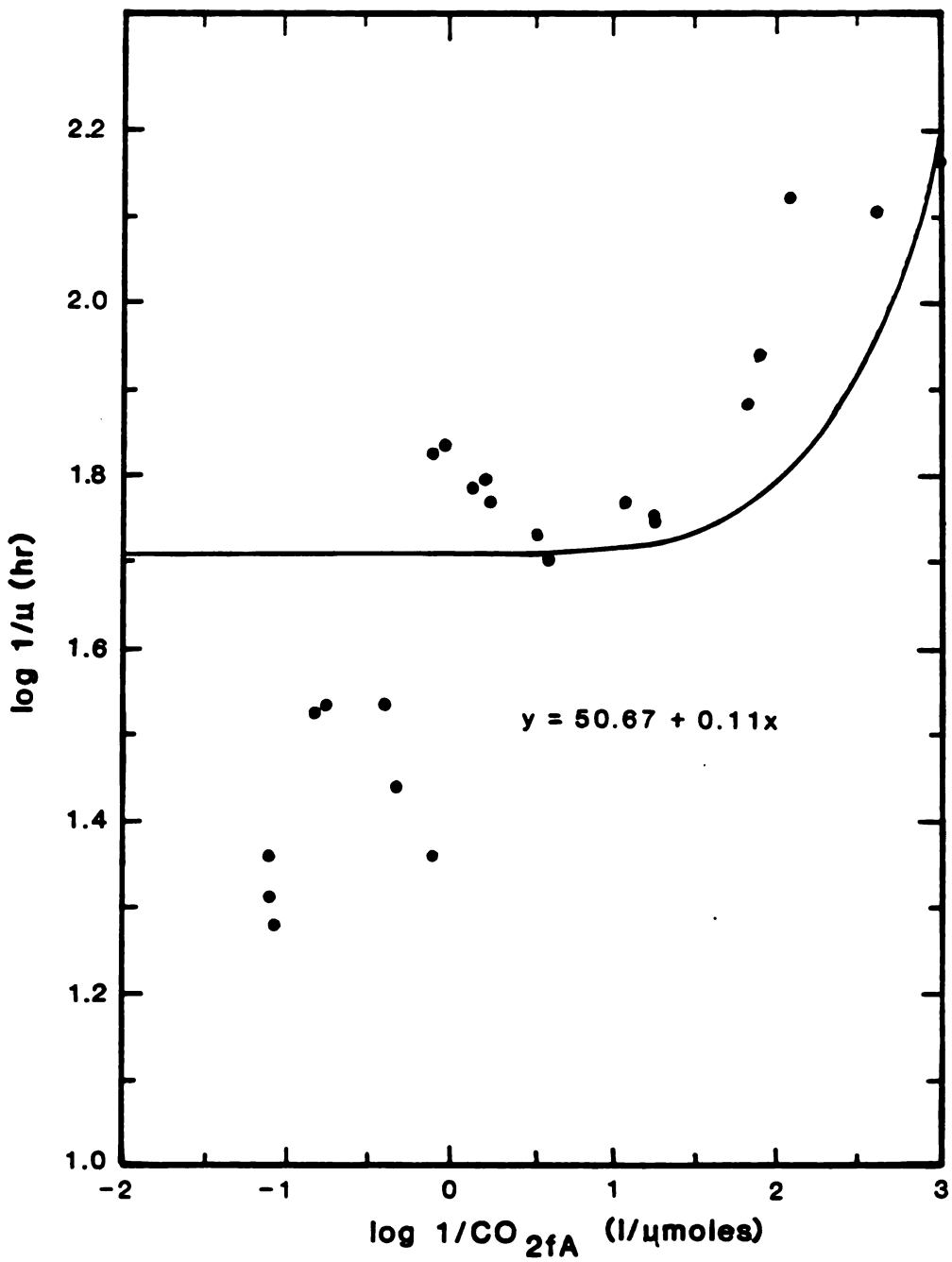


Figure 33. Reciprocal plot of the specific growth rates of the 0.10 mg Cu^{2+}/l treatment Chlorella vulgaris cultures in Experiment 5 as a function of the CO_{2fA} concentration of the medium.

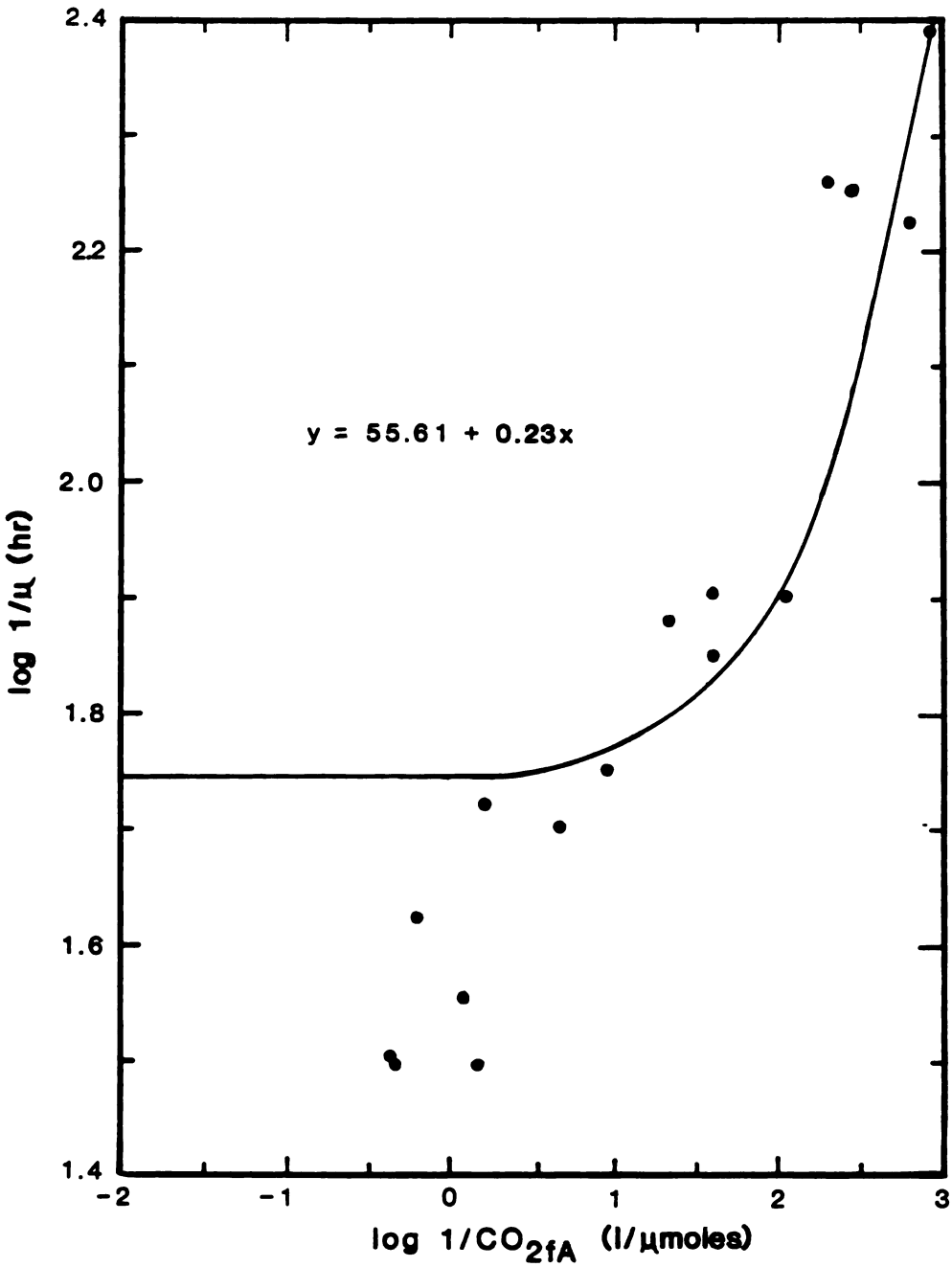


Figure 34. Reciprocal plot of the specific growth rates of the 0.125 mg Cu^{+2}/l treatment Chlorella vulgaris cultures in Experiment 5 as a function of the CO_{2fA} concentration of the medium.

With the pH method it was not possible to produce a model which estimated u at various cupric ion and CO_{2fA} concentrations of the medium because of the lack of fit to Michaelis-Menton kinetics. However, the effort expended in sampling and enumeration in the AAP is much greater than that in the pH method. For these reasons, the pH method was judged to be superior to the AAP in this test.

EXPERIMENT 6

The Effects of p-Cresol on *Chlorella vulgaris* Culture Growth

Purpose

This experiment was designed to assess the effects of three concentrations of p-cresol on the growth kinetics of *Chlorella vulgaris* cultures. The purpose was also to ascertain the mode of response of the algal cultures, to a compound of unknown toxicity, in the two bioassay methods used in this study.

Procedure

Light measurements of the assay platform were taken before and after the experiment. Twenty-six of the 64 available spaces on the assay platform were found to be in the acceptable range of 400 $\pm$40 footcandles of illumination (Figure 35). Twenty-four test vessels were each half filled with 50 ml of fresh nutrient medium (a = 1.96 meq CO_3^{2-}--HCO_3^- alkalinity/l) and these were assigned to spaces on the assay platform. The resulting positions, with respect to the photosynthetically active radiation falling on the assay platform, are shown in Figure 36.

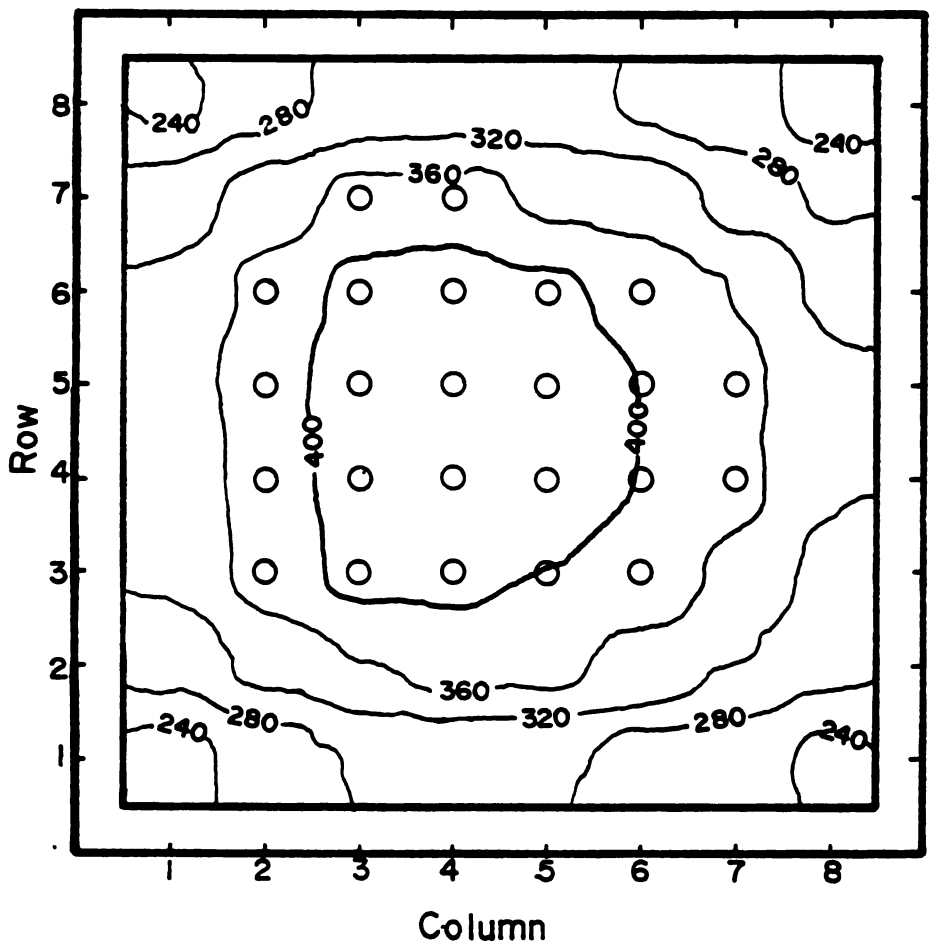


Figure 35. Contour map of the light intensity (footcandles) falling on the assay platform in Experiment 6. The position of each test vessel is indicated by a circle.

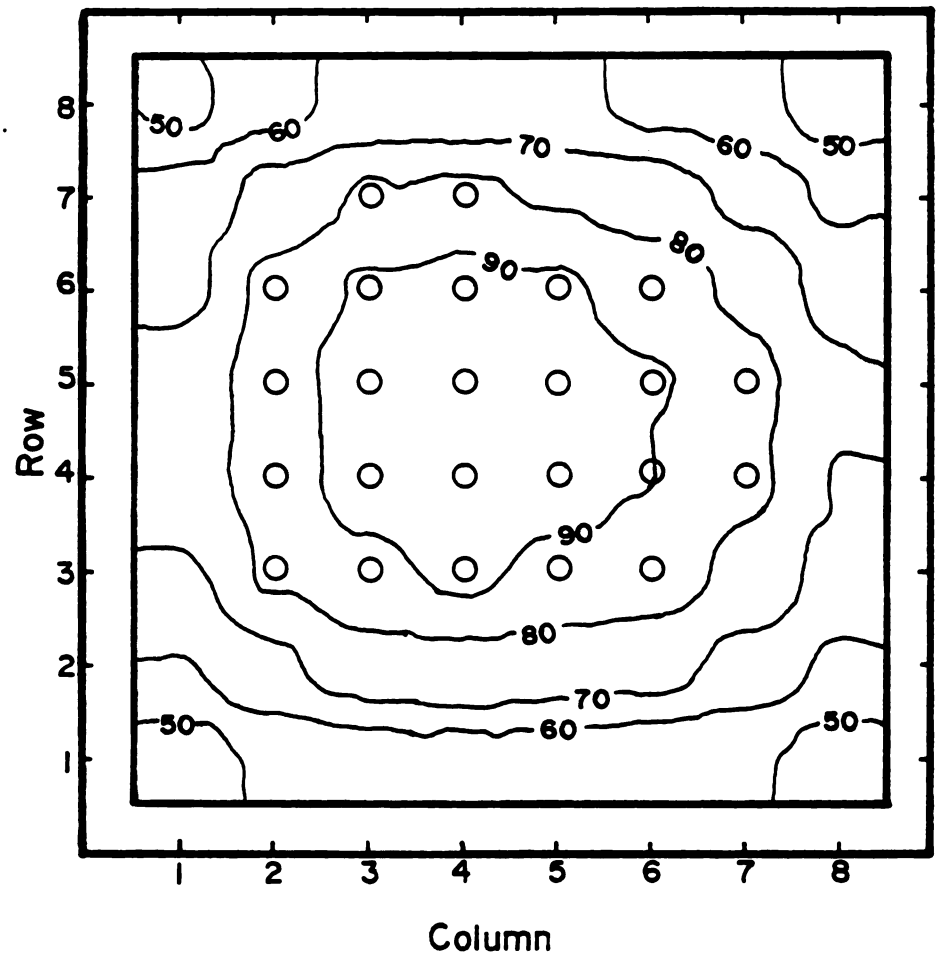


Figure 36. Contour map of the photosynthetically active radiation (uE/m^2/sec) falling on the assay platform in Experiment 6. The position of each test vessel is indicated by a circle.

Since the effects of p-cresol on algae had not been previously reported in the literature, the four p-cresol concentrations tested were based on information from fish and invertebrate bioassays. The stock p-cresol solution (1,000 mg/l) was added directly to the medium in the test vessels to give six replicates each of 5, 10, and 15 mg p-cresol/l. The remaining six replicates were used as controls. Half of the replicates at each p-cresol level were assigned to the AAP and half to the pH method.

Each flask was inoculated with 0.5 ml of 6-day-old Chlorella vulgaris stock culture to give a final concentration of 3,300 cells/ml (0.00975 mM organic C/l). Daily samples of the AAP cultures were taken and the number of cells/ml computed. These biomass estimates were tested for significant differences by a nested analysis of variance (Sokal and Rohlf, 1969) and a Student-Newman-Keuls multiple comparison procedure (Steel and Torrie, 1960). Samples of the pH method cultures were taken on a daily basis and the pH determined. The experiment was run at 27°C.

The p-cresol concentration of the test cultures was monitored during the test by gas chromatography methods. A Varian Model 3700 gas chromatograph, fitted with a 6-foot-long (2mm ID) glass column containing 80/100 Chromosorb 101 (John's Manville Co.), was used for this purpose. One sample of the 5 mg p-cresol/l treatment cultures and one of the 15 mg p-cresol/l treatment cultures from each of the two bioassay methods were taken at times 3, 42.5, and 114

hours. The samples were centrifuged for 5 minutes at 1,000 rpm to remove the algal cells. The samples were acidified with H_3PO_4 to pH 2 and refrigerated until analysis. Prior testing showed that these steps minimized p-cresol loss from the samples. One subsample (1 ul) from each sample was analyzed in the aqueous phase by flame ionization detection. The column was kept at 220°C., the dector at 275°C., and the injection port at 270°C. during the analysis. The p-cresol peaks (time = 5.5 minutes from injection) were measured by planimetry.

Results

The daily cell counts from the AAP cultures are tabulated in the Appendix (Table A8). The AAP growth curves are shown in Figure 37. The growth of the control cultures exceeded that of the three p-cresol treatments' cultures throughout the test; however, the results of the statistical analysis do not show a graded response of the test cultures with increased p-cresol concentration. The results indicated no significant difference between the three p-cresol treatments' cultures throughout most of the test. Furthermore, the order (in terms of increasing biomass) of the three treatments varied from sampling time to sampling time (Table 17).

A comparison of the observed u_{max} values from the treatment cultures shows little effect due to p-cresol on the observed u_{max} values attained by the treatment cultures. Although the values are dissimilar, the time at which each

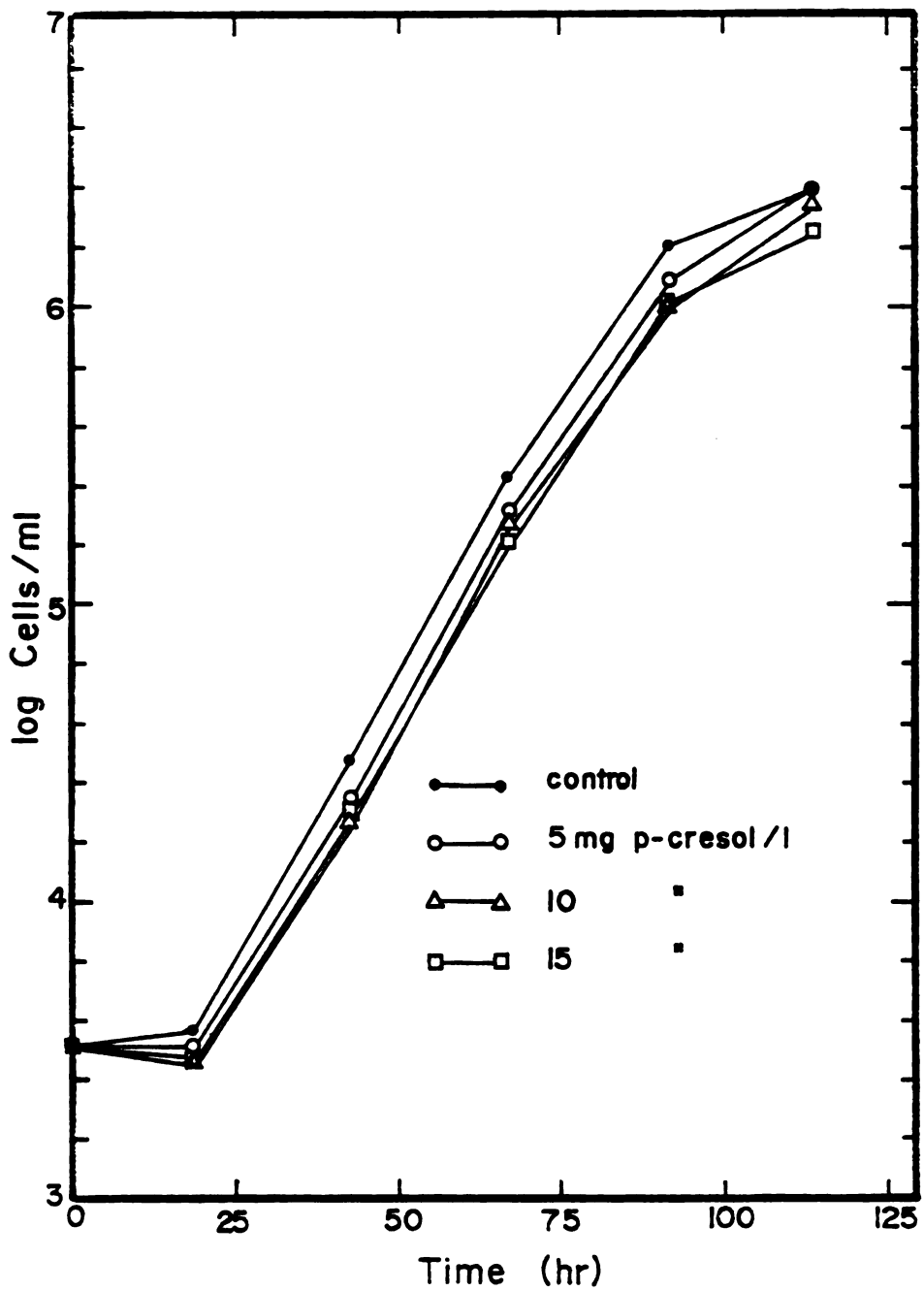


Figure 37. AAP growth curves for Chlorella vulgaris cultures in Experiment 6. Each point represents the mean of three replicates.

Table 17. Statistical analysis of the AAP results of Experiment 6. F test values and associated significance levels from a nested analysis of variance are listed for each sampling time. The treatments are listed in ascending order of biomass (cells/ml) with those means judged not significantly different, using a Student-Newman-Keuls multiple comparison procedure (P = 0.95), grouped by underlining.

Time (hr)	F value	Treatment (mg p-cresol/l)
18.5	4.82 **	10 15 5 0
42.5	27.73 ***	10 15 5 0
67.0	9.27 ***	15 10 5 0
91.5	2.66 +	15 5 15 0
114.0	5.46 **	15 10 5 0

*** P = 0.99
** P = 0.95
* P = 0.90
+ P = 0.80
ns P = 0.80 (not significant)

treatment's observed u_{max} occurred varied. The trend appeared to be that as the p-cresol concentration was raised, the observed u_{max} for each successive treatment occurred at a later time in the test. Since the observed u values were not statistically tested on a sequential basis, it is difficult to say whether or not the values preceding the observed u_{max} were significantly lower than the observed u_{max} (Table 18). In addition, no morphological differences between test cultures were noted. From the result obtained by the AAP, the evidence of sublethal effects of p-cresol on Chlorella vulgaris cultures was inconclusive.

The results of the daily pH measurements of the pH method cultures are recorded in the Appendix (Table A9). The daily rise in pH of the control cultures mimicked the results found in the preceding experiments. The pH of the p-cresol treatment cultures, however, decreased for the first few days of the test. The amount of pH decrease was proportional to the initial p-cresol content of the cultures. After the initial pH decrease, the pH of all the test cultures increased until all four treatments had virtually the same pH at 114 hours (Table A9).

It was hypothesized that the initial pH decrease of the test cultures might be due to algal respiration caused by the p-cresol. However, the amount of carbon present in the algal inoculum, 0.00975 mM C/l, could not account for the drop in culture pH that was noted. Clearly, some other proton source must have contributed to this pH decrease.

Table 18. Specific trowth rates (u) of Chlorella vulgaris in Experiment 6, as determined by the AAP. Maximum specific growth rates are underlined.

	Treatment (mg p-cresol/l)			
Interval	Control	5	10	15
1	0.0062	-0.0017	-0.0070	-0.0034
2	0.0652	0.0627	0.0607	0.0613
3	0.0650	0.0657	0.0668	0.0635
4	0.0599	0.0591	0.0571	0.0642
5	0.0196	0.0309	0.0340	0.0153

The gas chromatographic analyses of the initial samples (time = 3 hours) showed no decrease of the p-cresol concentrations of the 5 and 15 mg/l p-cresol treatment cultures. By the time of the second sampling (time = 42.5 hours), the p-cresol concentrations of the 5 and 15 mg/l p-cresol treatment cultures were below the limit of detectability for the gas chromatography column used (determined to be approximately 3 mg p-cresol/l). The p-cresol concentrations of the samples taken at 114 hours also were below the limit of detectability.

Several of the test cultures, in particular those of the 15 mg p-cresol/l treatment had a whitish cast. Microscopic examination of these cultures revealed a high level of bacterial activity, as compared to the controls.

Conclusions

The effect of p-cresol on Chlorella vulgaris cultures was not determined due to problems encountered in the test. The results of the gas chromatographic analyses indicated that the p-cresol concentration of the medium was decreased by some mechanism. The results of the pH method test indicated that the p-cresol may have been degraded into constituent parts which caused a decrease in culture pH. The results of both bioassay methods indicated that these constituent parts apparently have no effect on Chlorella vulgaris culture growth. This conclusion supports the findings of Barthauer and Alred (1980). These researchers

stated that of all the known breakdown products of p-cresol, none was reported as being toxic.

As previously noted, p-cresol may be degraded by a variety of bacteria. This appears to have been the case in this test. However, other breakdown mechanisms also may have contributed to the decrease of the p-cresol concentration of the medium. Photodegradation, volatilization, sorption onto algal cells, and biodegradation by algae could have contributed to the p-cresol loss. Further investigation into these possible pathways of p-cresol degradation was called for.

EXPERIMENT 7

Analysis of the Degradation of p-Cresol Under Algal Bioassay Conditions

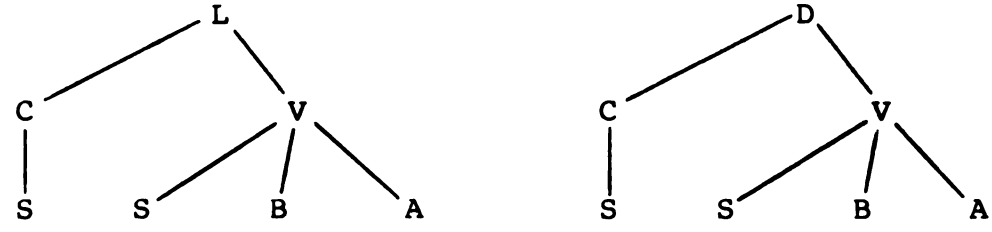
Purpose

This experiment was designed to ascertain the extent of p-cresol degradation under algal bioassay conditions due to four mechanisms. These were bacterial degradation, photolysis, sorption onto algal cells, and/or degradation due to the algae and volatilization from the medium.

Procedure

To test the extent of the effects of the four mechanisms listed above, the p-cresol concentration of eight treatments (with three replicates of each) was monitored by gas chromatographic methods for several days. The flowchart given in Figure 38 shows the setup of the test and the eight treatments. For brevity, acronyms are used when the treatments are discussed in the text. For example, a vented test vessel containing sterile medium and incubated in the dark has the acronym SVD.

All test vessels, closures, and other apparatus which came in direct contact with the medium were first washed, as previously described, and then autoclaved. The distilled water used in the medium preparation was membrane filtered



(SCL) (SVL) (BVL) (AVL) (SCD) (SVD) (BVD) AVD)

L incubated under 400 ±40 footcandles of continuous illumination

D incubated in the dark

C closed culture vessel

V vented culture vessel

S sterile medium

B medium with bacteria added

A medium with Chlorella vulgaris culture added (nonaxenic)

Figure 38. Flowchart of the experimental design of the p-cresol degradation test, Experiment 7. The acronyms for the eight treatments appear in parentheses.

(0.454 Millipore filter) into the autoclaved 18 l preparation bottle. The nutrient solutions also were filtered in the same manner and the filters washed with several rinsings of distilled water to minimize nutrient loss. The test vessels used for the SCL and SCD treatments were 1 l erhlenmeyer flasks fitted with Number 9 solid rubber stoppers and capped with Parafilm. The test vessels for the remaining treatments were 1 l erhlenmeyer flasks fitted with Number 9 one-hole rubber stoppers, in which the vent was placed. The vent was an inverted pasteur pipette containing a "glass wool" plug and capped with a foam plug. This allowed for the free exchange of gases with the atmosphere in all six treatments, while minimizing the chance of bacterial contamination of the medium in the SVL and SVD treatments.

Each test vessel was half filled with 500 ml of sterile medium (a = 1.940 meq CO_3^{2-}--HCO_3^- alkalinity/l) by following accepted sterile procedure (Levy et al, 1973). All test vessels were inoculated with a sterile 1,000 mg/l p-cresol stock solution to yield a final concentration of 15 mg p-cresol/l.

One hundred milliliters of nonaxenic Chlorella vulgaris stock culture were filtered through 1 u pore-size filter paper to remove the algal cells. The filtrate was examined microscopically and was found to contain only rod-shaped bacteria. The replicates of the BVL and BVD treatments were inoculated with 0.5 ml of this filtrate. The AVL and AVD treatment replicates were inoculated with 0.5 ml of nonaxenic 5-day-old Chlorella vulgaris culture, to yield a

final concentration of 4,200 cells/ml. The remaining treatments (SCL, SCD, SVL, and SVD) were inoculated with 0.5 ml of autoclaved distilled water.

Light measurements of the assay platform were taken, and 27 of the 64 spaces were within the acceptable range of 400 ±40 footcandles of illumination (Figure 39). The test vessels to be incubated in the light were then assigned to 12 of these 27 spaces. The positions of the test vessels in relation to the photosynthetically active radiation falling on the assay platform are shown in Figure 40. The remaining 12 test vessels were placed in a dark chamber. The temperature of all cultures was maintained at 27°C.

Samples of all treatment replicates were taken at times 0, 10, 22, 37.5, and 61 hours. The 10 ml samples were taken with volumetric pipettes by following sterile sampling procedure (Levy et al, 1973). The BLV, BVD, AVL, and AVD treatment replicate samples were centrifuged for 5 minutes at 1,000 rpm to remove bacteria and algae, respectively. All samples were fixed by the addition of H_3PO_4 to pH 2 and refrigerated until analysis.

Prior to analysis, 1 ml of a 55 mg phenol/l standard solution was added to each sample to give an internal standard concentration of 5 mg phenol/l. This step was intended to give greater precision in the estimation of the p-cresol concentration of the test cultures.

A Varian Model 3700 gas chromatograph equipped with a 6-foot-long (2 mm ID) glass column containing 10% SP-1200/1%

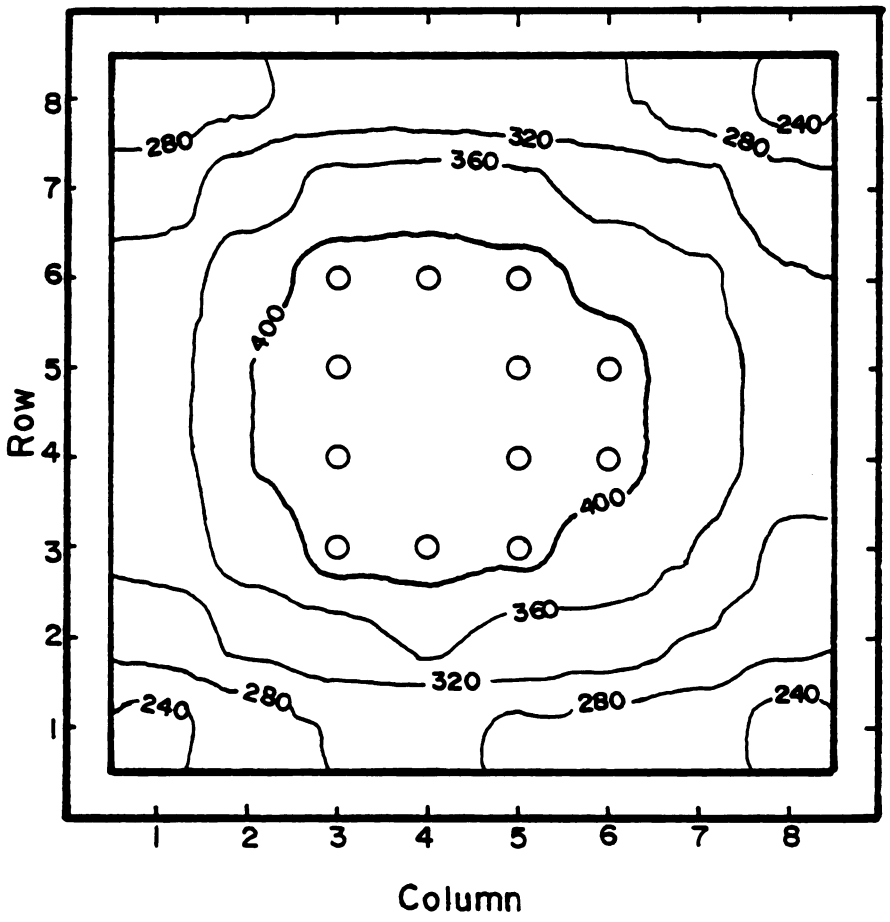


Figure 39. Contour map of the light intensity (footcandles) falling on the assay platform in Experiment 7. The position of each test vessel is indicated by a circle.

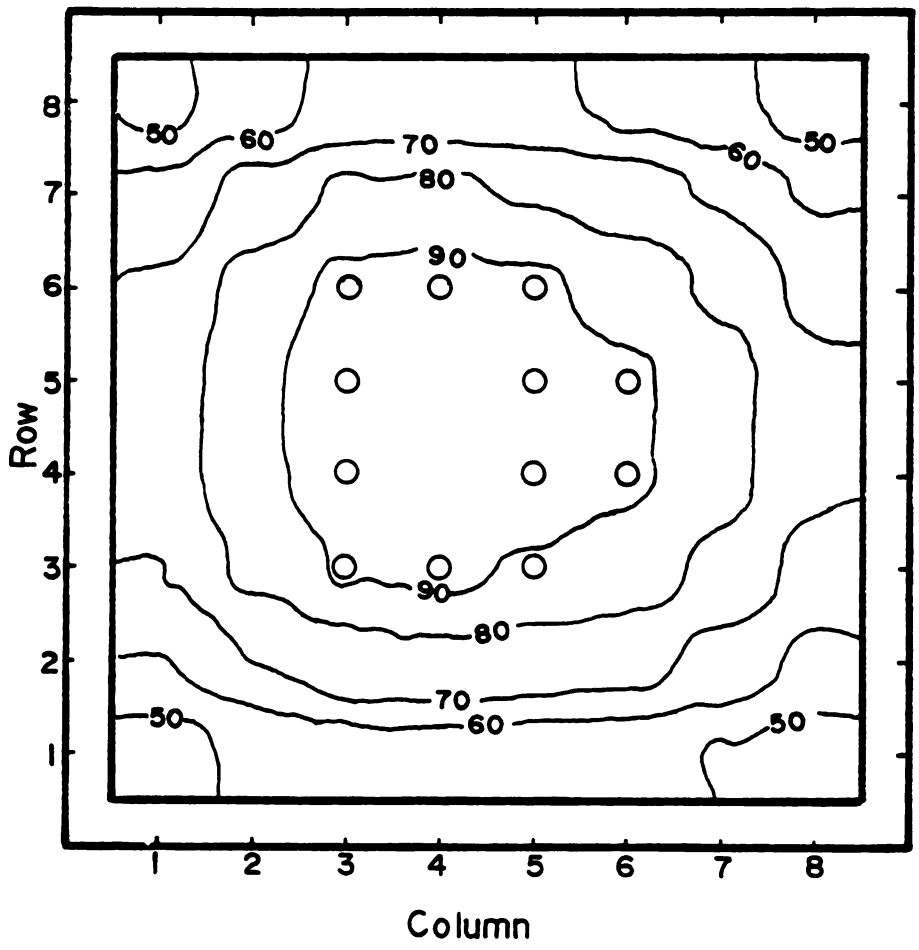


Figure 40. Contour map of the photosynthetically active radiation (uE/m^2/sec) falling on the assay platform in Experiment 7. The position of each test vessel is indicated by a circle.

H_3PO_4 on 80/100 Chromosorb W AW (Supelco, Inc.) was used for the analysis. One subsample (1 ul) from each sample was analyzed in the aqueous phase by flame ionization detection. The column was kept at 160°C., the detector at 250°C., and the injection port at 190°C. during the analysis. The phenol and p-cresol peaks (time = 2.2 and 3.4 minutes from injection, respectively) were measured by planimetry.

Prior to the initiation of the experiment, several possible sources of error were investigated. In the first test, the p-cresol concentration of centrifuged samples was compared to that of uncentrifuged samples. This was done to determine if any p-cresol was lost in centrifugation. The second test was designed to determine the conversion factor needed to convert units of phenol concentration to units of p-cresol concentration. This was determined by analyzing p-cresol standards of 0, 5, 10, and 15 mg p-cresol/l which contained a 5 mg phenol/l internal standard. The conversion factor was determined by the equation

$$f = \left(\frac{\text{p-cresol concentration}}{\text{area of p-cresol peak}}\right)\left(\frac{\text{area of phenol peak}}{\text{phenol concentration}}\right) \quad (26)$$

The average of the observed f values ($\bar{f}$) was used as the conversion factor in the following equation.

$$\text{mg p-cresol/l} = \left(\frac{\text{p-cresol peak area}}{\text{phenol peak area}}\right)(\text{mg phenol/l})(\bar{f}) \quad (27)$$

By measuring the phenol and p-cresol peak areas and using the known phenol concentration and $\bar{f}$, the unknown p-cresol concentration of the treatment replicates was determined.

Results

The centrifuging process was found not to affect the p-cresol content of the samples. Therefore, only samples of treatments BVL, BVD, AVL, and AVD were centrifuged prior to analysis. The conversion factor, $\overline{f}$, was determined to be 1.065 p-cresol units to 1 phenol unit.

The results of the experiment are presented in Table 19. The treatments are presented in the order which would be expected (lowest to highest p-cresol concentration remaining) if all four factors which were tested were effectively decreasing the p-cresol concentration of the medium. This does not appear to be the case, however, as none of the three sampling times where p-cresol remained in the medium of each treatment (10, 22, and 37.5 hours) shows this trend.

As in the previous experiment, most of the test cultures developed a whitish cast. Bacterial contamination was suspected not only in those treatments with known bacterial populations, but also in the treatments containing "sterile" medium. Microscopic examination of samples revealed that rod-shaped bacteria were present in all test vessels. These bacteria had a mean length of 0.8 u, but most had a cellular width of less than 0.45 u, the pore size of the "bacterial" filter paper used for the sterilization process. This finding complicates the analysis somewhat; however, several conclusions can still be made.

The results indicate little or no difference between the closed and vented flasks in terms of p-cresol loss. Also, as

Table 19. Results of the gas chromatographic analysis for p-cresol in Experiment 7. Each entry represents the mean p-cresol concentration (mg/l) of three replicates ±1 standard deviation.

Time (hr)*	Treatment							
	AVL	AVD	BVL	BVD	SVL	SVD	SCL	SCD
10.0	11.9 ±2.1	13.1 ±2.2	11.7 ±1.1	11.9 ±1.3	13.1 ±1.6	12.8 ±2.5	12.8 ±0.8	12.9 ±0.9
22.0	11.1 ±4.1	11.9 ±2.9	11.0 ±1.0	11.1 ±0.8	11.6 ±1.5	11.8 ±1.8	11.2 ±0.4	11.2 ±0.6
37.5	9.1 ±0.2	6.2 ±0.9	7.3 ±2.2	5.0 ±1.0	7.7 ±0.9	7.5 ±1.9	9.8 ±0.6	8.8 ±1.1
6.10	nd	nd	nd	nd	nd	nd	1.2 ±1.0	nd

* The initial p-cresol concentration at time = 0 hours was 15 mg/l for all replicates.

nd = none detected

previously reported, centrifugation did not contribute to the loss of p-cresol from the medium. This leads to the conclusion that volatilization is not a significant pathway of p-cresol loss from the medium when compared to microbial action.

The second hypothesis that photolysis could be causing the loss of p-cresol from the medium appears to be equally invalid. The data indicate that the test vessels incubated under continuous illumination did not have lower p-cresol concentrations than their counterparts in the dark.

The third hypothesis was that the p-cresol sorbed onto the algae cells or was degraded by them. When compared to cultures containing only bacteria, the p-cresol concentration of the algal culture containing vessels was not lower.

When compared to the biodegradation of the p-cresol by bacteria, the other three hypothesized pathways of p-cresol loss from the medium do not appear to be significant. It is apparent that the biodegradation of p-cresol by bacteria is the rate-determining factor in its loss from the medium in nonaxenic algal bioassays.

Conclusions

Due to the bacterial contamination of all of the treatment cultures, a statistical analysis of the experiment was not possible. However, the data indicate that bacterial degradation of p-cresol appears to be the most probable cause of the results found in the previous experiment. The observed results are in agreement with the findings of the

United States Environmental Protection Agency (1978). EPA's research indicated that under most conditions the bacterial degradation of p-cresol was the most significant factor in its loss from natural waters.

SUMMARY

The initial series of experiments were used to locate and eliminate various sources of error in the two algal bioassay methods utilized in this study. The majority of error in the AAP was found to be in the cell-counting procedure. The corrections made in the procedure and analysis helped to minimize experimental error. The modification of the McNabb (1960) method of enumerating algal cells which was used in this study, was found to be accurate. The procedures chosen for the pH control of the AAP cultures, sampling for the pH method, and analysis for the pH method were found to be acceptable.

Cupric ion was found to be acutely toxic to Chlorella vulgaris cultures at a concentration of 0.15 mg/l. This is lower than the level reported by McBrien and Hassal (1965) as being acutely toxic to Chlorella cells (0.27 ppm Cu^{2+}). Cupric ion concentration of 0.05 mg/l had little effect on Chlorella vulgaris culture growth. Sublethal concentrations of cupric ion between 0.05 and 0.15 mg/l inhibited algal growth by:

1. prolonging the lag phase of growth,
2. decreasing the maximum specific growth rate, and consequently
3. increasing the time needed to reach the maximum standing crop biomass level.

The extent of growth, the maximum standing crop biomass level, was not affected by sublethal concentrations of cupric ion. The results obtained and observations made are consistent with the previously reported findings that cupric ion inhibits algal photosynthesis (Cedeno-Maldonado et al, 1972), decreases cellular chlorophyll content (Gross et al, 1970), and increases cellular permeability (McBrien and Hassal, 1965).

The cupric ion analyses indicate that of the two algal bioassay methods tested, the pH method was more sensitive in detecting the effects of cupric ion on algal growth kinetics. In addition, the sampling and testing procedures for the pH method were less time-consuming than the procedures used in the AAP.

The test of the effects of p-cresol on algal growth kinetics was inconclusive. The p-cresol was rapidly degraded by bacteria present in the Chlorella vulgaris cultures. Further testing indicated that the bacterial degradation of p-cresol was the rate-determining factor in its loss from the algal nutrient medium, as compared to p-cresol loss due to photolysis, volatilization, and algal sorption and/or degradation of the p-cresol. The breakdown products of p-cresol had no effect on algal growth.

The p-cresol toxicity test also indicated an inherent difficulty in the pH method. A compound tested by the use of this method which affects the pH renders this method useless.

The affect on culture pH violates the assumption that any rise or fall of culture pH is due only to algal activity.

APPENDIX

APPENDIX

AAP and pH Method Data From the Experiments

Table A1. Cell counts as cells/ml from Experiment 1. Each entry represents the mean of 20 observations ±1 standard deviation.

Sample	Filter number	Cells/grid	Cells/ml
1	1	14.4 ±3.4	1,680,000 ±397,000
	2	15.3 ±4.4	1,780,000 ±513,000
	3	17.1 ±5.5	1,990,000 ±642,000
2	1	14.9 ±3.6	1,740,000 ±420,000
	2	17.5 ±4.2	2,040,000 ±490,000
	3	17.8 ±3.4	2,080,000 ±397,000
3	1	15.8 ±4.3	1,840,000 ±502,000
	2	16.1 ±4.4	1,880,000 ±513,000
	3	17.2 ±3.3	2,010,000 ±385,000

Table A2. Cell counts as cells/ml from Experiment 3. Each entry represents the mean of 20 observations ±1 standard deviation.

Time (hr)*	Replicate 1	2	3	4	5
21.0	7,800 ±1,600	7,960 ±1,300	5,850 ±1,600	7,870 ±1,170	8,770 ±2,880
44.5	91,000 ±16,000	94,300 ±13,700	89,700 ±18,800	89,700 ±18,800	92,900 ±18,100
68.5	368,000 ±156,000	487,000 ±108,000	6,64,000 ±95,500	421,000 ±80,500	495,000 ±85,200
93.5	1,670,000 ±615,000	1,750,000 ±408,000	1,940,000 ±621,000	1,790,000 ±498,000	1,780,000 ±390,000
116.5	2,070,000 ±596,000	2,710,000 ±1,400,000	3,060,000 ±1,280,000	2,890,000 ±945,000	2,200,000 ±802,000
140.0	2,410,000 ±512,000	2,530,000 ±615,000	2,900,000 ±481,000	2,730,000 ±481,000	2,530,000 ±647,000
164.0	2,910,000 ±635,000	3,000,000 ±562,000	3,240,000 ±1,030,000	3,370,000 ±615,000	2,760,000 ±671,000

* Each flask was inoculated with 5,300 cells/ml at time = 0 hours.

Table A3. pH values for cultures of Chlorella vulgaris in Experiment 3.

	Replicate				
Time (hr)*	1	2	3	4	5
21.0	8.4	8.5	8.4	8.3	8.6
44.5	9.1	9.2	9.3	8.9	9.2
68.5	10.1	10.3	10.0	9.7	10.1
93.5	11.1	11.2	11.2	11.2	11.2
116.5	11.2	11.4	11.6	11.7	11.6
140.5	11.2	11.2	11.5	11.4	11.5
164.0	11.2	11.0	11.3	11.4	11.3

* pH = 8.3 at time = 0 hours.

Table A4. Cell counts as cells/ml from Experiment 4. Each entry represents the mean of 60 observations ±1 standard deviation.

Time (hr)*	Treatment (mg Cu^{2+}/l) Control	0.05	0.10	0.15
20.0	3,300 ±1,200	3,200 ±1,100	3,400 ±850	3,200 ±1,100
44.0	12,300 ±3,700	9,300 ±2,400	3,700 ±1,600	2,700 ±1,100
68.0	91,700 ±25,400	53,000 ±14,900	11,500 ±9,140	3,100 ±1,100
91.5	661,000 ±260,000	391,000 ±123,000	43,700 ±35,800	2,300 ±1,100
140.5	2,510,000 ±914,000	2,490,000 ±1,310,000	597,000 ±795,000	560 ±460
164.0	2,570,000 ±819,000	2,760,000 ±857,000	873,000 ±1,110,000	***
188.5	3,140,000 ±880,000	3,270,000 ±1,490,000	1,510,000 ±1,230,000	
212.0	3,090,000 ±1,650,000	3,190,000 ±1,040,000	2,090,000 ±1,110,000	
236.0	3,690,000 ±1,110,000	3,380,000 ±725,000	2,770,000 ±831,000	
259.0	**	**	3,320,000 ±998,000	
331.0			3,530,000 ±905,000	

* Each flask was inoculated with 3,400 cells/ml at time = 0 hours.

** The previous day's result was the maximum standing crop biomass level for the control and 0.05 mg Cu^{2+}/l treatment.

*** These cultures were terminated following this sampling.

Table A5. pH values for cultures of Chlorella vulgaris in Experiment 4.

Time (hr)*	Replicate	Treatment (mg Cu^{2+}/l) Control	0.05	0.10	0.15
20.0	1	8.4	8.4	8.5	8.5
	2	8.5	8.5	8.4	8.5
	3	8.5	8.5	8.5	8.5
44.0	1	8 8	8.6	8.5	8.5
	2	8.8	8.7	8.5	8.5
	3	8.9	8.7	8.5	8.5
68.0	1	9.4	9.2	8.4	8.4
	2	9.4	9.1	8.6	8.4
	3	9.5	9.1	8.5	8.5
91.5	1	10.2	9.8	8.6	8.5
	2	10.3	9.9	8.7	8.5
	3	10.7	10.0	8.7	8.5
117.0	1	11.0	10.9	8.7	8.6
	2	11.3	11.0	9.0	8.5
	3	11.4	11.0	8.9	8.6
140.5	1	11.3	11.3	8.7	8.6
	2	11.4	11.3	9.0	8.5
	3	11.5	11.4	8.9	8.5
164.0	1	11.4	11.4	8.6	8.4
	2	11.5	11.4	9.1	8.4
	3	11.4	11.5	9.0	8.4
188.5	1	**	**	8.7	8.4
	2			9.2	8.4
	3			9.2	8.5
212.0	1			8.7	8.5
	2			9.4	8.4
	3			9.5	8.4

* pH = 8.4 at time = 0 hours.

** The previous day's pH was taken as the maxima or minima and used in subsequent comparisons between treatments.

Table A5 (cont'd)

Time (hr)*	Replicate	Treatment (mg Cu^{2+}/l) Control	0.05	0.10	0.15
259.5	1			9.2	**
	2			9.8	
	3			10.2	
306.5	1			9.8	
	2			10.8	
	3			11.2	
331.0	1			10.2	
	2			11.1	
	3			11.3	
355.0	1			10.6	
	2			11.4	
	3			11.5	

Table A6. Cell counts as cells/ml from Experiment 5. Each entry represents the mean of 60 observations ±1 standard deviation.

Time (hr)*	Treatment (mg Cu^{2+}/l)			
	Control	0.075	0.10	0.125
16.0	14,000 ±6,000	13,000 ±7,900	5,100 ±1,700	3,700 ±1,200
39.5	99,600 ±35,000	82,700 ±30,400	24,700 ±15,100	11,000 ±8,200
64.5	799,000 ±244,000	567,000 ±224,000	125,000 ±43,300	16,600 ±13,800
91.0	2,060,000 ±524,000	1,630,000 ±490,000	739,000 ±302,000	16,900 ±13,800
109.5	2,620,000 ±581,000	2,200,000 ±612,000	1,630,000 ±281,000	21,400 ±15,100
134.5	3,400,000 ±783,000	2,890,000 ±693,000	1,820,000 ±588,000	33,600 ±16,700
159.5	3,570,000 ±766,000	3,450,000 ±920,000	2,260,000 ±844,000	82,600 ±39,300
182.5	3,780,000 ±930,000	3,450,000 ±814,000	3,070,000 ±686,000	315,000 ±173,000
205.0	**	**	3,320,000 ±685,000	875,000 ±450,000
229.0			3,700,000 ±880,000	1,930,000 ±551,000
256.0			4,030,000 ±843,000	2,890,000 ±654,000
277.0			4,010,000 ±1,090,000	3,240,000 ±907,000
302.5				3,420,000 ±1,020,000

* Each flask was inoculated with 3,300 cells/ml at time = 0 hours.

** The previous day's result was the maximum standing crop biomass level.

Table A7. pH values for cultures of Chlorella vulgaris in Experiment 5.

Time (hr)*	Replicate	Treatment (mg Cu^{2+}/l) Control	0.075	0.10	0.125
16.0	1	8.6	8.6	8.4	8.5
	2	8.6	8.6	8.4	8.4
	3	8.6	8.5	8.3	8.4
39.5	1	9.4	9.2	8.7	8.5
	2	9.4	9.2	8.7	8.6
	3	9.4	9.2	8.6	8.5
64.5	1	10.4	9.8	9.0	8.6
	2	10.5	9.9	9.0	8.6
	3	10.5	9.8	9.1	8.6
91.0	1	11.2	10.7	9.4	8.7
	2	11.3	10.8	9.4	8.7
	3	11.3	10.7	9.6	8.7
109.5	1	11.3	11.0	9.6	8.7
	2	11.3	11.1	9.5	8.7
	3	11.3	11.1	9.8	8.8
134.5	1	11.3	11.2	9.8	8.7
	2	11.3	11.3	9.8	8.7
	3	11.4	11.3	10.2	8.9
159.5	1	**	11.4	10.2	8.7
	2		11.4	10.1	8.7
	3		11.4	10.7	9.0
182.5	1		11.4	10.7	8.7
	2		11.4	10.5	8.7
	3		11.4	11.0	9.1
205.0	1		**	10.9	8.7
	2			10.9	8.7
	3			11.2	9.2
229.0	1			11.1	8.8
	2			11.1	8.8
	3			11.3	9.2

* pH = 8.4 at time = 0 hours.
** The previous day's pH was taken as the maxima and was used in subsequent comparisons between treatments.

Table A7 (Cont'd)

Time (hr)*	Repli-Cate	Treatment (mg Cu^{2+}/l) Control	0.075	0.10	0.125
256.0	1		**	11.2	9.1
	2			11.2	9.1
	3			11.3	9.6
277.0	1			11.2	9.4
	2			11.3	9.5
	3			11.3	9.8
302.5	1			11.3	9.9
	2			11.3	10.0
	3			11.3	10.4
326.5	1			**	10.3
	2				10.4
	3				10.8
350.5	1				10.6
	2				10.8
	3				10.9
374.5	1				11.0
	2				11.0
	3				11.1
398.5	1				11.0
	2				11.1
	3				11.2

Table A8. Cell counts as cells/ml from Experiment 6. Each entry represents the mean of 60 observations ±1 standard deviation.

Time (hr)*	Treatment (mg p-cresol/l)			
	Control	5	10	15
18.5	3,700 ±1,000	3,200 ±900	2,900 ±1,100	3,100 ±1,100
42.5	30,400 ±9,400	22,600 ±11,600	18,500 ±9,800	20,300 ±9,500
67.0	268,000 ±61,500	209,000 ±71,900	185,000 ±72,900	162,000 ±51,900
91.5	1,640,000 ±427,000	1,230,000 ±356,000	991,000 ±525,000	1,250,000 ±414,000
114.0	2,470,000 ±638,000	2,540,000 ±663,000	2,220,000 ±508,000	1,770,000 ±563,000

* Each flask was inoculated with 3,300 cells/ml at time = 0 hours.

Table A9. pH values for cultures of Chlorella vulgaris in Experiment 6.

Time (hr)*	Replicate	Treatment (mg p-cresol/l)			
		Control	5	10	15
18.5	1	8.2	7.9	8.0	8.0
	2	8.2	7.9	8.0	8.0
	3	8.3	7.8	7.9	7.9
42.5	1	8.8	8.0	7.5	7.2
	2	8.8	8.0	7.5	7.3
	3	8.7	7.9	7.6	7.3
67.0	1	9.8	9.5	9.0	8.9
	2	9.8	9.5	9.0	8.9
	3	9.6	9.3	9.2	9.0
91.5	1	10.9	10.7	10.3	10.5
	2	10.8	10.7	10.3	10.4
	3	10.6	10.6	10.1	10.6
114.0	1	11.0	11.1	11.1	11.1
	2	11.1	11.1	11.1	11.1
	3	11.0	11.1	11.2	11.2

* pH = 8.2 at time = 0 hours.

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