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THE INFLUENCE OF SUBSTRATE NUTRIENT CONCENTRATION AND INDUCED TURBULENCE ON THE GROWTH OF ALGAL CULTURES

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INTRODUCTION

Significance of the Problem:

The acceleration of the aging process of natural waters, particularly lakes and ponds, by the addition of treated or untreated sewage is a source of great concern to the Sanitary Engineering profession. In this process of increased eutrophication, the problems caused by treated sewage are extremely challenging. This is due to the fact that even though such sewage has been subjected to the most modern conventional treatment processes, completely treated sewage still materially increases eutrophication rates.

The process of eutrophication is dynamic and involves a complexity of chemical, physical and biological factors. The initial step in the increased rate of eutrophication, as brought about by treated sewage, is the conversion of inorganic nutrients into organic matter. To accomplish such an interchange in nature requires the presence of a biological form and an energy source. Because of their ability to transform radiant energy into chemical energy, algae are the predominant bio-system involved in carrying out this interchange. The organic material produced is in the form of more algal cellular matter. Algae are, therefore, the first and possibly the most vital link in the chain of bio-reactions which bring about induced eutrophication.

That algae have such an important role in the acceleration of the aging process of natural waters, is of great significance to the sanitary engineer. Since algae do occupy such a

prominent position, it appears theoretically feasible to control the contribution of sewage to eutrophication by the use of algae in a treatment process. Such a process would involve the use of algae to extract the nutrients in a confined area followed by the extraction of the organisms themselves so that neither the nutrients nor the algae would enter the receiving water.

Algae certainly are not unknown to sanitary engineering, but it has only been in the last two decades that intensive investigation has been conducted concerning their potential as sewage treatment organisms. The most often encountered use of algae at present is in sewage lagoons. In such systems the algae live in symbiotic relationship with bacteria. This relationship provides a sewage treatment system. Sewage lagoons are roughly equivalent to primary and secondary sewage treatment and as such they are normally considered as stabilization devices. That is, their basic purpose is to oxidize the sewage to a high degree. Studies of lagoons have, therefore, been concerned with BOD reduction, solids removal and DO levels, but not particularly with nutrient extraction. Nutrient reduction through the application of biological tertiary sewage treatment is one possible process which could reduce eutrophication rates.

General Aspects:

Sewage lagoons produce as a basic product stabilized sewage effluents. Most investigations using algal cultures have been interested only in producing an abundance of algal cellu-

lar material. These investigations concerned with producing algal material are involved in the problem of "mass culturing" of algae. It seems as though a combination of sewage lagoons and mass culturing techniques will be necessary to successfully accomplish tertiary treatment.

The literature contains sufficient data to substantiate the fact that algae are capable of living in and extracting nutrients from a sewage substrate. This essentially means that tertiary treatment becomes a problem of culturing a sufficient mass of algae to produce a substrate nutrient limiting situation.

Studies concerned with mass cultures of algae usually maintain a high nutrient concentration so that growth is independent of this potential variable. If this is done then the two generally acknowledged variables affecting growth are light intensity and temperature. For tertiary treatment the nutrient concentration of the substrate also becomes a variable.

For successful nutrient extraction the nutrients must be incorporated into cellular material. This means that nutrient removal will be a function of the rate of production of algal cells. Productivity or yield is usually used to indicate the rate of production of cellular matter. Culture productivity is a function of the two related factors; growth rate and culture density.

At a constant nutrient level and constant temperature, growth rate is a function of light intensity. In general as light intensity increases growth rate increases up to the value of light saturation. Light intensity can be varied by changing

the intensity of the source or by altering the density of the culture. For all practical considerations growth rate will be a maximum when all algae are exposed to an intensity equal to the saturation value. As culture density is increased, the portion of algae receiving saturation intensity will decrease and consequently so will the culture growth rate. Thus, in a normal environment increasing culture density will result in decreasing culture growth rate.

The interdependency of growth rate and culture density is a distinct disadvantage in the use of algal cultures for tertiary treatment. Since nutrient extraction is directly proportional to yield, the most desirable condition would be to have the maximum culture growth rate occurring in conjunction with the maximum culture density. Such a condition would probably result in the optimum yield of the culture. As indicated previously, however, this condition is impossible under a normal culture environment. The literature indicates, however, that high growth rates can be obtained if algae are exposed to high intensity light for short periods of time. The intensity used is normally in excess of the saturation value. The reaction of algae to intermittent exposure of high intensity light is called the "Flashing Light Effect."

Most of the data in the literature pertaining to intermittent light exposure has been obtained by mechanically producing intermittent incident light in a low density culture. There is only sparce information as to whether turbulent stirring of a very dense culture will produce the same effect. Theoretically, moving an algal cell from the dark portion of

a very dense culture into the high intensity light portion of the culture then back into the dark portion should have the same effect as intermittent incident light on a low density culture. If the culture growth rates of very dense cultures can be increased by induced turbulence then a new plateau in yields can be expected. Accompanying this high productivity will also be a new high in the rate of nutrient extraction.

It appears that results from the flashing light effect can only be expected when the culture incident light is of very high intensity. There is an almost complete absence of data in the literature on the influence of induced turbulence on cultures subjected to low incident light intensity.

The preceding discussion indicates the dependency of culture productivity on light intensity when nutrient concentration is adequate and temperature constant. As previously noted, however, for tertiary treatment the nutrient concentration of the substrate also becomes a variable.

The influence of nutrient concentration on the continuous yield of an algal culture has received little attention. Lagoon studies have given this problem essentially little or no consideration because of the secondary role of the algae. Investigations of algal mass culturing techniques have, however been concerned to some degree with the ramifications of nutrient concentrations. This concern has been primarily related to establishing nutrient concentrations such that growth is independent of substrate quality.

To properly evaluate the potential of tertiary treatment utilizing algae as a biological extraction device requires

that the effect of a nutrient poor substrate on algal culture productivity be known. This knowledge is necessary because the purpose of such a treatment process would be to reduce the nutrient level of sewage to the extent that it would not be able to support algal growth. The problem then is, can an algal culture be maintained in a reasonably healthy state, while reducing the nutrient content of its substrate to such a degree that the substrate cannot further support an active algal growth?

An additional factor in this regard relates to the abilities of starved and non-starved algal cells to extract nutrients. Starved cells being defined as those that have been growing in a nutrient deficient environment. Information of this nature is necessary in order to decide what type of system will give the best overall nutrient extraction. That is, a system in which growth takes place in a continually poor substrate or a system which provides facilities for varying the substrate quality.

In accordance with the nature of the problems previously outlined, the literature was evaluated with respect to information pertaining to the influence exerted by induced turbulence and substrate nutrient concentration on algal culture productivity. This evaluation indicated that more experimental data in these two areas might prove of considerable value in determining the practicality of using algal cultures as a method of tertiary sewage treatment.

<u>Proposed Investigation:</u>

As a result of the literature search, the following studies were outlined: (1) using secondary sewage treatment plant effluent as a nutrient source, determine the influence of substrate nutrient concentration on the growth rate, productivity and nutrient extraction of an algal culture of constant density, and (2) determine the influence of induced turbulence on the growth rate of algae grown in batch cultures.

It is believed that these investigations are necessary to the eventual solution of the engineering problems relating to the use of algal cultures in the tertiary treatment of sewage. It is further hoped that the results of these studies will point to other pathways of investigation.

I. GENERAL

Algae

Algae is the common name applied to that heterogenous group of simple plants, classified under the sub-kingdom Thallophyta, and which are able to carry on photosynthesis. Algae are either photolithoautotrophic or photolithoaetero-trophic organisms whose natural habitat is water (1). The water environment of these organisms may vary widely in physical, chemical and biological characteristics. The ability to carry on photosynthesis differentiates the algae from the bacteria and fungi. The algae, by being able to synthesize most or all their food, occupy a very distinct role in an aquatic community. They can convert the inorganic materials entering a water into organic cellular material. This organic material serves as a food source for other members of an aquatic community. (2) (3) (4)

Under normal conditions a water ecosystem tends to receive and produce more organic material than it can consume or eliminate. There is, therefore, over a period of time an increase in nutrients and sediments due to inflow, decomposition and settling. The overall process producing the increase in nutrients and sediments is called eutrophication. The process is usually most noticeable in ponds, stream pools, dams and lakes. Eutrophication is a natural occurrence, initiated at the time of formation of a relatively quiescent body of water and continuing until the body of water is rendered extinct. The natural rate of eutrophication occurring in a

water basin is not usually considered as being alarmingly high. The addition of undue quantities of organic or inorganic substance can, however, produce a significant increase in the rate of eutrophication. Such an addition, intentional or unintentional, is called fertilization (2). Hasler (5), referring to lakes, states "That unwitting fertilization not only causes nuisances, but hastens the extinction of the lake by encouraging more rapid filling." Fertilization can be responsible for increasing the rate of eutrophication, by being of such character that it increases, directly or indirectly, the food content of the body of water. The addition of organics can result in a direct increase in the quantity of food available. Introduction of inorganics primarily nitrogen and phosphorus usually increases the food level indirectly. The presence of inorganics usually results in an increase in the components of the food chain initiated by algae. The algae can convert the inorganics into energy sources for other members of the aquatic community.

Of the various types of fertilization that may take place, the addition of secondary sewage treatment plant effluents is of most concern to Sanitary Engineering. The inorganic nitrogen and phosphorus contained in secondary treatment plant effluents is thought to be the reason for its stimulatory effect on algae. Lackey (6) points out that in addition to nitrogen and phosphorus sewage also contains hormones, vitamins and trace elements which are necessary for algal growth. He lists reoxygenation, mineralization and production of a food chain as benefits derived from algal

growths stimulated by sewage fertilization. Algal toxicity, asthetic harm, tastes and odors and increased BOD are listed as detrimental effects resulting from such algal growths.

Lackey concludes that the detriments of "admission of sewage treatment plant effluents to receiving waters may seem to outweigh the benefits" and that, "Perhaps what is needed is treatment so complete as to remove all nitrogen, or all phosphorus." Lackey (7), in a study of Wisconsin lakes, concluded "That lakes receiving domestic sewage or biologically treated domestic waste to any marked degree will never lack for phosphorus."

That sewage can serve as a suitable growth substrate for algae has been well established by Witt (8), Ludwig (9) and others. It appears, however, that treated sewage is a somewhat better substrate than raw sewage. Sawyer (10) found that the nutrients in treated sewage were more readily available than those in raw sewage. In addition Reid (11) discerned that greater algal growths occurred in treated sewage stabilization ponds than in raw sewage lagoons.

Chick (12) in 1903 isolated an alga which she named Chlorella pyrenoidosa from sewage. This alga showed a striking preference for ammonia and ammonical forms of nitrogen. Chick surmised that the preference of this alga for ammonia was the reason for its occurrence in sewage. Witt (8) presents a summary table listing various types of algal environments and the genera of algae most often reported to exist in each habitat. He indicates that Chlorophyta is the most widely represented phylum in sewage affected habitats. Hasler (5)

and Bartsch (13), however, point out that the seasonal appearance of members of the phylum Cyanophyta and in particular Oscillatoria rubescens are highly indicative of sewage pollution in lakes.

Analysis of algal cellular material shows that carbon, nitrogen and phosphorus represent 50 to 70 per cent, 1 to 11 per cent and 1 to 1.5 per cent respectively of the total dry weight of algae. Oxygen and hydrogen account for 24 to 34 per cent of the dry weight (14). Assuming that oxygen and hydrogen are not likely to be in short supply; it is then apparent, that the supply of carbon, then nitrogen, and finally phosphorus might determine the extent of algal growth in a water. Ketchum (15) concludes, however, that only rarely would the production of plant material in a natural aquatic habitat be limited by the inorganic carbon supply. The same thought cannot be applied to sewage lagoons and mass cultures of algae (8)(16) (17). In mass cultures carbon in the form of CO_2 is supplied to the cultures (18). In any case if carbon is present or is supplied in sufficient quantities so as not to limit growth then nitrogen and phosphorus are left as the most probable limiting factors. Bartsch (13) indicates that the reason, for reduced algal growths in surface waters during the most favorable growing season, is that both nitrogen and phosphorus are in limited supply in such waters that are unpolluted. Sewage contains relatively large quantities of nitrogen and phosphorus (19). It is, therefore, not surprising that waters fertilized with sewage should produce better algal growths than nonfertilized waters.

The relative abundance of nitrogen and phosphorus may be the cause of the type and quantity of algae present in an environment, but other factors should not be ignored. Lack of knowledge demands that consideration be given to micro-nutrients, vitamins and other growth factors when attempting to discern what causes a particular algae to grow in a given environment. The most important factor is the presence or absence of the biochemical process photosynthesis. Whether the physical and physiological differences of the various biological forms embodying this process are critical in terms of practical applications has not as yet been definitely determined. For treatment of domestic sewage photosynthesis is the required basic reaction.

The synthesizing of organic matter is a combination of photosynthesis and metabolism. The end product of these two activities is more organisms and some excretory materials. The production of organisms requires chemical elements supplied by a growth substrate which can be sewage. The nutrients (chemicals), principally nitrogen and phosphorus, which are used to synthesize cellular material are removed from the substrate, with the consequence that as growth continues the substrate becomes increasingly deficient in the required nutrients. This process is not basically different from the types of biological methods now used for sewage treatment.

The potential of algae to extract nutrients from sewage, as evidenced by Chick's work in 1903, was not further investigated for several years. The reason for this might have been that either the effect of sewage on receiving waters was not

fully appreciated or that the effect was not considered critical (20). Not until the relatively recent interest in sewage lagoons and stabilization ponds had algae received much consideration as sewage treatment organisms. Intensive studies have been conducted on the various factors of oxidation ponds (9)(16)(21)(22)(23)(24)(25)(26)(27). Pipes (28) presents a summary of the biological factors associated with stabilization ponds. However, not until the most recent studies of Witt (8), Bogan (29), Bush (30), and Fitzgerald (31) had algae been considered only with respect to stripping nutrients from sewage.

Although, the problems and potentials of algae as related to Sanitary Engineering have not received excessive attention, algae have not gone unnoticed. In the fields of plant physiology, plant nutrition, taxonomy and food and feed production, algae have been an often used and investigated group of organisms. It is from these scientific fields that Sanitary Engineering can and must draw knowledge in order to expedite the determination of the most advantageous role for algae in sewage treatment (1)(32)(33)(34).

Physical Factors Influencing Algal Growth

Photosynthetic growth of algae has been shown to be affected by a number of chemical, physical and biological factors. Photosynthetic growth being that growth which takes place utilizing energy derived from radiant energy and carbon supplied in the form of carbon dioxide or one of its hydrated

forms. The most influential physical factors presently recognized are light quality and intensity, temperature and stirring of the culture.

Influence of Light on Algal Growth:

The wave length (quality) and the intensity of light both affect the growth of algae. The visible range of the light spectrum, 400 to 700 millimicrons wave length, has been found to be the best quality light for photosynthesis. Emerson and Lewis (35) using Cholorella pyrenoidosa and Tanada, as reported by Fogg (1), using Navicula minima determined the photosynthetic efficiency of the respective organisms as a function of wave length. Photosynthetic efficiency is the number of molecules of oxygen produced per quantum of energy absorbed. Except for a slight decrease at about 480 millimicrons the photosynthetic efficiency is relatively independent of wave length in the range from 420 to 680 millimicrons. Above and below these two limits the efficiency drops off rapidly with increasing or decreasing wave lengths. Investigators have not been able to resolve their differences as to what values represent the average and maximum values of the quantum efficiency for algae. The maximum efficiency reported is 4 quanta per molecule of oxygen produced, while more commonly recorded values fall between 8 and 12 quanta per molecule.

McLeod and McLachlan (36) determined the sensitivity of several algae to ultra-violet light of 253.7 millimicron wave length. The degree to which the ultra-violet light impaired

photosynthesis was found to be dependent on the growth phase of the algae. Low sensitivity was exhibited by the algae when in the lag and stationary growth phase. The maximum sensitivity was observed during the logarithmic growth phase. Gerloff et al (37) obtained bacterial free cultures of Nostoc by irradiating the cultures for 20 to 30 minutes with ultra-violet radiation of 275 millimicron wave length. The longest exposure time killed both algae and bacteria. Subcultures of the cultures with an intermediate exposure time produced algae but no bacteria. The short exposure time did not totally kill either the algae or the bacteria.

The overall efficiency of algal utilization of absorbed light is the ratio of the energy contained in the algal cells to the useable radiant energy absorbed by the culture while producing the algal cells. Krauss (38) reports that the overall efficiency may range from 35 to 70 per cent in red light to a maximum high of 45 per cent in sun light. He suggests, however, that 20 to 25 per cent appears to be the best estimate for calculations. Myers (39) assuming that 40 per cent of the sunlight is available for photosynthesis found a 23 per cent overall efficiency for Chlorella. A value of 5.8 kilo-calories per gram of Chlorella produced was used to determine the efficiency.

Light intensity, with respect to algal growth, has at least three critical values: (1) the compensation intensity which is the intensity just sufficient to allow the organism to maintain its basic metabolism using photosynthetic products, (2) the intensity which produces the maximum growth rate and

is designated as the saturation intensity and (3) the intensity which is sufficient to inhibit growth (40).

The compensation intensity is rather difficult to determine and in many instances can only be estimated by extrapolation of data. For Chlorella the compensation intensity is reported as being 24 foot-candles (38)(41).

Sorkins (42) reports that the saturation intensity for several Chlorella species is 500 foot-candles, but one species had a saturation value of 250 foot-candles and another had a value of 1400 foot-candles, all values were determined at 25°C. Sorkins (43) determined that the thermophilic Chlorella Tx 7-11-05 has a saturation intensity of 1400 foot-candles at 39°C. Because of the dependency of growth rate on temperature, the saturation light intensity should always be accompanied by the temperature at which it was determined. If this temperature is not given then the light intensity reported should be used with caution.

The intensity of light that produces inhibition is not well defined, but it seems to vary with the genus and species of the algae. Krauss (44) indicates that inhibition occurs between 1000 and 2000 foot-candles. Bartsch (45) finds the range of inhibition to be between 1000 and 4000 foot-candles. Phillips and Myers (41) found that growth rate increased slightly from that at saturation intensity up to at least 5000 foot-candles. Tamyia (46) finds that inhibition intensities were temperature dependent. The lower the temperature the lower the intensity required for inhibition.

Efficiency of light utilization, culture growth and

culture productivity are all interrelated. The efficiency indicated here is not necessarily the same as the overall efficiency of light utilization by the algae. The efficiency of incident radiation use is a method of rating the quantity of incident radiation absorbed and to distinguish it from the other efficiencies it might be referred to as the absorption efficiency. As culture density increases for a constant depth culture or as the depth of a constant density culture increases the amount of light attenuated increases. Light attenuation by algal suspensions tends to follow Beer's Law, therefore, theoretically it is impossible to attain 100 per cent absorption but in practice this value is normally considered attainable (16). A discussion by Myers (40) suggests that when maximum absorption efficiency occurs in an algal culture very little culture growth will result. The problem then is one providing as many cells as possible with the light required for maximum growth while accomplishing, as nearly as possible, total attenuation of the light by the culture. This problem has prompted reconsideration of the studies of intermittent illumination of algae by Phillips and Myers (41), Kok (47) and Semenenko (48). All of these investigators were interested in determining whether algal utilization of light was a function of intensity or of intensity and duration. Kok exposing a dilute algal culture to flashing incident light found that within a certain limit algal light utilization was a function of the intensity of exposure and duration of exposure. Using a light source of approximately 7000 foot-candles it was found that a light exposure of one to three milliseconds

with a dark period of about 10 milliseconds was required to produce photosynthetic activity equal to that attainable in continuous illumination of the same average energy per unit time. Studying exposure times of 1, 4, 17 and 67 milliseconds to a light source with an intensity of approximately 5700 foot-candles Phillips and Myers found some degree of light integration by a dilute algal culture for each exposure time. Light integration indicates the ability of algae to grow at a given rate when exposed to a given average light intensity, when the light intensity is obtained by sucessive light and dark periods. The one millisecond exposure time with various dark periods up to 41 milliseconds gave a growth curve identical to that in continuous illumination. At the longer exposure times integration was not so complete, but was still considered significant. As the degree of light integration decreased the growth rate for an average light intensity per unit time was less in flashing light than in continuous light. At zero integration growth rate becomes a linear function of the average intensity of the flashing light per unit time. For the condition of no integration growth occurs at the maximum rate in the light, but no growth takes place during the dark periods.

Inducing algal growth through intermittent illumination is based on the concept that photosynthetic growth depends on two basic reactions. One reaction is the fixing of radiant energy in a chemical form. The second reaction is the formation of a carbon dioxide complex. The second reaction is assumed to take place in presence or absence of light, while

the first reaction takes place only in the presence of light. The products of photosynthesis are the result of a reaction between the products of the two basic reactions. It is also important to note that both reactions are presumed to be independent of each other but that either reaction can determine the rate of photosynthesis. It is thought that the chemical compound or compounds entering in the photochemical reaction (fixing of radiant energy) are in limited supply and that it or they are recycled back to capture more radiant energy after having reacted with the carbon dioxide complex to form photosynthetic products. The fundamental concept of intermittent illumination is, in the presence of high intensity light, to rapidly convert the photochemical reaction to the product form, then in the dark the photochemical product and the carbon dioxide complex react somewhat more slowly to produce photosynthetic products. The maximum light exposure time is that required to just drive the photochemical reaction essentially completely to the product form. For intermittent illumination to be functional, the intensity of light should be such that the time required to drive the photochemical reaction to the product form is instantaneous as compared to the reaction time of the photochemical product and the carbon dioxide complex. As the rate of formation of photochemical product approaches the reaction rate between the product and the complex, the quantity of photosynthetic products formed in the light will increase while those formed in the dark will decrease due to lack of photochemical product. When the two rates are equal photosynthetic products will be formed only in the light, this

being the condition of zero integration mentioned by Phillips and Myers. The optimum dark time is that time required for all the photochemical product to be consumed in the subsequent reaction. Because of the cyclic nature of the chemical compound involved in the photochemical reaction a dark time shorter than optimum will result in inefficiency of the process. A dark time longer than optimum will, again because of the cycling process, result in periods of no photosynthetic activity and consequently a less than maximum use of the mechanism of photosynthesis (35).

Davis et al (49) approached the problem of intermittent illumination by another method. Instead of using dilute cultures and an intermittent incident light, a denser culture subjected to a high degree of turbulence was exposed to continuous illumination. The density of the culture was initially adjusted so that 99 per cent of the light was absorbed in 1/10 the depth of the culture. A rotor located in the culture chamber, with a wall clearance of 1/4 inch, was turned at various speeds to create different degrees of turbulence. The turbulence moved the algal cells into and out of the light, producing the same effect as flashing the light source. Turbulence produced a culture growth rate 70 per cent greater than that attainable without turbulence.

Myers and Graham (50) find that "For mass culture under sun light illumination, attempts to increase yield become attempts to minimize or circumvent the limitations due to light saturation." Rather than attempting to utilize the concept of intermittent illumination to resolve this problem

these investigators sought to provide wider distribution of the incident light. Solid lucite cones and hollow glass cones placed base up in the culture were used to distribute the light throughout the depth of the culture. The conical surface area of the cones was about 10 times the base surface area. The cones more than doubled the yield of the cultures as compared to cultures not equipped with the cones. Also of significance is the fact that the maximum yields occurred at higher culture densities than in cultures without cones.

Effects of Temperature on Algal Growth:

Photosynthetic activity of algae, as previously indicated, involves at least two general steps. The photochemical step is generally considered to be insensitive to temperature within the range of temperatures that is not destructive to some component of the system. The carbon dioxide fixation step is temperature sensitive, experiencing an increasing reaction rate with increasing temperature. If light is limiting the rate of photosynthesis, then temperature changes will have little effect on the rate. And, in addition, if the carbon dioxide supply is insufficient the overall rate of photosynthesis will probably remain unaffected by temperature changes. Only when the intensity of light and the concentration of carbon dioxide are sufficient will the rate of photosynthesis be influenced by temperature variations (51). The Q_{10} value for photosynthesis is approximately 2 between 20° and $30^{o}C$. Below $20^{o}C$ it increases and above $30^{o}C$ it decreases. The value also seems to vary from species to

species (52).

Witt (8) lists the commonly accepted temperature ranges for the various phylum of algae. Diatoms are considered to like cooler temperatures, green algae slightly warmer temperatures and blue greens require the highest temperatures. Most of the algae which have been used for experimental study have optimum growth rates between 20° and 30° C. Unfortunately it is very difficult to maintain temperatures of 20° to 30°C in dense outdoor cultures using sunlight as an energy source (53). This fact has lead to the search and isolation of several so called thermophilic green algae whose optimum temperature is above the 20° to 30°C range.

Sorkins and Myers (53) isolated a *Chlorella* strain growing in warm waters. The alga *Chlorella* *Tx7-11-05* has an optimum temperature for growth of 39°C. The maximum temperature at which growth will take place is 41°C. Holton (54) reports on the isolation of the bluegreen alga *Hapalosiphon luminosus* from a hot spring. This alga can maintain its maximum growth rate up to 50°C. Dyer and Gafford (55) have isolated a blue green from the hot springs in Yellow Stone National Park. The alga has been tentatively identified as *Synechococcus lividus*. The maximum growth rate for the organism occurs between 50° and 55°C. Katz and Myers (56) studying the blue green algae *Anacystis nidulans*, *Anabaena variabilis* and *Nostoc muscorum* found that these algae had optimum temperatures of 41°, 35° and 32.5°C respectively. These values are considerably higher than the optimum temperatures of the green algae most commonly used for mass culture studies. Tamyia (57) lists the temperature ranges for the

thermal classification of algae as: (1) psycholophylic 10° to 15 C, (2) mesophilic 5° to 35°C, and (3) thermophilic 35° to 42°C.

The temperature range for the thermophiles is obviously too low in view of the already mentioned findings of Holton, Dyer and Gafford. Tamyia (18) makes a comparison of the average growth rates of a mesophilic and a thermophilic Chlorella species for different months of the year. The mesophilic species experienced growth during all months while the thermophilic species grew only during the warmer months. Even in the summer months the growth rates of the two species were about the same.

Influence of Stirring on Algal Growth:

As noted in the discussion of the effects of light on algal growth Davis (49) seemingly obtained the condition of intermittent illumination by inducing a high degree of turbulence into an algal culture. Many other investigators have mentioned but not substantiated that such a condition exists to a slight degree in any well mixed dense culture. Ippen (58) made a study to determine the relation of intermittent illumination to turbulent flow in a channel.

In general the stirring, mixing or shaking of algal cultures has not received much attention beyond recognition that some motion is required to prevent the algal cells from settling. Brannon and Bartsch (59) found that agitating their algal cultures for 5 minutes out of each hour increased both the density of the cultures and the efficiency with which the

cultures used the substrate. They conclude that the agitation enhanced algal growth by facilitating release of auto spores, providing more rapid gas diffusion and preventing accumulation of deleterious substances around the algal cells. Pratt (60) shows a difference in growth rate and in final culture density, between cultures stirred twice a day and cultures stirred continuously. Zehender and Gorham (61) found that shaking a culture continuously increased the yield approximate 200 mg/l over that of an unshaken culture. Improved gas exchange brought about by the shaking is credited with producing the increase in yield. Gotaas et al (16) by inducing periodic vertical mixing improved algal growth in experimental lagoons. The present status of culture mixing is summarized by Cook's statement (62) that "This (turbulence) serves three primary purposes: (1) To keep cells in suspension and, therefore, at uniform density; (2) To maintain equilibrium conditions between the necessary nutrients and each cell, and (3) To remove cells under high light intensity conditions to lower light intensities to prevent their injury and thus maintain them at maximum growth rates."

Chemical Factors Influencing Algal Growth

The major portion of investigations dealing with algae have dealt in some aspect, with algal nutrition. The early studies of algal nutritional requirements were necessary in order to be able to determine suitable artificial nutrient

substrates which would permit culturing of these organisms. More recent studies have been concerned with how to provide nutrients in the quantities required for mass culturing of algae. Studies have also been made of the inhibitory action that algae exhibit toward each other and bacteria. Evidence is also presented in the literature that at least one algal species is inhibitory towards itself.

Autotrophic growth of algae requires, for the most part, only inorganic nutrient substances. Heterotrophic growth requires the same chemical elements as autotrophic but the form in which they are supplied has to be of the organic chemical form. Probably the most satisfactory definition of nutrients is that they are the chemical elements required for algal growth. These nutrients can be roughly divided into macronutrients and micronutrients. Such a break down is based on the relative quantities of the nutrients required for algal growth. The classification of nutrients into the two groups will vary, to some degree, depending on the type of algae. There seems to be general agreement, however, that elements O, H, C, N, and P are macronutrients while the remaining nutrients required by an algae for growth would fall into the micronutrient classification. Ketchum (15) lists four types of nutritional requirements: (1) absolute nutrient requirement indicates that growth cannot take place without the particular nutrient, (2) normal requirement, the quantity of nutrient in actively growing cells with no nutrient in limited supply and (3) minimum requirement being the quantity of nutrient contained in a growing cell when the particular

nutrient is limiting.

Pringheim (63) has isolated many algae in pure culture and has devised substrates for them. Many algal nutrition studies have been carried out to determine the optimum nutrient concentration for growth or photosynthesis. The optimum conditions for these two functions may or may not be the same. Chu (64)(65), Rhode (66), Osterlind (67), Gerloff et al (68)(69) and Katz and Myers (53), have determined optimum nutrient conditions for several algal species. Myers (70) studied the influence of varying the media concentration as a whole rather than varying the concentration of each individual nutrient. He used a constant density culture for this study.

In order to produce a more comprehensible presentation of the literature related to the chemical factors influencing algal growth the various chemical parameters are discussed individually.

Carbon:

The major portion of the energy used in metabolism comes from carbon compounds or carbon containing compounds. Carbon fixation is commonly used to denote carbon dioxide reduction resulting from the overall process of photosynthesis. Carbon so reduced comprises the major portion of the carbon and carbon containing compounds necessary for algal metabolism Carbon is, therefore, fundamental to the photosynthetic growth of algae. This element exists in both the organic and inorganic form in the natural habitat. For the growth of algae, as for the growth of any organism, an element must not

only be present in the environment but it must also be in a biochemically available form before it can be assimilated. The question of availability of different carbon forms has received considerable attention.

Inorganic carbon exists in water as carbon dioxide, bicarbonate and carbonate. Since these three forms are in chemical equilibrium with each other, it is impossible to obtain a solution containing only one of the forms. The relative quantities of the three forms existing in a given condition depends on the pH and total concentration of inorganic carbon. The interrelation between inorganic carbon forms and pH has made it difficult to evaluate the effect of the various forms of carbon on growth.

Osterlind (71) using Scenedesmus quadricauda found optimum growth occurred at pH 8.1. At this pH the CO_2 concentration is almost zero and all the carbon is essentially bicarbonate. At a constant bicarbonate concentration an increase of either carbon dioxide or carbonate limited the algal growth. Carbonate inhibition appeared to start at about 4 micromoles per liter. Osterlind (67) using Scenedesmus produced the same growth with 80 micromoles of carbon dioxide per liter as with 10 to 20 micromoles of bicarbonate per liter. Osterlind (72)(73) studying cultures of Scenedesmus and Chlorella concludes that algae can be placed in two different groups depending on the forms of inorganic carbon they can assimilate. One group would include those algae such as Scenedesmus which can utilize both carbon dioxide and bicarbonate. The other group would be composed of those algae

similar to Chlorella in that they can only assimilate carbon dioxide. The method and rate of carbon dioxide assimilation was found to be essentially identical. Further investigation of Scenedesmus revealed that a 10 day old culture had lost its ability to assimilate bicarbonate. Carbon dioxide assimilation by Scendesmus starts immediately on exposure to light, whereas, a lag period of about 10 minutes occurs before bicarbonate assimilation will start. This fact has led to speculation that some component requires photoactivation before bicarbonate assimilation can proceed (74). Bicarbonate assimilation is favored by low pH values and low carbon dioxide concentrations (75). Nielsen (76) using dilute suspensions of Chlorella pyrenoidosa demonstrated that growth was independent of carbon dioxide concentration when the concentration was above 0.01 to 0.03 per cent. There is evidence that bicarbonate can penetrate the Chlorella cell and can under special conditions be used in photosynthesis. It is doubtful however, that this source of carbon is significant under normal conditions of photosynthetic growth of Chlorella (77).

Witt (8) cultured Scenedesmus at pH 8.3 using a 5 per cent carbon dioxide in air mixture to maintain the pH. The carbon supply was shut off and growth rate immediately dropped to a lower, but constant value. Twelve hours after stopping the carbon feed the growth rate had increased until it was equal to that at pH 8.3. After this increase the growth rate decreased curvilinearly until it reached zero at pH 10. The organism concentration during the experiment

was 1500 per cubic millimeter. In another experiment using 12,000 Scenedesmus per cubic millimeter carbon dioxide was fed to the culture until growth stopped. The pH at this point was 5.5. After growth had ceased the carbon dioxide supply was shut off and growth allowed to proceed. Growth continued for about 35 hours and growth rate was constant for approximately 18 of the 35 hours. Growth ceased at pH 11. This experiment showed no dip in growth rate at pH 8.3 as was previously described. Witt indicated that the heavy concentration might have masked the dip in growth rate at pH 8.3.

The general conclusion is that about 1,000,000 Scenedesmus per milliliter can be supported by secondary sewage effluents without use of a supplementary carbon source. Ludwig (9) finds that the addition of carbon dioxide would have increased the yield of Euglena cultured in sewage from 0.08 to 0.26 grams per liter per day. Gotaas (21) obtained maximum growth of algae with an 0.5 per cent carbon dioxide concentration. He suggests that "carbon dioxide fortification may be a feasible method of increasing algal and oxygen yield if an economical source of carbon dioxide were available."

The concentration of carbon dioxide required to maintain optimum growth seems to be from 0.01 to 0.03 per cent (18) (78). Higher concentrations are normally supplied in order that the minimum requirement will exist in the immediate environment of the organism. Ketchum (15) indicates that probably a 3 to 5 per cent carbon dioxide in air mixture

bubbled through a culture is necessary to maintain the required concentration. Krauss (38) reports high concentrations of carbon dioxide to be toxic, but this seems to be open to question (18). Witt (8) finds that the concentration of carbon dioxide is probably incidental as long as it is sufficient. In addition he speculates that the best results would be obtained if the supply and demand of carbon dioxide were balanced. Golueke (79) found optimum carbon dioxide concentrations to be between 1 and 3 per cent with some inhibition taking place at 4 per cent. His test results also show that carbon dioxide concentrations of 2 per cent or greater favored *Scenedesmus* growth while concentrations of 1 per cent or less favored *Chlorella* growth.

The inorganic carbon supply requirements for blue green algae appear to be the same as for *Scenedesmus.* The photosynthetic quotients for three blue green species were found to be the same whether carbon dioxide or bicarbonate was the carbon source (80). Both the blue green algae (*Coccochloris peniocystis* and *Microcystis aeruginosa*) experienced maximum growth at pH 10 (68)(69)(81).

Organic carbon sources are quite plentiful in the natural environment (82). Interest is focused on whether algae can use organic carbon sources to supplement the inorganic carbon or if there is ever a preference for organic carbon during photosynthetic growth. Pipes (83) finds that when carbon dioxide is limiting the rate of photosynthesis, the presence of compounds such as sugars, organic acids, amino acids and some nitrogen compounds will increase the photosynthetic rate.

He also points out that some of the organic compounds of sewage will accelerate growth even in the presence of sufficient carbon dioxide and concludes that some algae can use sewage organics directly as a carbon source. Pipes and Gotaas (84) used sterile cultures to determine if Chlorella pyrenoidosa could assimilate organic carbon from the substrate. The extent of organic carbon assimilated was inversely proportional to the amount of carbon dioxide supplied. There was also some indication that organic carbon assimilation was dependent, to some degree, on growth rate.

The results of Briston Roach's investigation of the effect of various organic substances on the growth of Scenedesmus costulatus var. chorelloides are summarized by Fogg (1). Algae vary from those having very specific organic substrates to those that are able to assimilate a wide variety of organic compounds. Some flagellates utilize only acetate and are found in abundance in the presence of decomposing organic matter such as sewage. In general phototrophic and chemotrophic assimilation, under conditions of photosynthetic growth, are dependent on each other. When illumination and carbon dioxide are sufficient to maintain maximum growth the addition of organic substances will not increase the rate. With a culture exposed to either or both a light intensity slightly limiting or insufficient carbon dioxide the addition of an organic carbon source can increase the growth rate up to its maximum. Addition, however, of glucose to a culture of Chlorella pyrenoidosa subjected to rather severely limiting light produced a growth nearly

equal to that attainable with glucose in the dark(1). Studies of the blue green algae Analystis nidulans, Anabaena variabilis and Nostoc muscorum, showed that organic carbon sources would not support the growth of these algae (56). Brannon and Bartsch (59), report that the reproduction of Chlorella vulgaris and Coccmyxa simplex was stimulated by the addition of several different monobasic organic acids to the substrate. The growth of Mesothenium caldanorum, however, was unaffected by the acids.

The inorganic carbon content of sewage is determined by alkalinity determinations. The organic carbon content of sewage is normally evaluated in terms of the biochemical oxygen demand (B.O.D.), however, the chemical oxygen demand or C.O.D. may be used in some instances. The B.O.D. value of sewage varies from several hundred parts per million in raw sewage to a few parts per million in the effluents of secondary sewage treatment plants. During the sewage treatment process the organic carbon is for the most part oxidized to carbon dioxide by bacterial action. A portion of the carbon dioxide so produced escapes as a gas while the remainder resides in the sewage as free carbon dioxide or one of its hydrated forms. There does, however, seem to be sufficient correlation between algal growth in sewage and sewage B.O.D. to make B.O.D. a useful parameter for culture growth calculations (16)(23).

Nitrogen:

The nitrogen in algal cells is usually present in the

form of amino acids and protein. Protein has been indicated as possibly being the most important single constituent of protoplasm. In the natural environment nitrogen is present in the inorganic form as elemental nitrogen, ammonia, nitrite and nitrate. Organic forms of nitrogen such as urea, amines, amino acids, polypeptides and protein may also be found in the natural habitat. The protein content of algae has been found to vary from 9 to 58 per cent (85).

The form of nitrogen most readily assimilated by algae has been of great interest to investigators. For a considerable period of time it was generally accepted that nitrates were the most desirable form of nitrogen for algae. Intensive nutritional and physiological studies have prompted a re-evaluation of this concept. Syrett (86)(87)(88)(89) using nitrogen starved cells of Chlorella vulgaris has clarified the conditions associated with nitrate and ammonia assimilation. He found that ammonia nitrogen is assimilated about four times as fast as nitrate nitrogen. With ammonia assimilation amino acid production greatly exceeded protein synthesis while for nitrate the rate of formation of protein approximately equals the rate of amino acid production. Accompanying the assimilation of either nitrogen form there was a rapid disappearance of cellular carbohydrate. Carbon dioxide production increased significantly with the uptake of either form but oxygen consumption accompanying nitrate assimilation was lower than that for ammonia uptake. The assimilation of ammonia continued after nitrate uptake had ceased. In either case when uptake ceased there was still

intracellular polysaccharide remaining; however, addition of extracellular glucose initiated further nitrogen assimilation. Myers (90) discusses the relation between photosynthesis and nitrogen assimilation. It is suggested that nitrate utilization does not take place if ammonia is present. Witt (8) found that approximately 80 per cent of the ammonia nitrogen in the substrate was consumed before any uptake of nitrate was observed. Nitrogen starved cells when placed in a nitrogen sufficient substrate exhibit a high rate of nitrogen assimilation and do not start growing until the carbon to nitrogen ratio is restored to that of normal cells (1)(91)(92). Krauss (38)(93) contends that nitrogen must be present in a substrate if algal cells are to maintain their protein content. In the absence of substrate nitrogen the cellular protein content will continually decrease.

Nutrition studies have been directed at determining the effect of the nitrogen form and concentration on algal growth. Chu (64)(65) made an extensive study of the quantities of various forms of nitrogen required to produce optimum growth and to initiate growth inhibition. In this study most of the algae grew equally well with either ammonia or nitrate so long as the concentrations were optimum. The optimum range of nitrogen was narrower when supplied as ammonia than when nitrate was used. The range for ammonia nitrogen was from about 2 to 13 ppm while for nitrate the range was 0.3 to 17 ppm. The use of nitrate also favored a wider range of optimum phosphorus concentrations. Within the optimum ranges of nitrogen and phosphorus the algal growth was not influenced

by the ratio of nitrogen to phosphorus. Pennington (94) investigated the utilization of various forms of nitrogen by mixed cultures of algae and bacteria. Significant interchanges among the various nitrogen forms were found but control was not sufficient to allow concise conclusions to be made. Oxidation-reduction potentials were one of the parameters determined in this study.

A very inclusive study of various nitrogen compounds in relation to their availability to serve as nitrogen sources for algae was conducted by Ludwig (95). Thirty six different nitrogen compounds were considered. Most of these were nitrogen containing organic compounds. A *Chlorella* used as the test organism demonstrated better growth utilizing several of the organic nitrogen sources than with a nitrate source. Rhode (66) determined that at least 5 ppm of nitrogen was required for the maximum growth of *Scenedesmus quadricauda*. Osterlind (67) also using *S. quadricauda* found 0.1 to 5 ppm ammonia nitrogen to 10 to 50 ppm nitrate nitrogen provided good growth. Myers (96) used 6.25 grams per liter of KNO_3 without producing inhibitory effects, but notes that 10 grams of KNO_3 per liter is inhibitory.

Either type of nitrogen source, ammonia or nitrate, normally found in sewage or employed in artificial substrates will cause pH changes in the cultures. In cultures without pH control these changes can be quite dramatic. It is possible that pH changes have to some extent affected conclusions drawn from studies of the relative merits of nitrate and ammonia nitrogen sources. Pratt and Fong (97) considered

the relative suitability of nitrate and ammonia as nitrogen sources. The total nitrogen concentrations used was 350 ppm. Various ratios of ammonia and nitrate, from 100 to 50 per cent ammonia were used to supply the nitrogen. Chlorella vulgaris was the test organism. Ammonia utilization resulted in a pH drop while nitrate assimilation produced a pH rise. When ammonia and nitrate were supplied together the pH first dropped then rose indicating that first ammonia then the nitrate was consumed. During the course of the experimental runs the nitrogen concentration was reduced from 350 ppm to 2 to 5 ppm. Ammonium bicarbonate has been suggested as a nitrogen source which will prevent a pH decrease as the ammonia nitrogen is assimilated (81).

The suitability of using nitrite as a nitrogen source is not agreed upon. Ketchum (15) summarizing the literature concluded that nitrite has been found to be both toxic and non-toxic. Flaigg and Reid (98) evaluating the relative merits of ammonia, nitrite and nitrate as nitrogen sources found nitrite to be superior to the other two forms at low concentrations while the reverse was true at high concentrations. Ammonia and nitrate were essentially equal at all concentrations. Ammonia, nitrite and nitrate all exhibited an equal ability to support the growth of Microcystis aeruginosa if pH adjustment was used when ammonium chloride was the nitrogen source (69). The high nitrogen requirement of Microcystis aeruginosa was demonstrated by the fact that a nitrogen to phosphorus ratio of 75 to 1 provided maximum growth.

Evaluation of the influence of various concentrations of

nutrients on the growth of *Coccochloris peniocystis* revealed that the dependency of growth on nitrogen content could be divided into two regimes (68). At concentrations of 6.8 ppm nitrogen or less, growth was highly dependent on the nitrogen supply. Above 6.8 ppm nitrogen growth showed only a slight dependency on the concentration of nitrogen in the substrate. The maximum concentration of 20.4 ppm nitrogen supplied as nitrate did not inhibit growth. Gerloff et al (99) measured cellular nitrogen and algal yield as a function of nitrogen supplied in substrate of *Microcystis aeroginosa* cultures. The maximum yield was attained at an initial concentration of 8.0 ppm nitrogen. This concentration is not significantly different than that previously mentioned for *Coccochcoris peniocystis*. Maximum cellular nitrogen was not reached, however, until the initial nitrogen concentration was raised to 17 ppm. The nitrogen content of the cells above that required for maximum yield is referred to as luxury consumption. Algae having experienced this luxury consumption were able to grow for 12 days in culture with a nitrogen deficient substrate. During this 12 day period the number of cells doubled. Nitrogen content of the cells was not reported for the growth in the nitrogen deficient media. Fogg (92) maintains, however, that nitrogen cannot be stored.

Reid and Assenzo (100) found that the removal of nitrogen from a batch culture of attached algae took the form of a monomolecular reaction. The velocity constant of the reaction was 0.128 $\log_{10}$ units per hour at 50 C. This is roughly equal to 55 per cent removal per 2 hours. Using an artifi-

cial sewage substrate, Gour et al (101) found that the growth rate of Oscillatoria was directly related to substrate nitrogen content for short detention periods (high growth rates). Witt (8) concluded that at least a 24 hour dentention period would be required to provide acceptable nitrogen removal from secondary sewage plant effluents. The progressive removal of nitrogen through four oxidation ponds operated in series reduced the nitrogen concentration from 15 to 1 ppm (102). Lackey and Sawyer (4) report that three Wisconsin lakes removed 48, 51 and 46 per cent of the incoming nitrogen even though it was in the ratio of 1:3:2 respectively.

The value of nitrogen removal from sewage is complicated by certain algae which have the ability to fix atmospheric nitrogen. At present only the Cyanophycae have shown beyond a doubt that they can fix nitrogen. There has been much speculation that nitrogen removal would not prevent algal blooms due to the potential existence of the nitrogen fixers (103). Nitrogen fixation seems to take place only when other nitrogen sources have been essentially depleted (104). The extent to which the primary productivity of a water is influenced by algal nitrogen fixation is not well understood at this time.

Phosphorus:

The participation of phosphorus in the energy transfers included in photosynthesis and metabolism establishes the need for phosphorus in any growth substrate (105). Phosphorus normally occurs in the form of orthophosphate, polyphosphate

or organic phosphate in natural waters. Distinction is also usually made between the soluble and insoluble form of phosphate. The low degree of solubility of many phosphate compounds makes such a breakdown desirable.

Of the macronutrients phosphorus is the least plentiful, but fortunately or unfortunately, depending on the point of view, algae do not require large quantities of this element. Alkoly (106) determined that the minimum phosphorus content of an alga cell which would still support growth was 1×10^{-6} micro grams of phosphorus. The maximum content was 1.5×10^{-6} micro grams of phosphorus per cell. The addition of phosphorus to a phosphorus deficient culture resulted in a lag of about one day before growth started. For the diatom *Asterionella*, the minimum phosphorus requirement was found to be 5×10^{-8} micro gram atoms of phosphate (107). The cell phosphorus content for this diatom increased linearly with increasing media phosphorus concentration. The additional observation that the reproduction time of *Asterionella* was related in some way to cell phosphorus content was made.

Chu (64)(65) determined the optimum concentrations of phosphorus for several algae. He found that the range of optimum phosphorus concentrations to be dependent on the associated nitrogen source. The most favorable range of phosphorus was 0.018 to 17.8 ppm when nitrate was used as the nitrogen source. Using an ammonia nitrogen supply reduced this favorable range to 0.018 to 1.79 ppm phosphorus. Osterlind (67) reports that 0.1 to 10 ppm phosphorus did not noticeably affect the growth of *Scenedesmus quadricauda*.

Another study of Scendesmus quadricauda shows that better growth was attained with 1 microgram of phosphorus than 10 to 40 micrograms of phosphate per liter (66).

Chu (108) suggests that plants of the sea may use organic phosphate. This means that organic phosphate may be used either directly or converted to orthophosphate. This suggestion that algae use organic phosphate directly is supported by Odum (109). Benoit and Curry (111) report that 10 ppb of phosphorus is thought to be the critical level for blue green algae.

The uptake of phosphorus by phosphorus deficient cells is very similar to nitrogen assimilation by nitrogen deficient cells. Deficiencies are overcome by rapid assimilation in either the light or dark. Uptake in the light being the more rapid (1). Reid and Assenzo (100) ascertained that phosphorus removal, like nitrogen removal, from a batch algal culture could be represented by a monomolecular reaction. The velocity constant was found to be 0.103 $\log_{10}$ units per hour at 34°C. Secondary sewage treatment plant effluents were used as the substrate for these studies. Gerloff et al (68) relates that the growth of Cocochloris peniocystis was independent of phosphorus concentration in the media down to a level of 0.45 ppm of phosphorus. Growth in a media containing no phosphorus was 12 per cent of that obtained with 1.8 ppm phosphorus concentration. How or why such growth took place is not explained. The influence exerted by the initial phosphorus content of the media on the growth of Microcystis aeruginosa was explored by Gerloff et al (99). Maximum yield of this algae was attained with an initial phosphorus

concentration of 0.025 ppm phosphorus. The cellular phosphorus content did not reach a maximum until the initial concentration was raised to 0.80 ppm phosphorus. The maximum cellular phosphorus content was four times that at the point when maximum yield was obtained. This algae when transferred from a phosphorus sufficient to a phosphorus deficient media increased its density 300 per cent in 12 days.

Bogan et al (29) investigated the possibility of using algae to remove phosphate from secondary sewage treatment plant effluents. The algae forms considered were <u>Chlorella</u>, <u>Scenedesmus</u>, and <u>Stiglecolonium</u>. The removal of phosphate was accomplished through assimilation and coagulation by the algae. Removal was more a function of pH than of cellular material production. Removal of phosphorus by pH adjustment results in coagulation of the phosphorus on the exterior of the algae. How removal of this type compares with assimilatory removal from secondary sewage plant effluents was a function of temperature and light intensity. The uptake of both organic and ortho phosphate was found to take place at the same time. Witt suggests that maximum removal of phosphorus would require a reaction time of about 40 hours.

Other Nutrients and Growth Factors:

The macronutrients carbon, nitrogen and phosphorus are of major importance to the tertiary treatment of sewage. The role of micronutrients and growth factors can not, however, be ignored. The stumbling block at present is the lack of sufficient evidence of the absolute need of many of these

substances for maximum algal growth. Krauss (44) lists the micronutrients required by algae based on the present level of knowledge. Provasoli (111) summarizes the available information on the vitamin requirements of the algae. Of the 154 algae considered, 56 demonstrated no need for vitamins. Based on these findings, it is pointed out that the presence or absence of vitamins may have a profound effect on the algal community that can exist in a given environment.

Iron has been one of the most studied micronutrients and can probably be considered as representative of the problem of micronutrients. Myers (96) indicated that one serious problem of mass culturing is to provide adequate micronutrients and maintain their availability while not exceeding the toxic limits. The toxic concentration of iron appears to be relatively high (70). The relative insolubility of many ferric compounds however, complicates the task of maintaining iron in an available form.

Gerloff et al (68) compared the suitability of ferric citrate and ferric chloride for supporting algal growth. Using three day old solutions of each, growth was equal with 1.12 ppm iron, however, with an iron concentration of 0.03 ppm the ferric chloride supported only 75 per cent of the growth maintained by ferric citrate. A 20 month old solution of ferric chloride supported considerably less growth than the fresh solutions of ferric citrate or ferric chloride. When compared to ferric citrate the 20 month old solution of ferric chloride supported relatively more growth at low concentrations than at high ones. Harvey (112) studying marine

diatoms, found that they had in and on them quantities of iron greatly in excess of that required for growth. Ferric hydroxide was observed to be readily absorbed on the surface of the diatoms. The proposition is advanced that the iron absorbed on the cell surface is subsequently put in solution and taken into the cell. Goldberg (113) using radioactive iron shows that the marine diatom *Asterionella japonica* can assimilate only colloidal or particulate iron. Ionized or complexed iron is not bio-chemically available to the organism. The minimum cellular iron content needed to promote cell division was 10^{-7} micromoles per liter.

Greenfield (114) evaluated the toxic effects of most of the inorganic compounds which are normally considered as necessary micronutrients in algal culturing substrates. These compounds were categorized according to their ability to: (1) inhibit the dark reaction, (2) inhibit the light reaction or (3) inhibit both the light and dark reaction.

In order to maintain the micronutrients in solution and to supply them in concentrations sufficient to support extensive growth, yet not be toxic, chelating agents are regularly added to synthetic substrates. One of the earliest chelating agents employed was citrate (37). This is added to the nutrient solution to keep the iron in a more available form. Myers (96) applying the findings of Hunter used EDTA to sequester, at least to some degree, all the micronutrients. The addition of EDTA increased the yield of *Chlorella* cultures 100 per cent. Tanyia (46) suggests using nitroso R salt or nitrilo triacetic acid instead of EDTA. Nitroso R salt is reported to be more

economical than EDTA. Pravosoli (111) reports that the humic acids produced in nature undoubtedly provide a certain amount of chelating action in natural waters. An extracellular polypeptide produced continually during the growth of Anabaena cylindrica has demonstrated complexing properties (115). This polypeptide has exhibited ability to form complexes with copper, zinc, ferric ions, phosphate and some organics. Copper is normally toxic to Anabaena cylindrica at a concentration of 2 ppm, however, the presence of the polypeptide was found to raise the copper concentration required for toxicity to 16 ppm.

Growth Inhibition:

The occurrence and action of algal products which are toxic to the same algae, other algae and bacteria are not fully understood or appreciated. Probably the classic study of growth inhibitors is the one conducted on Chlorella vulgaris by Pratt (60)(116)(117)(118)(119)(120). The study was initiated by the observation that the size of the inoculum influenced the rate of growth of the algae but not the total attainable population. The smaller the inoculum the greater was the growth rate. The data shows that inoculums of 100 to 10 cells per cubic millimeter produced no highly significant difference in growth rate; inoculums, however, of 1 and 0.1 cells per cubic millimeter gave significant increases in growth rates. The techniques used in this initial study allowed Pratt to deduce that the inhibitory substance was transferred to fresh substrate within the algal cells. Additional study deomonstrated

that the inhibiting substance was released to the substrate during growth. Actively growing cultures were filtered and the filtrate used as a media for other cultures. The growth rate and maximum population were inversely proportional to the percentage of filtrate in the media and directly proportional to inoculum size.

The inhibitory substance of Chlorella has been designated as chlorellin. This substance has been found to be heat liable, oxidation resistant and ether soluble. Extremely low concentrations of chlorellin have demonstrated beneficial effects on growth. Using collodion sacs and Norite A, Pratt was able to show that if chlorellin was continuously removed from cultures that the maximum population was three times that attainable without chlorellin removal. Myers (42) commenting on Pratt's work observes that the growth curves used to evaluate the effect of chlorellin on growth also demonstrate light limitations. The true influence of chlorellin is, therefore, not readily discernible. Myers further notes that no algae except the one used by Pratt has ever shown chlorellin like inhibition.

Jorgensen and Nielsen (121) observed that the filtrates of _Chlorella vulgaris_ cultures could either inhibit or accelerate the growth of _Staphylococcus aureus_. In one experiment the degree of inhibition of the filtrate on the bacteria was reduced by heating the filtrate. The filtrate obtained in another experiment was found to have two inhibitors. After one inhibitor was inactivated by illumination and the other removed by adsorption and Norite A the filtrate

stimulated the growth of Staphylococcus aureus.

Experiments using Asterionella formosa and Nitzschia palea demonstrated mutual inhibition, with the extent of inhibition being dependent on the conditions of the experiments. The Nitzschia also produced autotoxic substances. Asterionella was found in some cases to be stimulatory to both itself and Nitzschia. Filtrate from a Scenedesmus quadricauda culture was highly toxic to Nitzschia. The degree to which Chlorella pyrenoidosa filtrates inhibited Nitzschia was dependent on the age of the Chlorella culture. The older the culture the more inhibitory was its filtrate. The filtrate from an old culture of Nitzschia, however, was found to accelerate Chlorella and Scenedesmus growth. Chlorella and Scenesdesmus were both inhibited by filtrates from old (21 day) Scenedesmus cultures. Scenedesmus was, however, stimulated by the filtrate of a 21 day old Chlorella culture (122).

Rile (123) demonstrated that the extent of the inhibition that Chlorella and Nitzschia exhibited toward each other was directly proportional to the inoculum size of the inhibiting organism. The auto and mutual inhibitory properties of the filtrates of cultures of both algae could be eliminated by adsorption on Norite A followed by autoclaving. The growth rates of both Nitzschia and Chlorella were inhibited when pond water which had supported excessive growth of Pandorina was used to prepare the growth substrate.

Talling (124) studying Asterionella formosa and Fragilaria crotonensis found no evidence that these algae produced any substance capable of modifying their own or the others growth.

This author also considered the literature on the topic of inhibitory substances and suggests that there is no definite evidence of inhibitory substances in natural phytoplankton populations. It appears possible that in both his own experment and in review of the literature he did not give due consideration to the potential masking effects of limiting light.

The content of the literature on inhibitory substances provides little comfort to the reader, but it does emphasize that this problem needs a large amount of additional investigation before clarification will be possible.

Mass Culturing of Algae

The term mass culture refers basically to organism concentration or density rather than volume of a culture. Culture volumes range from a few hundred milliliters for laboratory cultures to several hundred gallons for large scale outdoor culture units. The upper limit of size might be extended to several thousand gallons if sewage lagoons are considered as mass cultures. Mass culturing techniques have been developed from studies in two different areas of interest. Of the two areas, the one concerned with obtaining algal material for biological and bio-chemical investigations is probably the more advanced. Many of the investigators initially active in this area have moved on to the problem of producing algae for food and feed. The other area of investigation responsible for providing knowledge about algal mass culturing is the use of algae for sewage treatment. The evolution of knowledge

in these two areas has been very different.

Investigations of algae with reference to production of algal cellular material have followed, in general, an orderly line of development. The cultures initially used were rather dilute and had small production rates. Knowledge gained from these cultures was applied to other cultures with resulting increases in both attainable density and yields. The highest density attained has been the 55 grams per liter obtained by Myers (96) using a 6 millimeter deep annular chamber exposed to sunlight. The mass culturing of algae for sewage treatment started with the large and essentially unregulated sewage lagoons. Algal cultures used for this purpose did not receive any real scientific or engineering evaluation until the study of Gotaas, Oswald and others at the University of California.

The fundamental difference in the two approaches to mass culturing lies in the treatment of the substrate. The optimum production of algae requires that certain minimum concentrations of nutrients be present at all times. Substrate quality is increased when ever the minimal nutrient levels are encountered. The purpose of sewage treatment is to produce an effluent with as low a solids content as possible. The desired quality of the product essentially eliminates the possibility of supplementing the substrate to produce good algal growth at all times. There have, however, not been sufficient studies using algae to provide tertiary sewage treatment to allow a generalization about the treatment of the substrate.

Tamyia (13) lists adequate CO_2 and nutrients, sufficient

illumination, favorable temperatures and sufficient agitation to provide even distribution of light, nutrients and CO_2 and to prevent settling of cells as basic requirements for mass culturing of algae. The type of unit used will, to some degree, affect the factors mentioned by Tamyia. Glass tubes, small flasks and bottles, carboys standing vertically or lying horizontally, variously shaped plexiglas chambers, polyethylene tubing and open ponds or ditches are some of the containers that have been used for growing algae. Agitation has been provided by bubbling air or carbon dioxide, shaking, rolling, pumping and mixing with stirrers (34)(40)(57)(67).

Illumination has been accomplished with high and low intensity incandescent and fluorescent lights and with the sun. Solar illumination has been assumed by some to be the only feasible means of providing radiant energy (96), whether this is a wholly practical observation still remains to be verified.

The methods, units and terminology used to measure and report mass culture density, production of cellular matter and growth rate provide an almost insurmountable obstacle for the average reader. Culture density is measured in grams per liter, grams per culture, packed cell volume (cubic milliliters per liter) determined at various centrifuge speeds, transmittance of algal suspensions using various wave lengths and light paths and number of organisms per unit volume. Growth rate determinations are based on any of the parameters used for measuring culture density. Growth rate is reported in terms of $\log_e$ units per hour or day, $\log_{10}$

units per hour or day, number of doublings per day, reproduction time of the organism, detention time of the culture unit, grams per hour or day and number of organism produced per hour or day. Usually no distinction is made between growth taking place in the log phase and that in the arithmetic phase. (8)(23)(55)(62)(83)(116)(125).

The most widely accepted method of expressing culture growth is in terms of the velocity constant of a monomolecular reaction. As indicated by Phillips and Myers (125), the velocity constant as determined for constant density cultures could result from either exponential or arithmetic growth. Whether growth in a batch culture is exponential or arithmetic can be determined by plotting the growth measuring data on graph paper. Exponential growth gives a straight line on semi-log paper.

Although, the velocity constant of a monomolecular reaction is most often used, Robertson (126) suggested that Pratt (116) applied the velocity constant of an autocatalyzed monomolecular reaction to rate growth. The velocity constant for this type of reaction depends on both the population existing at a given time and on the ultimate population.

The rate of production of cellular material is usually indicated by yield or productivity. Yield or productivity is normally measured in terms of grams per liter per day or grams per meter square per day. Oswald and Gotaas (127) have used the unit of tons per acre per year.

Development of practical applications for mass cultures of algae requires consideration of the best strain or species

of organism to use. Factors to be considered are the response of the organisms to temperature and illumination control and the other environmental parameters which when combined will result in maximum culture productivity under a given regime. The isolation of the thermophilic strains of Chlorella with their high light saturation intensities has reduced the problem of temperature control that was anticipated with the use of the mesophilic species in outdoor cultures (55). Possibly even of more importance than the isolation of the thermophilic Chlorella species is the finding of Katz and Myers (53) that several of the more common blue green algae had significantly higher optimum temperatures than the mesophilic Chlorella species. Furthermore, the same authors (56) conclude that except for the limited ability of blue green algae to utilize exogeneous organic matter there is no real difference in the physiological characteristics of green and blue green algae. It is possible that blue green rather than green algae will ultimately be the organisms of choice for outdoor culturing.

The increasing amount of data pertaining to culture yield is making it quite obvious that this is a highly variable and extremely dependent parameter of algal cultures. Ketchum et al (128) surmised that algae should obey "the theory of optimum catch." This theory being that every population has a density which is optimum for the propagation of the population. Studying Stichococcus bacillaris they found yield to be a curvilinear function of culture concentration. Low culture density provided low yields as density increased the

yield increased to a maximum, further increases in density resulted in decreased yields. The theory of optimum catch was thus supported. It was also found that the optimum catch or yield was obtained when the algae were growing at less than maximum specific growth rate. Evaluation of several species showed that smaller species produced greater numbers but the weight produced was nearly the same for all algae, demonstrating that all the algae carried on assimilation at about the same rate under similar culture conditions. Myers and Graham (129) using Chlorella ellipsoidea, light intensity equal to sunlight and culture densities from 71 to 390 mg per liter found that yield was very sensitive to culture density. The maximum yield of 140 mg per day was obtained with a culture density of 130 mg per liter. Further investigation showed that the irradiance curve of photosynthesis (oxygen production versus light intensity) was different for each culture density. This is thought to be dependent upon the fact that chlorophyll content varies linearly with the average irradiance of an algal cell. Thus for each culture density there is a different chlorophyll content per cell because the average irradiance per cell will decrease with increasing density. The photosynthetic response at any given time was dependent, therefore, on the amount of irradiation the cell was receiving at that time and the irradiance curve associated with the particular culture density. Myers and Graham (50) using Chlorella ellipsoidea and Chlorella TX 7-11-05 continued investigations of yield as a function of culture density. Again yield was found to be

highly dependent on culture density. The maximum specific growth rate of the thermophilic C. TX 7-11-05 is double that of Chlorella ellipsoidea but its maximum yield was only 15 per cent higher. This higher maximum yield was attained at an approximately 50 per cent lower culture density. Using the previously mentioned cones for distributing the light increased the maximum yield two fold and more than doubled the culture density associated with the maximum yield as compared to cultures without cones. The conclusion is that yield is governed by efficiency of light utilization and not specific growth rate and that the irradiance curve of photosynthesis is not a fixed characteristic of an algal species, but is dependent on at least the average irradiance of the algal cell in a culture. Cook (62) also found yield to be very sensitive to culture density. A maximum yield of 0.48 grams per liter per day (12.2 grams per meter square per day) was attained in a 4" glass column culture using artificial light at a culture density of 0.36 grams per liter. The yield of algae in sewage oxidation ponds was found to be dependent on retention time, being a maximum between 0.5 and 1 day (21). Algae grown in continuous cultures on synthetic sewage were found to attain maximum yield at about one-sixth the retention period required to produce a maximum population (22).

The depth of an algal culture measured perpendicular to the illuminated surface also affects the yield of the culture. The use of different culture depths introduces the variable representing the relation between culture surface area and culture volume. Milner (130) proposes the idea that maximum

yield might be a function of the total mass of cells per unit area rather than culture density. This implies that for increasing culture depths the maximum yield would occur at decreasing culture densities assuming a constant illumination. That such an assumption might be valid is demonstrated by Gotaas and Oswald (23), whose work indicated that the culture density decreased with increasing depth while the yield remains approximately constant for depths of 2, 6 and 12 inches. Data from a laboratory culture (13) show that it might be possible to attain constant maximum yields at various depths without decreasing the culture density. This data was obtained using extensive culture stirring and 650 foot-candles of illumination. Although the influence of depth has not been fully investigated it seems plausible that the depth effect might, as with density effect, be a function of the irradiance curve of photosynthesis for algae growing at various depths.

The parameters determining the most desirable culture yields for sewage treatment have not been ascertained. Oxidation ponds require conditions such that sufficient oxygen will be produced to satisfy the sewage B.O.D. Algae yields in excess of this may be detrimental to the overall treatment of process (16). Tertiary treatment will require at least a consideration of the balance between the overall efficiency of light utilization and the yield measured possibly by the energy contained in the algal cellular material (23).

Techniques used for mass culturing of algae can generally be classified as either batch or constant density procedures. Each group possesses certain inherent desirable and undesirable

characteristics. The technique of choice for a particular application depends on the purpose for mass culturing the algae. The term batch culture means that all organisms produced during the culture period are retained in the culture. The constant density culture technique maintains some index of the culture population constant. This is usually accomplished by adding fresh substrate to the culture to wash out some organisms and dilute the culture or in some instances to just dilute the culture. The organism concentration at the beginning and end of the culture period is the same, the organisms produced having been removed or the culture volume increased (8)(62)(135)(132)(133).

Harvesting algae, particularly for laboratory investigations is easier using batch cultures than constant density cultures because of the smaller liquid volume to be handled. The three least desirable attributes of batch cultures are the continual decrease in substrate quality, the continual increase in culture density and the opportunity for toxic metabolites to concentrate. With a constant density culture the substrate quality, organism density, effective light, temperature and pH can be kept constant, also toxic materials will not be able to accumulate. The batch culturing technique seems to be most valuable for defining the gross ranges of influence of certain variables on algal growth. Detailed studies of the effect of a variable on growth are not readily accomplished with batch cultures. Successful use of constant densities cultures on the other hand, assumes that certain parameters governing the growth of a culture under selected

conditions are known.

The practical application aspects of the two culturing methods also merit some attention. The production of algal material for food and feed might be successfully accomplished using either technique. Tamyia (57) has carried out the mass production of algal material using 2000 liter batch cultures. Wide spread use of algal cultures for sewage treatment would appear to depend on whether or not the objective can be attained using a constant density culture. The detention times presently used in most lagoons and oxidation ponds precludes their use in all but the relatively sparsely populated areas (25)(26)(27). Mass culturing of algae for food is concerned with algal production and the rate at which the overall process takes place depends only on productivity. Sewage treatment has two different component rates which must be suitably combined to produce an overall process rate. The hard fact of tertiary sewage treatment is that so many gallons of sewage have to be treated per day. The ultimate need then is to determine if the methods of mass culturing of algae can be adapted such that productivity will be adequate to remove sewage nutrients without the use of storage ponds of excessive area.

There are two generally accepted systems for maintaining constant density cultures. The simplest way to distinguish between these two systems is to designate one as a continuous dilution system and the other as a controlled dilution system.

The continuous dilution system provides a constant flow rate of fresh substrate into the culture with the excess algae

being continuously washed out in the effluent. The flow rate of the effluent equaling that of the influent. If the displacement time of the culture volume is 1.44 times or greater, assuming log growth, than the minimum generation time of the organism, under the given conditions, an equilibrium population will be reached. The equilibrium population is that organism density which produces new organisms at the same rate as they are lost in the overflow. This principal of culturing has often been used for culturing bacteria.

With the continuous dilution system when nutrients are not limiting the equilibrium population and associated growth rate are dependent only on the culture dilution rate. If a nutrient is limiting then the growth rate is dependent on both the concentration of the limiting nutrient and the flow rate. This system has been termed the "Chemostat" and its advantage is that the concentration of the limiting nutrient in the growth chamber is dependent on the dilution rate and is independent of the nutrient concentration in the influent (134)(135)(136)(137). A possible objection to this type of unit is that with high density cultures the separate effects of light and nutrients could not be easily delineated. The experimental oxidation ponds and laboratory cultures at the University of California (21) are operated on a slight modification of the chemostat principle. The difference being that the nutrient concentration in the feed is constant irrespective of the flow rate. For these laboratory cultures and oxidation ponds dilution rate is measured in terms of retention time and load or nutrient concentration is reported in terms of

B.O.D. Retention time and B.O.D. loading influence bacterial growth while retention time, B.O.D. and illumination influence algal growth. The controlled dilution technique commonly utilizes a photometric method to control dilution. The transmittance of the culture is poised at a given value, when growth takes place the transmittance decreases and dilution is initiated to return the transmittance to the desired level (8)(62)(125). This is the only culturing system which allows separation of all the variable parameters. This method of density control has not been applied to large mass algal cultures because of certain engineering problems. The most basic of these problems is how to insure that all the contents of a large culture are equally distributed.

The fundamental difference between the two dilution techniques is that continuous dilution technique forces the algae to adjust to the environment while the controlled dilution allows the algae to vary several factors in their environment as is required for their gorwth.

This review and consideration of the literature reveals that there is a vast but rather heterogeneous collection of information pertaining to algae. The major portion of the literature is concerned with nutrients, growth rates and productivity as they pertain to algal growth. The enthusiastic speculation of sanitary engineering about the use of algae has far out distanced its active, constructive investigations of potential applications of algae. The investigations of the algal nutritionists and physiologists have revealed the many similarities and differences that exist between both

species and genera of algae. Whether the differences among the algae with relation to light quality, light intensity, temperature and nutrient requirements are of importance in connection with tertiary sewage treatment has yet to be determined. However, before any such decisions can be made more information related to the growth of various algae in sewage substrates must be obtained. Consideration must also be given to determining how an algal sewage treatment process can be operated under controlled conditions. To make a rational evaluation with regard to bringing algal sewage treatment into the fold of a controlled process will require much more data than now exist on how the various factors influencing algal growth affect nutrient extraction from the growth substrate. The problem of reducing the surface area now generally considered necessary for lagoons and oxidation ponds appears to be directly related to determining if stirring of relatively deep dense algal cultures can induce growth to the same extent that now exists in shallow and rather dilute culture units.

The study, as proposed in this thesis, of the influence of substrate nutrient concentration on growth and nutrient extraction of algal cultures should provide valuable information as to what type of controlled algal tertiary treatment process will provide the best sewage treatment. This study also has the possible potential of explaining some of the conditions which occur in lagoons and oxidation ponds. The study of the effect of induced turbulence on the growth of algal cultures, as proposed in this thesis, will add to the

rather meager information existing in this area. In addition it will give an indication of whether mixing algal cultures can reduce the surface area requirements of mass algal cultures thus providing a more practical treatment process.

II. APPARATUS AND PROCEDURES

Culturing Facilities

Both types of algal culturing methods, batch and constant density, were used for the experiments reported on in this thesis. Evaluation of the effect of induced turbulence was made employing the batch culture method. Appraisal of the influence of substrate nutrient concentration was carried out using a constant density culturing method.

Batch Culturing Unit:

The culture unit used by Witt (8) was modified so it could be used as the batch culture unit. This culturing unit was a symmetrical two chamber unit constructed of clear plexiglas. The chambers were each 25 cm. wide and 30 cm. high on the interior. At a normal culture height of 25 cm. the surface area exposed to direct lighting was 625 cm.^{2} for each chamber. The depth of the cultures could be varied from 1.25 cm. to 25 cm. by predetermined increments. Depth being the dimension parallel to the path of light.

Certain modifications were made on Witt's chambers in order that they could be used for batch culture experiments. The moveable backs were replaced with one back fixed at a culture depth of 25 cm. The only appurtenances on the new back were those required for carbon dioxide addition to the cultures. The unit being made of clear plexiglas allowed light to enter the top, bottom and sides. Light could also pass from one culture to the other. A preliminary investiga-

tion was pursued in an attempt to evaluate the influence of this additional light. The results of this investigation indicated that at conditions of high culture densities and greater culture depths significant increases in growth rates might be experienced. Because of this potential effect the top, bottom, sides and panel between the chambers were covered with a black opaque material. There was no light source behind the chambers thus the only light entering was through the culture face.

Constant Density Culturing Unit:

The constant density culture unit was of the same basic design as Witt's (8). It consisted of two symmetrically designed chambers 25 cm wide and 30 cm high. The depth was variable, in predetermined increments, from 2.5 to 40 cm. Because of the previously indicated short-comings of clear plexiglas this unit (Figs. 1 and 2) was constructed of black opaque plexiglas. Clear plexiglas was used for the fronts and backs of the chambers.

Experimental results indicated that a control chamber was not necessary. The validity of these results was substantiated by Witt (138). Both chambers of the culture unit were, therefore, used as culturing chambers.

Carbon dioxide was added to the cultures through fritted glass diffusers located at the bottom center of the backs of the chambers. Inflowing substrate was introduced through the back of the chambers above the culture surface. The culture overflow was taken out through the middle of the back of the

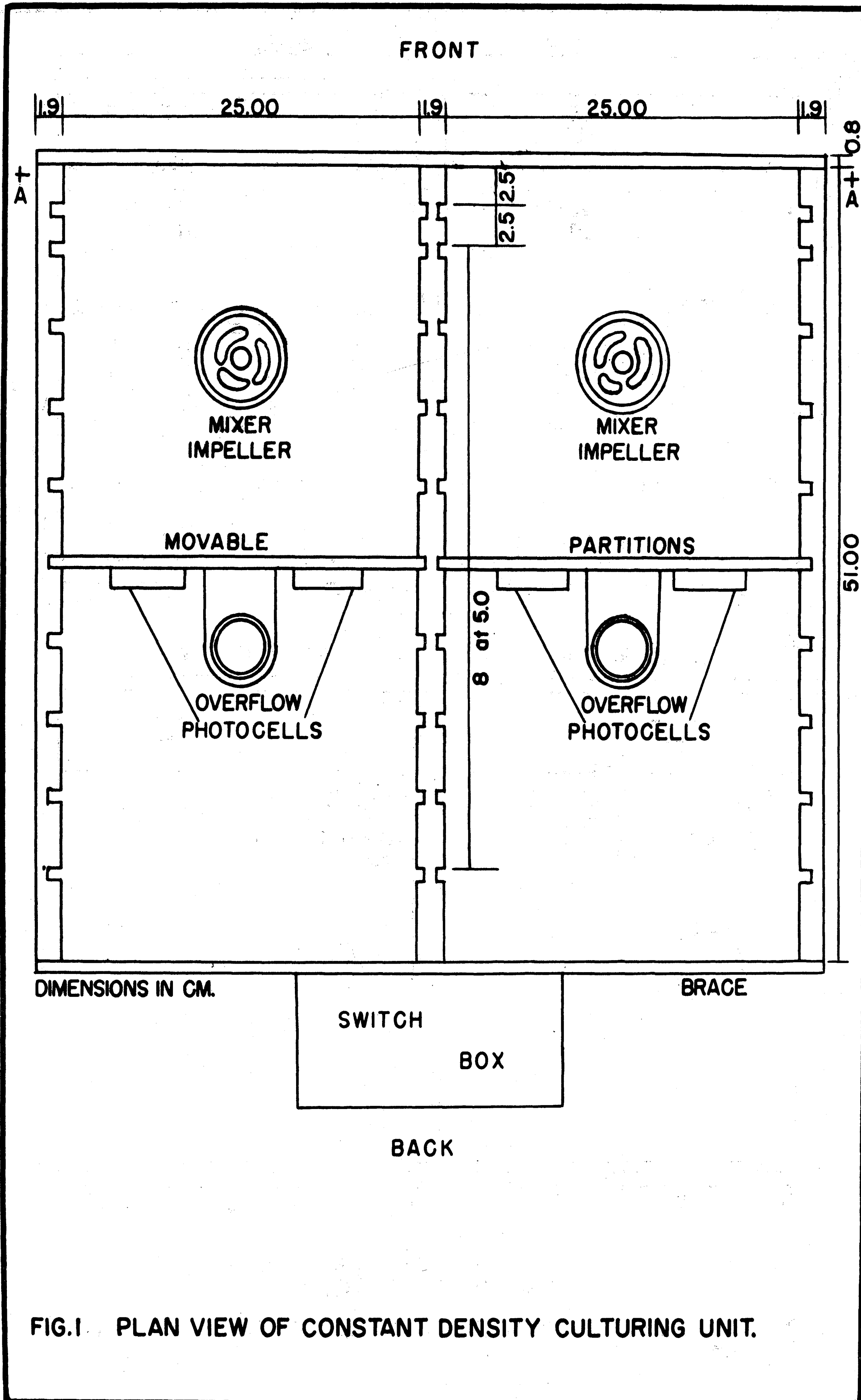
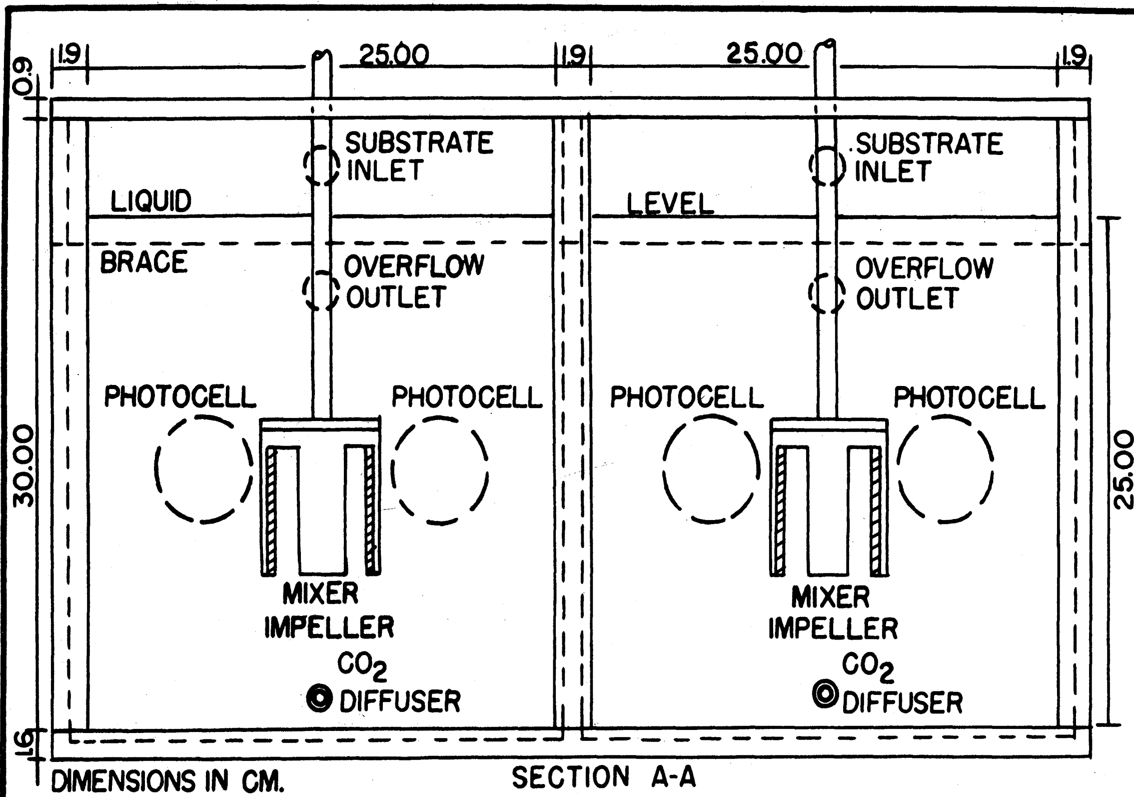


FIG.1 PLAN VIEW OF CONSTANT DENSITY CULTURING UNIT.



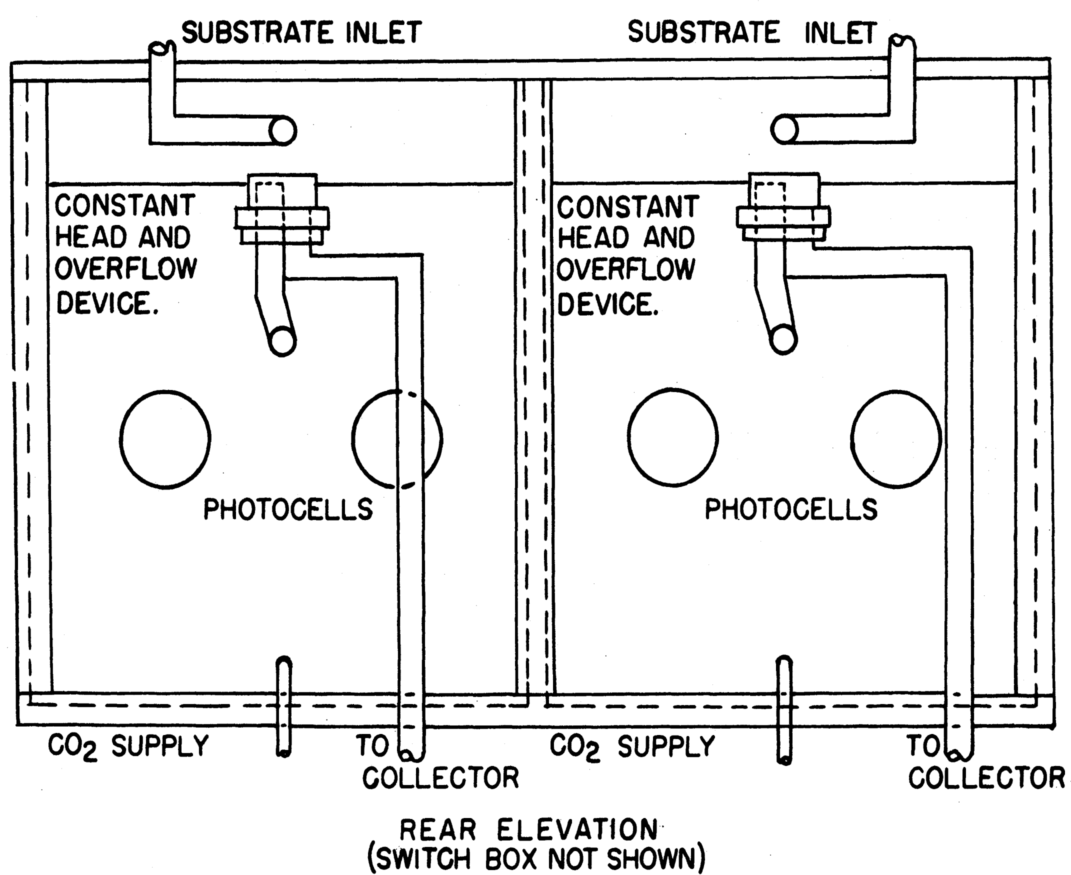


FIG. 2 CONSTRUCTION DETAILS OF CONSTANT DENSITY CULTURING UNIT.

chamber. The culture volume was maintained by a constant head device in the overflow arrangement (8)

Light Sources:

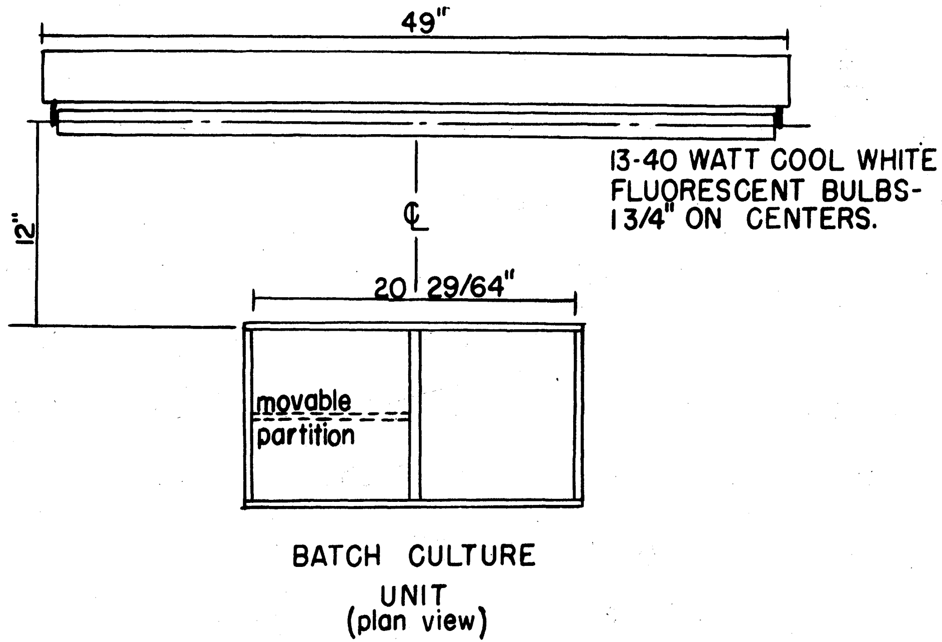
The light sources were identical to those of Witt (8). They consisted of 13, 40 watt cool white flourescent tubes placed 1-3/4 inches center to center. The relative energy spectrum of these tubes is shown by Witt (8). Each tube had its own control switch. Each of the light sources had a white back board which reflected the light from the back side of the tubes. Light intensity was varied by varying the number of tubes in operation. The center tube of the bank was located on the horizontal center line of the culture when the culture liquid height was 25 cm. The vertical center line of the culture unit intersects the vertical center line of the bank of lights.

In order to obtain relatively balanced intensity distribution on the culture faces, the same number of tubes had to be operating on both sides of the center tube. Each light source (Fig. 3) could produce a maximum intensity at the culture faces of about 1200 foot candles. The culture faces were located 12 inches from the light sources.

Culture Density Control Arrangement:

As already indicated the batch cultures had no density control or density measuring devices.

The terminology "Constant density algal culture" implies that some factor, usually numbers of cells or culture light adsorption, is maintained constant in the culture. For any



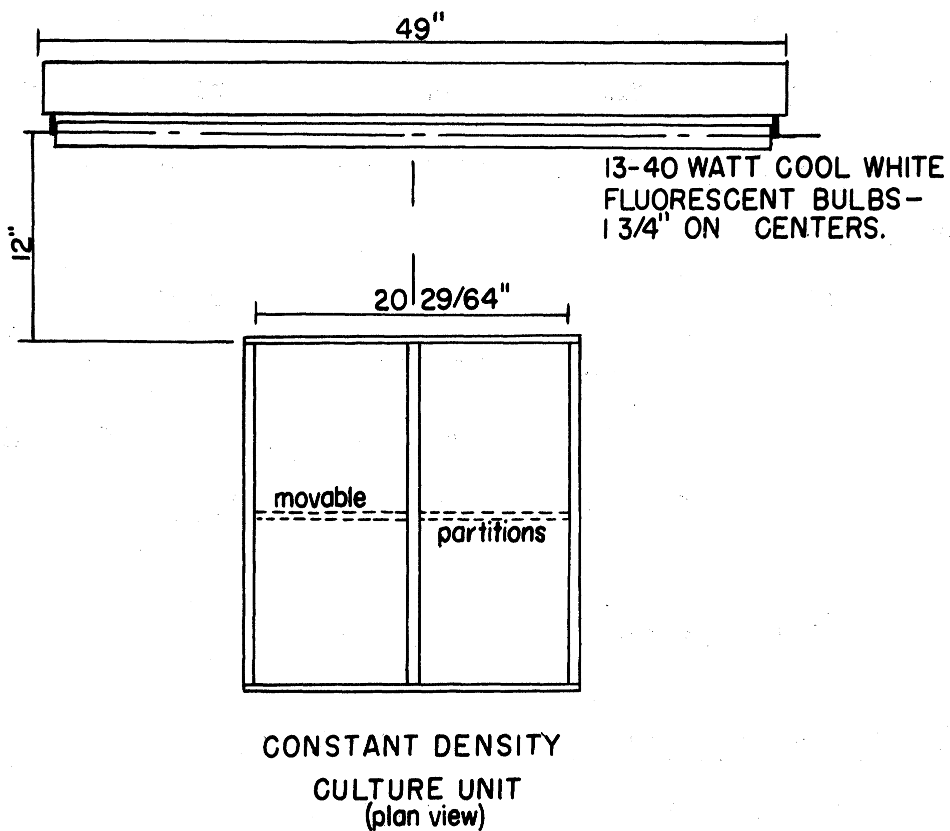


FIG. 3 LOCATION OF CULTURES WITH REFERENCE TO THE LIGHT SOURCES.

given condition, light attenuation is a function of cell numbers. Any algal cells produced in excess of those necessary to maintain conditions constant must, therefore, be removed. Removal of the excess cells in constant density, constant volume cultures is most readily accomplished by adding fresh substrate. When fresh substrate is added some of the algal cells are carried out in the overflow. The density of the culture is maintained constant by a combination of culture dilution and algal cell removal (Fig. 4).

The basic principles of the control system used for the experiments reported herein are the same as those employed by Witt (8). The control systems were developed around the ability of an algal culture to attenuate light in some proportion to its density. The basic operation of the control system involved bringing a photoelectric circuit into balance at a desired culture density; when density increased due to growth the circuit was unbalanced and new substrate was introduced until the photoelectric circuit was again balanced.

The two constant density cultures each had their own control system. The one chamber had, with the exception of the number and location of the photoelectric cells, the same control system as used by Witt (8). As noted previously a control chamber was not necessary, therefore, the control photoelectric cell was placed exterior to the culture unit at the same distance from the light source as the front of the unit. Two photoelectric cells were required at the rear of the culture to insure sufficient output to operate the control system and to minimize interference from the mixing

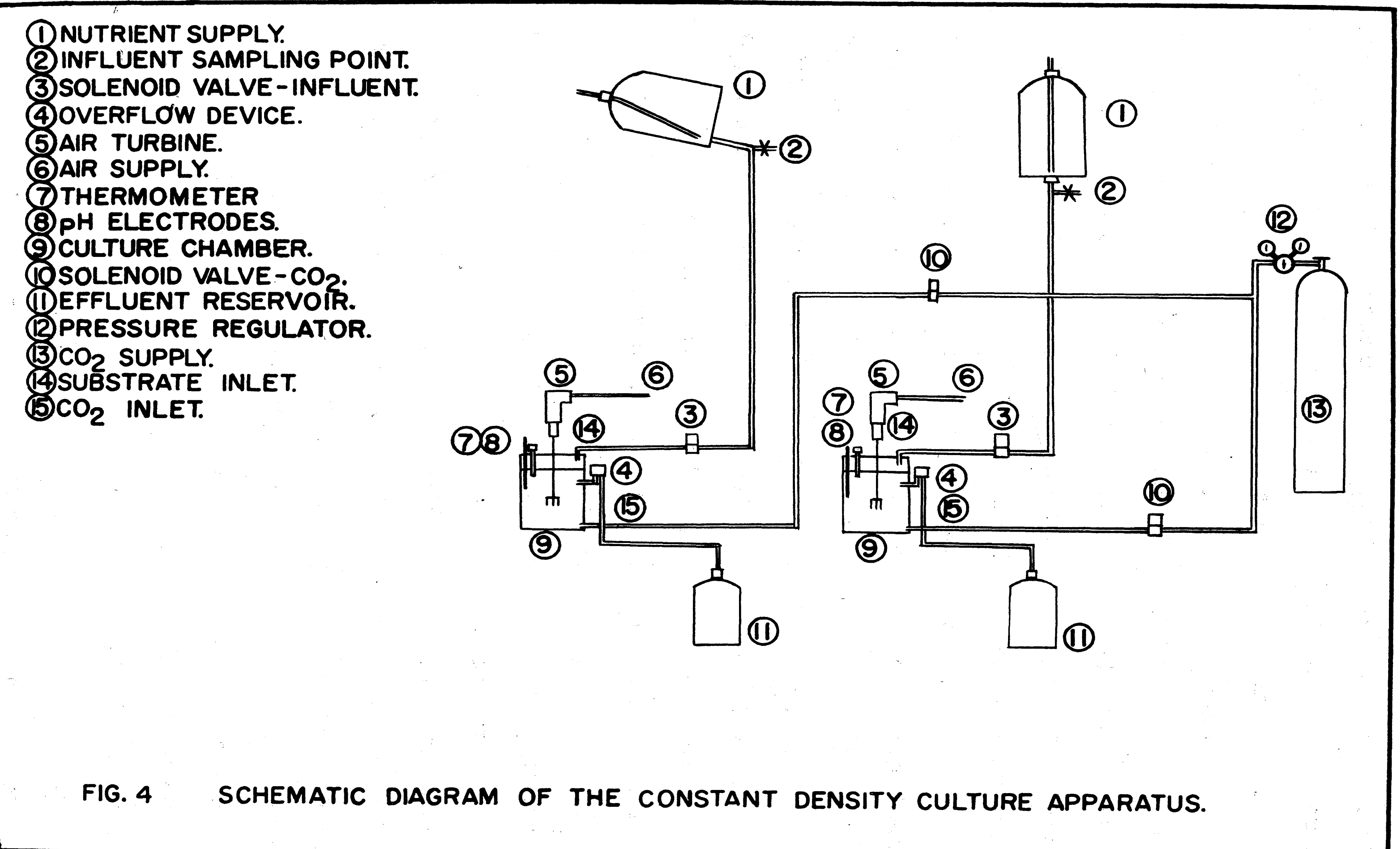


FIG. 4 SCHEMATIC DIAGRAM OF THE CONSTANT DENSITY CULTURE APPARATUS.

device.

The control system for the other chamber (Fig. 5) operated on the same principle but was constructed of different components. The number and location of the photoelectric cells were the same as those just described. The two culture photo-cells were connected in parallel, like terminal to like terminal. The combined culture cells were then connected opposite terminal to opposite terminal with the control cell. A Weston Model 1092 Sensitrol Relay, a sensitive electrical relay, was connected in parallel with the photoelectric cells. The sensitrol unit operated a Weston Model 712 small power relay. This power relay controlled the action of a solenoid valve and a drum recorder. The valve governed the flow of fresh substrate into the culture. A continuous plot of overflow versus time was provided by the drum recorder. The recorder rotated at a constant rate during any time interval in which substrate was flowing into the culture.

For any given culture density the current outputs of the control and culture photoelectric cells were brought to near balance by use of neutral filters of various densities. Final adjustment was accomplished by use of a micro-potentiometer. When growth occurred the balance of the photoelectric cells was upset and the sensitrol unit's contacts were closed. When these contacts closed the small power relay was activated. The relay opened the solenoid valve and started the drum recorder rotating. Unbalancing of the photoelectric cell circuit closed the sensitrol unit contacts and initiated culture dilution. Returning the culture to its original density and rebalancing

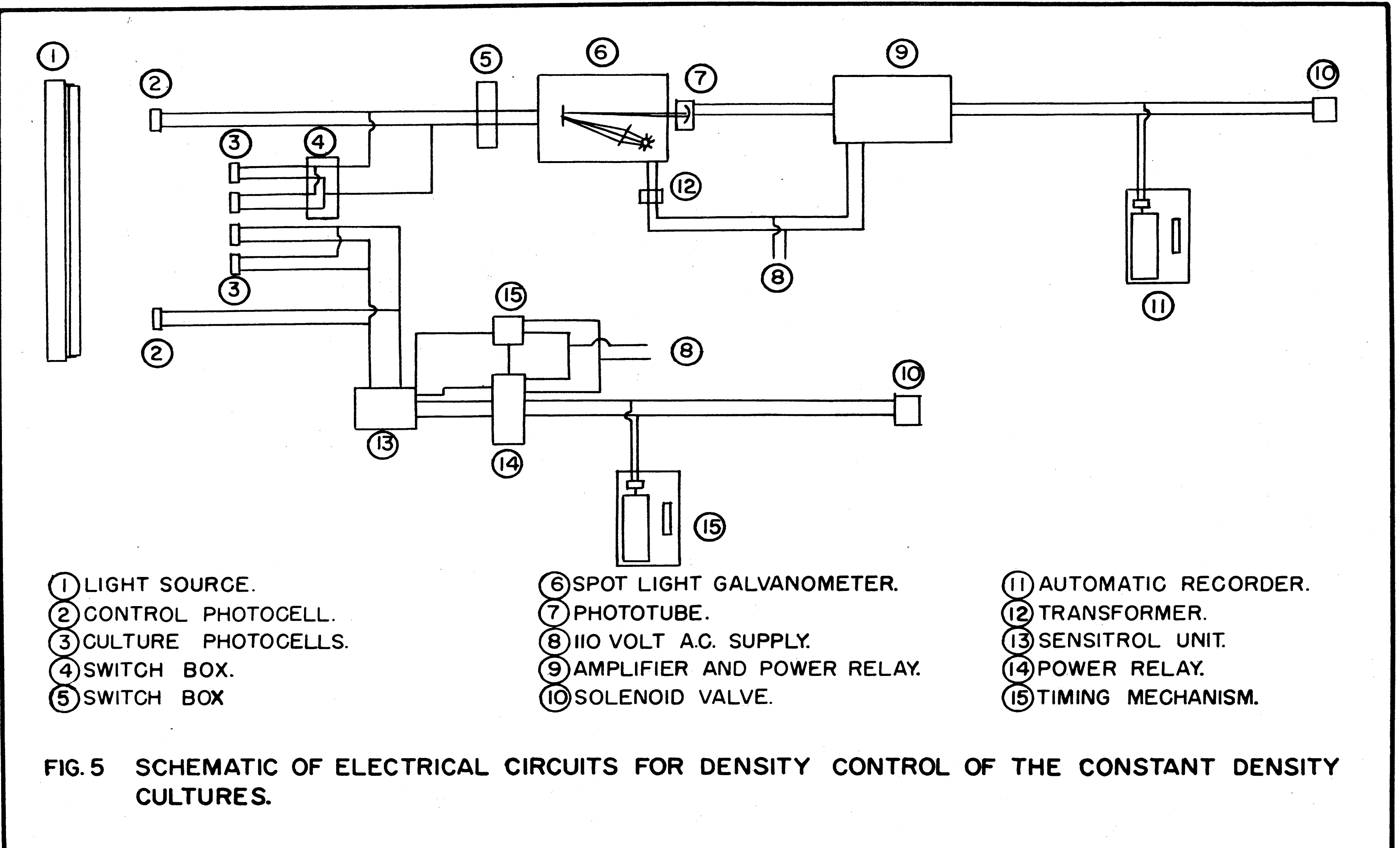


FIG. 5 SCHEMATIC OF ELECTRICAL CIRCUITS FOR DENSITY CONTROL OF THE CONSTANT DENSITY CULTURES.

the photoelectric cell circuit would not, however, open the contacts. Once closed these contacts could only be opened by activating a reset circuit. Advantage was taken of this arrangement to provide time for the entering substrate to be throughly mixed with the culture between dilutions. A timing mechanism was incorporated into the reset circuit. This system provided a maximum dilution time of approximately 6 seconds followed by a mixing time of about the same duration. If the culture was still too dense after the mixing, dilution would take place again. However, if the density had been sufficiently reduced no further dilution would occur at that time. Since dilution could be started at any time during the dilution period, but could not be stopped till the end of that period, careful control of the inflow rate was necessary to prevent excessive overdilution.

Fresh substrate flowed by gravity from a constant head supply container through the solenoid valve into the culture. To keep algal growth in the influent line to a minimum the substrate was introduced above the surface of the culture.

pH Control Mechanisms:

pH rather than CO_2 content was used to control the amount of inorganic carbon supplied to the cultures. Carbon dioxide and air mixture or just carbon dioxide was used to maintain the desired pH. A five per cent carbon dioxide and 95 per cent air mixture and 100 per cent carbon dioxide were used to supply inorganic carbon to the batch cultures.

The carbon dioxide and air mixture or pure carbon dioxide

flowed continuously into the batch cultures. Carbon supply adjustments and pH measurements for these cultures were accomplished manually. Samples were drawn from the cultures and the pH was determined with the aid of a line operated pH meter. Raising or lowering of the culture pH was done by manually decreasing or increasing the flow rate of the carbon dioxide and air mixture.

The pH of the constant density cultures was monitored continuously and CO_2 was added only when pH adjustment was necessary. For pH control, as with culture density control, each culture had its own control and supply system. The only common component between the two arrangements was the carbon dioxide supply tank.

The control and carbon dioxide supply system of one of the constant density cultures was the same basic system as that used for density control and culture dilution. A pH meter and pH electrodes provided the signal to operate a Weston Model 1092 Sensitrol Unit. A power relay operated by the sensitrol unit controlled a solenoid valve. The solenoid valve regulated the flow of carbon dioxide into the culture. The sensitrol unit could be adjusted to close the electrical circuit for the pH meter output associated with any given pH value. When the pH of the culture was less than the desired value, the electrical circuit was open and the solenoid valve was closed, thus no carbon dioxide entered the culture. If the pH of the culture equaled or exceeded the pre-selected value the electrical circuit was closed and the solenoid valve was opened allowing the carbon dioxide to

enter. The sensitrol unit contacts which controlled the electrical circuit operated in the same manner as the sensitrol contacts of the density control unit. Once closed the contacts could not be opened except by activation of a reset system. The reset system for the pH control arrangement was operated by the same timing mechanism that regulated the density control system reset device. This then provided a maximum time interval of 6 seconds for continuous carbon dioxide addition to the culture. This interval was then followed by a six second mixing period. If after the mix time the pH was still too high carbon dioxide was added for another six seconds. However, if the pH had been sufficiently lowered by the carbon dioxide already added to the culture no further addition occurred after the mix interval. Carbon dioxide addition could be initiated at any time during the six second addition interval, but could only be terminated at the end of the interval.

The other constant density culture had a slightly different type of pH control system. A line operated pH meter and pH electrodes were used to determine the pH of the culture. The output signal of the pH meter was used to operate a Brown Electronic strip chart recorder. A micro switch attached to the recorder controlled a solenoid valve which in turn regulated the flow of carbon dioxide to the culture. The micro switch could be adjusted to close at any desired pH. Except for not providing a mix interval between carbon dioxide additions, the system functioned in the same manner as the other pH control arrangement. A culture pH value equal to or greater than a

preselected value resulted in the admission of carbon dioxide to the culture. When the pH dropped below the selected value carbon dioxide flow was stopped.

The carbon dioxide supply tank was equipped with a pressure reduction valve which maintained a constant outlet pressure while the tank pressure varied from 1800 psi to zero. Normally the outlet pressure, depending on the condition of the fritted glass diffusers in the cultures, was maintained between 12 and 20 psi.

The pH electrodes of the control systems (Fig. 6) were located as far as possible from the carbon dioxide inlets. This was done to prevent the accumulation of carbon dioxide on the glass electrode. Such an accumulation produced pH readings that were lower than actual values.

Stirring Devices:

Each culture was equipped with its own stirring apparatus. All the stirring devices (Fig. 7) were of the same general type differing only in the construction used for propellers. The components of each stirring apparatus were the propeller, connecting shaft and air turbine (air driven hand drill). The turbines were connected through hoses and valves to a high pressure (approx. 100 psi) air supply. The range of speed of the turbines was 500 RPM to 3000 RPM. Below 500 RPM the turbines were not reliable in their operation. By proper adjustment of the valves the turbines could be operated at any speed between the maximum and minimum values.

The turbines were mounted above the cultures with the

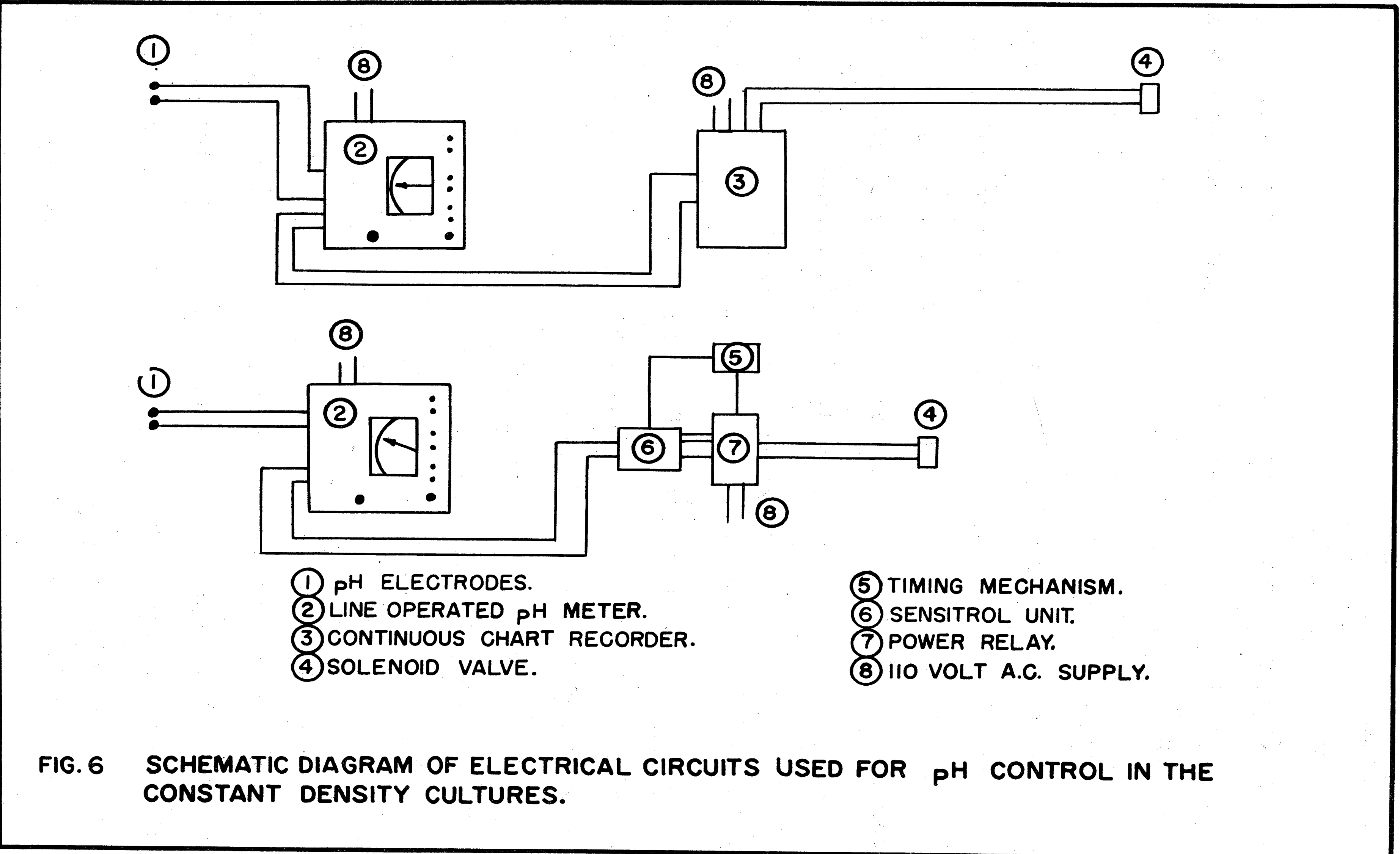


FIG. 6 SCHEMATIC DIAGRAM OF ELECTRICAL CIRCUITS USED FOR pH CONTROL IN THE CONSTANT DENSITY CULTURES.

drive shafts penetrating down through the cultures from the top. The propellers were located in the center of the horizontal plane of the cultures. The tops of the propellers were placed 6 inches above the bottom of the culture chambers. The propellers were made of clear plexiglas. Shafts were made of stainless steel or aluminum and coated with plastic.

Temperature Control:

The two constant density cultures and the two batch cultures were located in a constant temperature room (8). The temperature of each culture depended on its density, mixing speed and intensity of light source. Since each of the four cultures in the room were operating at different values of these factors it was not possible to keep all the cultures at the same temperature.

The temperature control of the room was set so that the temperature of one of the constant density cultures was maintained at 20° C. The other such culture was allowed to seek its own constant temperature which, as will be shown later, did not differ greatly from 20° C.

The temperature of the constant temperature room required some supplemental temperature control devices for the batch cultures. At high speeds of stirring and high algal densities, the batch cultures would reach temperatures of 27° to 29°C, when the room temperature was that required to maintain the previously mentioned 20°C. At the same room temperature, low stirring speeds and low algal densities would produce culture temperatures of 20°C, or slightly less. In order,

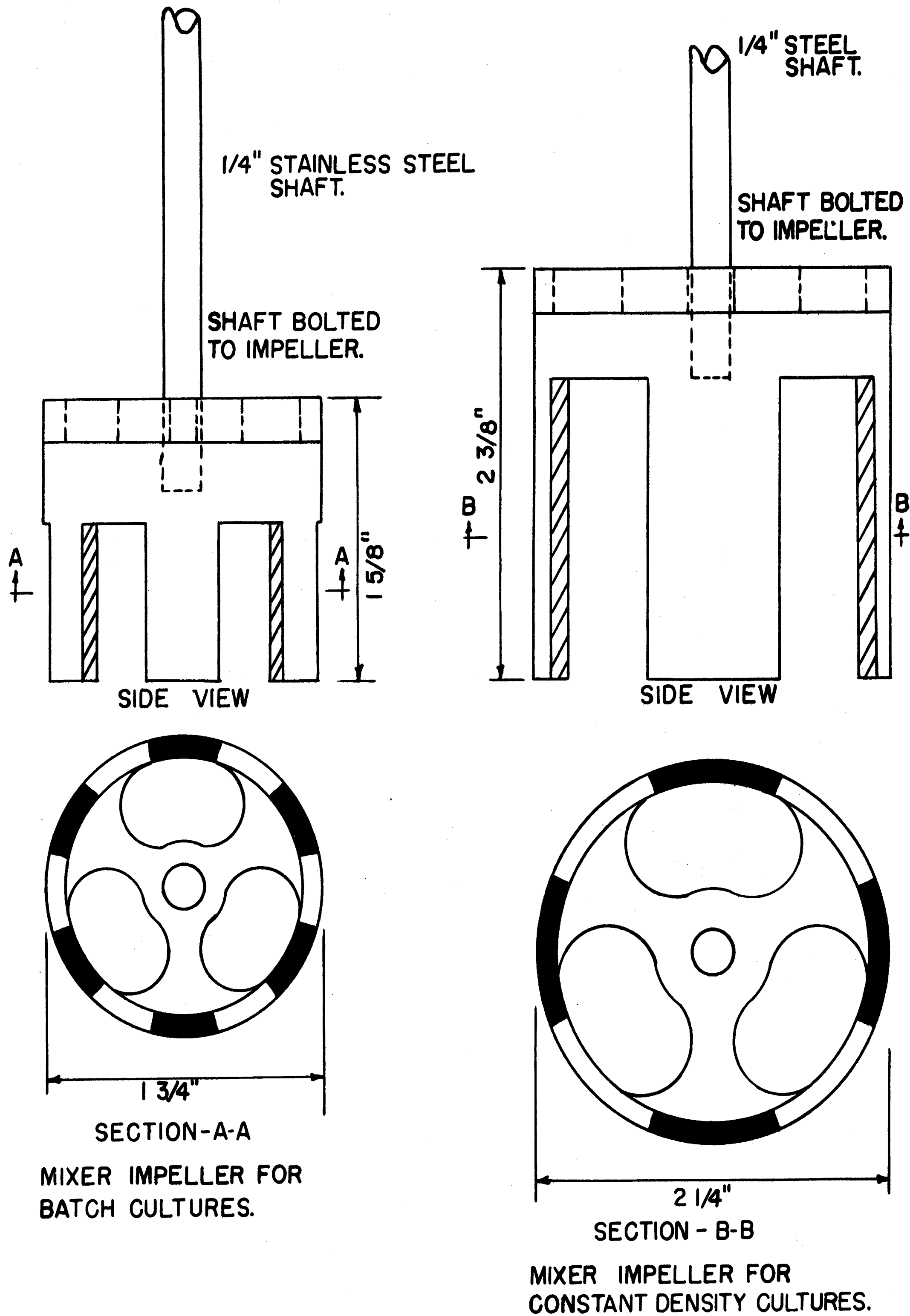


FIG. 7 MIXER IMPELLERS

therefore, to maintain a nearly constant culture temperature, it was necessary to locate a 60 watt and a 25 watt incandescent light bulb underneath each batch culture. Judicious operation of these light bulbs made it possible to maintain a nearly constant temperature for all combinations of stirring speed and culture density encountered. The cultures were completely shielded from the light of these bulbs.

Testing Procedures

Physical Tests:

Stirring speed.- The stirring speed was determined by use of a stroboscope. The instrument was calibrated in 10 RPM increments from 250 to 1150 RPM and in 50 RPM increments from 1000 to 4500 RPM. Preliminary evaluation of the variation in mixing speeds indicated that only a daily measurement was required.

Light intensity.- Light intensity was measured with a Weston Model 756 illumination meter and a Weston Illumination Target equipped with a viscor filter. The target with the viscor filter measured light intensity from 420 mμ to 710 mμ.

The general distribution of the light across the faces of the cultures with the fluorescent tube light sources is shown in Figure 8. The light intensity of these cultures was measured at the central point of the cultures on the outside of the chamber. To determine the intensity at the culture liquid face the corrective factor of 0.95 for the transmission of plexiglas, as determined by Witt (8), would have to be

applied. Light intensity readings were made daily.

pH determinations.- As stated previously these were made continuously for the constant density cultures. Additional determinations were made daily to check the functioning of the continuously monitoring pH meters.

The pH values of the batch cultures were determined at least twice a day. Determinations were made more often if the pH was either varying widely or changing constantly in one direction. The measurements were made by sampling the culture and using a line operated pH meter for the actual measurements.

Algal density determinations.- The algal densities were determined at least once a day by four methods: (1) enumeration of the cells or particles using a haemacytometer, (2) enumeration of the cells or particles using a Coulter Counter, (3) dry weight determinations, and (4) the per cent transmission of the culture.

Using the haemacytomer both the number of algal cells and the number of algal particles were determined. For filamentous algae, counts were made only of the number of algal particles. Only the center 1 $mm.^2$ field of each of the counting chambers was used. A fresh sample was taken for filling each chamber. A total of 1000 cells or particles or 10 of the above described fields, whichever came first, were used to determine the algal concentration. The results were reported in cells or particles per milliliter.

The Coulter Counter (Appendix I) provided a rapid accurate method for determining the number of algal particles

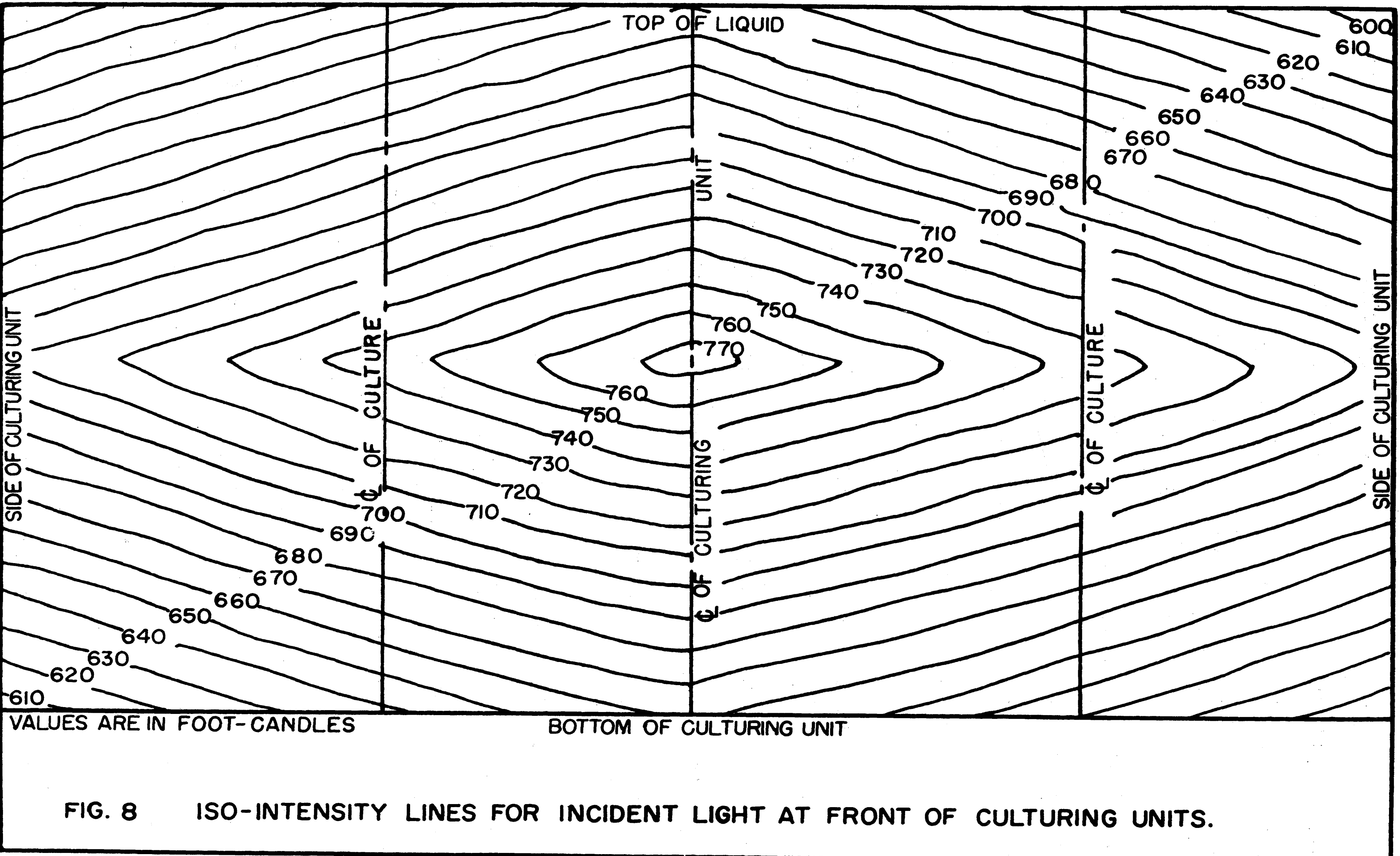


FIG. 8 ISO-INTENSITY LINES FOR INCIDENT LIGHT AT FRONT OF CULTURING UNITS.

in a culture. This enumeration procedure determines only the number of particles in a sample, the number of algal cells can only be determined if the algae is unicellular. Results were reported in particles per milliliter.

Dry weights were determined by use of Millipore filters type RA. The filters were pre-dried at 103°C for 24 hours, then stored in a dessicator until needed. To determine the dry weight a stored filter was weighed and suitable volume of algal suspension passed through it. The filter and the algae were then placed in an oven and dried for 24 hours at 103°C. After 24 hours, they were removed from the oven, cooled and weighed. The weight of the algae in the sample volume used was determined by difference. Results were reported as ppm 24 hour dry weight.

Light transmittance of the cultures was determined by use of a Coleman Model 14, Spectrophotometer; a 20 mm. light path was used for the constant density cultures, and a 13 mm. path for the batch cultures. The shorter light path made it possible to obtain readings at the higher densities encountered with these cultures. All transmittance determinations were made at a wave length of 425 millimicrons. The results were reported in per cent transmittance (%T).

<u>Specific extinction coefficient</u>.- The specific extinction coefficient (Equation I) is a function of length of light path concentration of algae and light transmitted.

$$\log \frac{I_{dw}}{I_{sus}} = A_1 bC.$$

$$\frac{I_{sus}}{I_{dw}} = 10^{-A_i bC}.$$

$$A_i = \frac{2-\log\%T}{bC}. \quad (I)$$

Where: A_i = Specific Extinction Coefficient (L/mg-cm).

b = Length of lightpath (cm).

c = Concentration of Algae (mg/L).

I_{dw}, I_{sus} = Light transmitted by distilled water and algal suspension respectively.

Equation I has been derived from the Lambert-Beer Law. For the value A_i to be entirely correct, the light should be monochromatic and the incident light and light path used to determine I_{dw} and I_{sus} should be equal.

The A_i values reported in this thesis are an average of the A_i's of the light wave lengths from 420 millimicrons to 710 millimicrons inclusive. The per cent transmittance of the algal suspensions was determined every 25 millimicrons from 425 millimicrons to 700 millimicrons inclusive and at 420 millimicrons and 710 millimicrons. The light path was 13 mm. The average per cent transmittance was then determined. Using this average value and the 24 hour dry weight the A_i value was found. The A_i value so determined is the average specific extinction coefficient for the range of wave lengths whose intensity was measured with the light intensity meter.

Growth rates.- The growth rates of the constant density cultures were determined by the method of Phillips and Myers (125) and Witt (8). The overflows of the cultures were collected in carboys. The volume of overflow was measured

periodically. A plot was then made of cululative overflow in liters versus cumulative time in hours (Fig. 9). The slope of the line of this plot was the rate of overflow in liters per hour. The growth rate can then be expressed as:

$$\underline{K} = \left(\frac{\Delta V}{\Delta T}\right)\left(\frac{24}{V}\right).$$

Where:

$\underline{K}$ = Growth rate in liters/liter/day which is numerically equal to $\log_e$ units/day.

$\frac{\Delta V}{\Delta T}$ = Flow rate in L/Hr.

V = Volume of culture in liters.

24 = Hrs./day.

The batch cultures having no overflow required that the growth rates be determined in some other manner. The velocity constant of a culture growing under batch culture conditions may be expressed as:

$$\frac{dN}{dT} = \underline{K}N.$$

$$\frac{dN}{N} = \underline{K}dT.$$

$$\log_e \frac{N_2}{N_1} = \underline{K}(T_2 - T_1).$$

$$\log_{10} \frac{N_2}{N_1} = K(T_2 - T_1).$$

Where $K = \underline{K}/2.303.$

$$K = \frac{1}{T_2 - T_1} \log_{10} \frac{N_2}{N_1}.$$ Slope of Semi-log plot.

Then $\underline{K} = 2.303\ K.$

Where: $\underline{K}$ = Growth rate in $\log_e$ Units/Day.

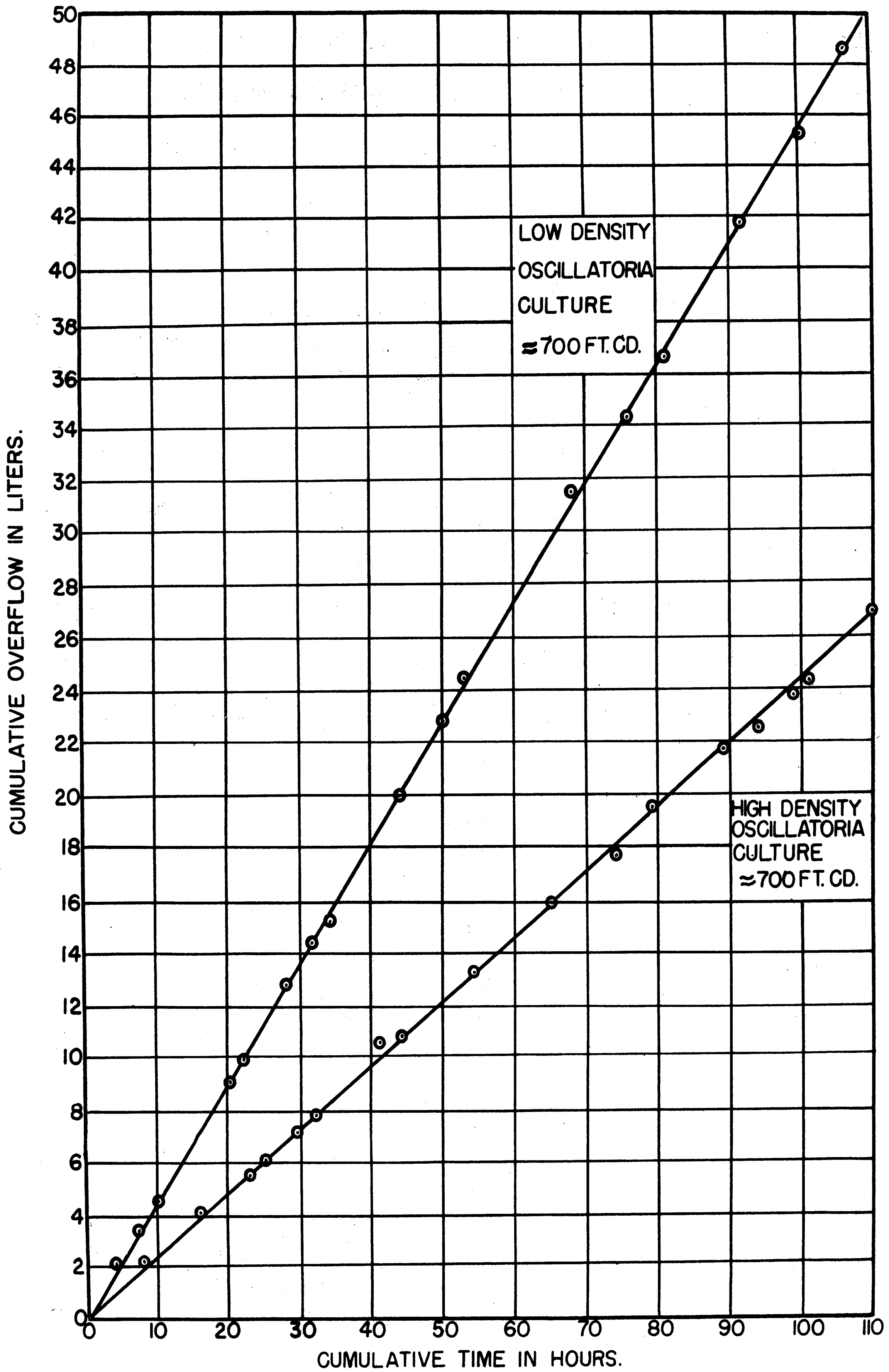


FIG. 9 CUMULATIVE OVERFLOW AS A FUNCTION OF TIME.

N_1 = Some measure of algal concentration at time T_1.

N_2 = Some measure of algal concentration at time T_2.

The concentrations of algae in terms of weight and numbers were determined once or twice daily. A cumulative plot of both of these factors versus cumulative time was made using semi-log paper. The algal concentration factors being plotted on the log scale. The slopes of these plots were used, as indicated above, to determine the growth rates of the cultures. Thus a growth rate for each culture was obtained using both weight and numbers as measures of culture concentration. All growth rates were reported in terms of $\log_e$ units per day.

Chemical Tests:

Chemical determinations were routinely made for NH_3, NO_2, NO_3, TON (Total Organic Nitrogen), PO_4(orthophosphate), TP (Total Phosphate) and alkalinity. The test procedures for NH_3, NO_2, NO_3, TON with a slight modification, PO_4 and alkalinity are the same as those used by Witt (8). Total phosphate was tested for using the method outlined by the technical advisory committee of the ASSGP (139). All the procedures used are presented in Appendix II.

Chemical analyses were routinely conducted only on the constant density cultures. Samples of the influents and effluents of the cultures were analyzed daily. The effluents of the culture were passed through millipore filters to remove the algae. The filters being used to determine the dry

weight concentrations of cultures, the filtrate produced by the filtration was used for chemical analysis. The difference between the concentration of the various chemical components in the influent and in the filtrate represent the quantity removed by the algae culture. Composite samples were used to determine the TON and TP of the influents, filtrates and algal suspensions of the cultures.

III. EXPERIMENTAL RESULTS AND DISCUSSIONS

The Influence of Nutrient Concentration on Algal Culture Growth

To evaluate the effect of nutrient concentration on the growth rate and the nutrient removal capabilities of algal cultures four series of experimental test runs were made. Basically two different organism concentrations and two different incident light intensities were considered in addition to the varying quantities of nutrients in the substrate. The per cent transmittance of the culture as measured at a wave length of 425 millimicrons with a 20 millimeter light path was used as the control parameter during the operation. The transmittance value and the incident light intensity are used to identify the various culture conditions in the discussion and on the graphs. The average values of incident light intensity in foot-candles (ft.cd.), per cent transmittance (%T), dry weight in ppm, mixing speed in RPM and pH are given in Table 1.

Table 1

The Basic Parameters of the Culturing Conditions Investigated.

%T	Dry wt. in ppm	Incident Light Intensity in ft. cd.	Mixing Speed in RPM	pH
45.1	42.2	760	1000	7.6
45.6	45.1	1090	1000	7.6
21.5	92.6	820	1020	7.5
22.1	98.8	1110	1030	7.4

The nutrient source used for the constant density cultures was effluent collected from the Ann Arbor, Michigan, Sewage Treatment Plant. This plant uses the activated sludge process

for secondary treatment. In order to have sufficient nutrient supply to make at least one and possibly several runs using the same basic substrate, 200 liters of the effluent were collected at one time. The maximum volume was determined by the available storage facilities. During the course of the investigation several 200 liter portions of secondary effluent were used.

Previous experience with this particular sewage plant effluent had shown that it was subject to chemical and biological change when stored unless it was stored at $0^{o}C$ or sterilized before being stored. Since cold storage space was not available each 200 liter portion was sterilized before it was stored. The sewage effluent placed in stainless steel buckets was heated under pressure to $121^{o}C$. and held there for one hour. During sterilization approximately 50 per cent of the ammonia nitrogen and 20 to 40 ppm of alkalinity as ppm $CaCO_3$ were lost from the sewage. In addition the pH increased from about 7.0 to 9.0 or 9.5 during sterilization. Ammonium chloride and sodium carbonate, reagent grade, were used to replace the ammonia nitrogen and alkalinity lost during the sterilization process. Reagent grade sulfuric acid was used to re-establish the pH to the range of 7.0 to 7.8. This adjustment was not absolutely necessary since the pH could have been lowered in the culture chamber with CO_2 but the use of sulfuric acid was more direct. Storage after sterilization produced no changes in the sewage effluent except for one 200 liter portion in which after about one month of storage all the inorganic nitrogen was converted to the nitrite form.

When necessary the sewage plant effluent was filtered through glass wool to remove turbidity before sterilization. The chemical composition of the various 200 liter portions of sewage plant effluent, before sterilization, is given in Appendix III. The sterilized effluent was stored in the constant temperature room housing the algal cultures.

The average temperatures in the two culturing chambers during the seven and one-half months of testing were 19.8°C and 20.2°C. Both of these temperatures are referred to as a nominal value of 20°C on all the graphs.

The organism used for these studies was the blue green alga *Oscillatoria*. The species has been tentatively identified as *O. rubescens* by Dr. Beeton, Sanitary Biologist, at the University of Michigan. The cultures were not pure cultures but *O. rubescens* was the dominant algal species. The *Oscillatoria*, obtained from the secondary sewage effluent, was capable of keeping the contaminants to a minimum. The major contaminant was *Chlorella* which never represented more than 10 per cent of the total number of algal particles. The contribution of the *Chlorella* to light attenuation and dry weight was much less than the 10 per cent value due to the vast difference in size between a *Chlorella* cell and an *Oscillatoria* particle.

To obtain various nutrient concentrations for the algal culture influents the sterilized secondary sewage effluent was diluted with distilled water. Since it was not possible to conduct all the necessary runs with one portion of sewage plant effluent it was necessary to measure the nutrient con-

centration by some means other than volume dilution. Inasmuch as it has been indicated that nitrogen and not phosphorus limits algal growth in secondary sewage plant effluents total inorganic nitrogen (TIN) was selected as the major parameter to measure the nutrient concentration (140). Total inorganic nitrogen is defined as the sum of the ammonia, nitrate and nitrite nitrogen all expressed in terms of nitrogen. Total inorganic nitrogen was used, therefore, to determine the dilutions of secondary effluent to be used and to express the results of the investigation. This decision made the TIN in the influent of the cultures the basic independent variable for the investigation. Inorganic phosphorus determinations were made but these did not represent a controlling parameter.

Data were collected for a particular test run only after the cultures had reached stability in terms of both growth rate and nutrient concentration in the substrate-effluents of the cultures. The minimum acceptable stability time was set at three days, although, data for any of the test runs were never collected for less than a week.

Both culturing chambers and their associated cultures were used to collect data for the four different culturing conditions. This was done to eliminate any possible differences in the cultures from the data. After the first few runs it was found that the cultures reached stability much more quickly if changes were made from low to high influent TIN values than if the reverse procedure was employed. In any event at least two and possibly as much as three days are required for stability to be attained.

For a constant density, semi-continuous dilution culture there are three nutrient concentrations to be considered. These three are the nutrient content of the culture influent, substrate-effluent and the quantity of nutrient removed by the culture organisms. The term substrate-effluent is used to indicate that the substrate and effluent of such cultures have the same physical, chemical and biological characteristics.

Figure 10 shows the growth rates of the four different cultures as a function of the influent-TIN expressed in terms of ppm N. Although the two different culture densities differ in absolute value the general relationship is the same. At the low value of influent-TIN, relatively speaking, there is a critical region where growth rate was directly proportional to influent-TIN. Increasing the influent-TIN above the critical region decreases the dependency of growth rate on influent-TIN since the relationship becomes curvilinear in nature. This same general relationship has been found by others to express growth rate as a function of temperature and light intensity (1)(8)(33).

It is apparent from Figure 10 that both light intensity and culture density influence the relationship between growth rate and influent-TIN. It would be anticipated that temperature would also affect this relationship, however, this can not be determined here since all the cultures were operated at the same temperature.

The combination of light intensity and culture density dictated that maximum growth rate which the cultures could attain. The span of the critical region, that portion of

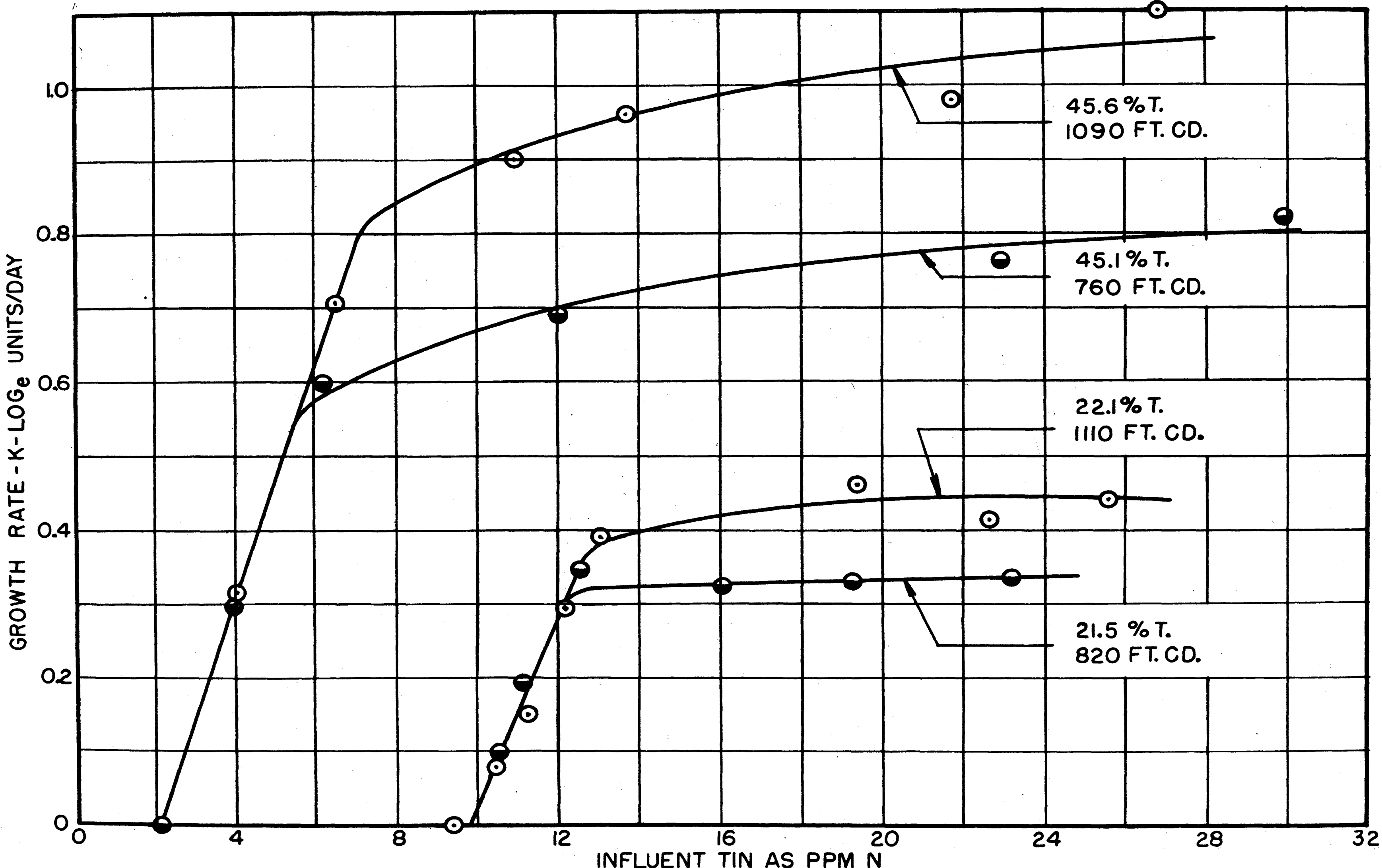


FIG.10 GROWTH RATE AS A FUNCTION OF INFLUENT TIN FOR OSCILLATORIA CULTURES.

the curve where growth rate is directly proportional to influent-TIN, depends, also, on light intensity and culture density. An increase in light intensity for a given culture density increased the span of the critical region. The same effect was obtained when culture density, for a given light intensity, was reduced. This can be interpreted to mean that the span of the critical region is a function of the maximum possible growth rate for the given environmental conditions.

The slope of the curve or the extent of the dependency of growth rate on influent-TIN, in the critical region, was independent of both light intensity and culture density. In Figure 10 then it is seen that the slopes of the two curves in the critical region are the same. The absolute influent-TIN concentration required, however, to produce a given growth rate in the critical region was about five times higher for the high density cultures than for the low density ones. The high density cultures had approximately twice the organism concentration of the low density cultures.

The rate of change in growth rate, beyond the critical region, with increasing influent-TIN values increased as the culture photosynthetic activity increased. The growth rate of the 21.5%T-820 ft.cd. culture was essentially independent of influent-TIN above the critical region. Growth rate increase for a given increase in the influent-TIN was greatest for the 45.6%T-1090 ft.cd. culture.

Figure 10 in summary then shows that growth rate of an algal culture is dependent on the nutrients supplied to the culture. The quantity of nutrients required to support a

given growth rate, when nutrients are limiting, increases as culture density increases. The nutrient requirement is not a linear function of organism density. For a given culture density when nutrients become limiting the growth rate is independent of light intensity. Although the amount of nutrients required to produce growth increases with culture density, an incremental increase in the nutrients supplied, above the base requirement, produces an increase in growth rate which is independent of light intensity and culture density. As the amount of nutrients supplied to a culture increases the dependency of growth rate on nutrients supplied approaches zero.

For tertiary sewage treatment it is necessary to know not only the influence of nutrients on the growth of algae but also the quantities of nutrients removed from the substrate during growth. Figure 11 shows the removal of TIN as ppm N as a function of the influent-TIN. For the two low density cultures the TIN removed was directly proportional to the influent-TIN. The 45.6%T-1090 ft.cd. culture was slightly more effective in the removal of nitrogen than the 45.1%T-760 ft.cd. culture, particularly at the higher concentrations of influent-TIN.

The relationship between TIN removal and influent-TIN was identical for the two high density cultures. The proportionality is linear up to about 19 ppm TIN-N in the influent. Beyond this value the quantity of nitrogen removed decreased curvilinearly as the influent-TIN increased. At the high culture density the incident light did not influence

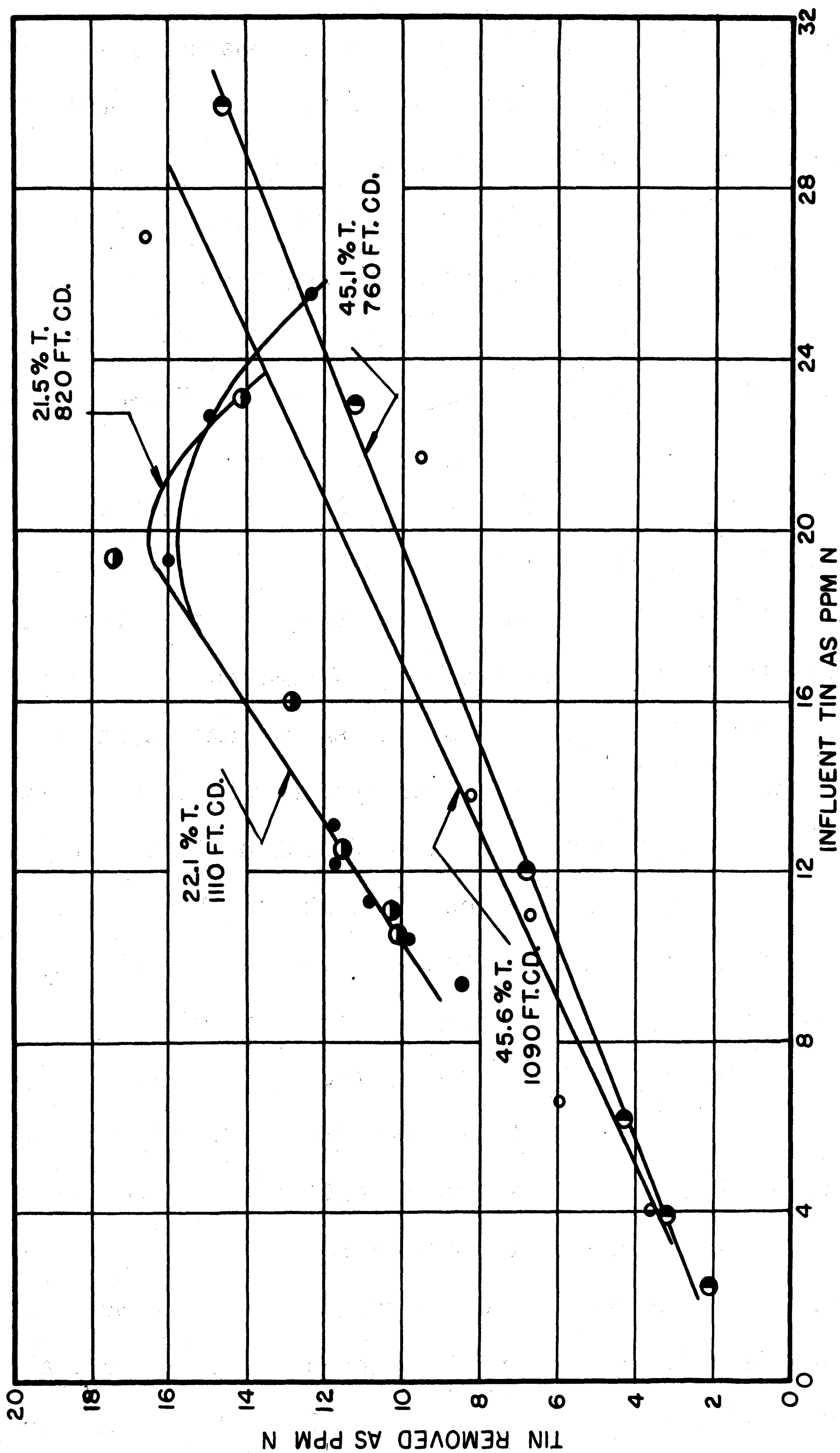


FIG.11 TIN REMOVAL AS A FUNCTION OF INFLUENT TIN FOR OSCILLATORIA CULTURES.

the amount of TIN removed by the organisms.

For high culture densities there is apparently an optimum influent nutrient concentration which results in a maximum quantity of nutrients being removed from the substrate. No such relationship seems to exist for low density cultures. In addition, at low culture densities increased light intensity increases slightly the amount of TIN removal. Light intensity has no significant effect, however, at high culture densities.

Since the TIN removed is a function of the TIN supplied to the culture it was of interest to determine the effect the quantity of TIN removed, has on growth rate. Figure 12 shows growth rate as a function of TIN removed.

Comparison of Figures 10 and 12 shows some definite differences in the relationship of growth rate as a function of TIN removal and growth rate as a function of influent-TIN. Growth rate as a function of influent-TIN went from a linear to a curvilinear relationship with increasing influent-TIN concentration. Growth rate as a function of TIN removed for each of the four culturing conditions, however, is represented by two linear functions. One linear function, having a rather steep slope, represents conditions when the cultures were experiencing relatively low TIN removal. The other linear function, having a lower slope, shows the proportionality between growth rate and TIN removed when TIN removal, relatively speaking, was high. It is also interesting to note that growth rate as a function of TIN removed does not become independent of light intensity as was the case with

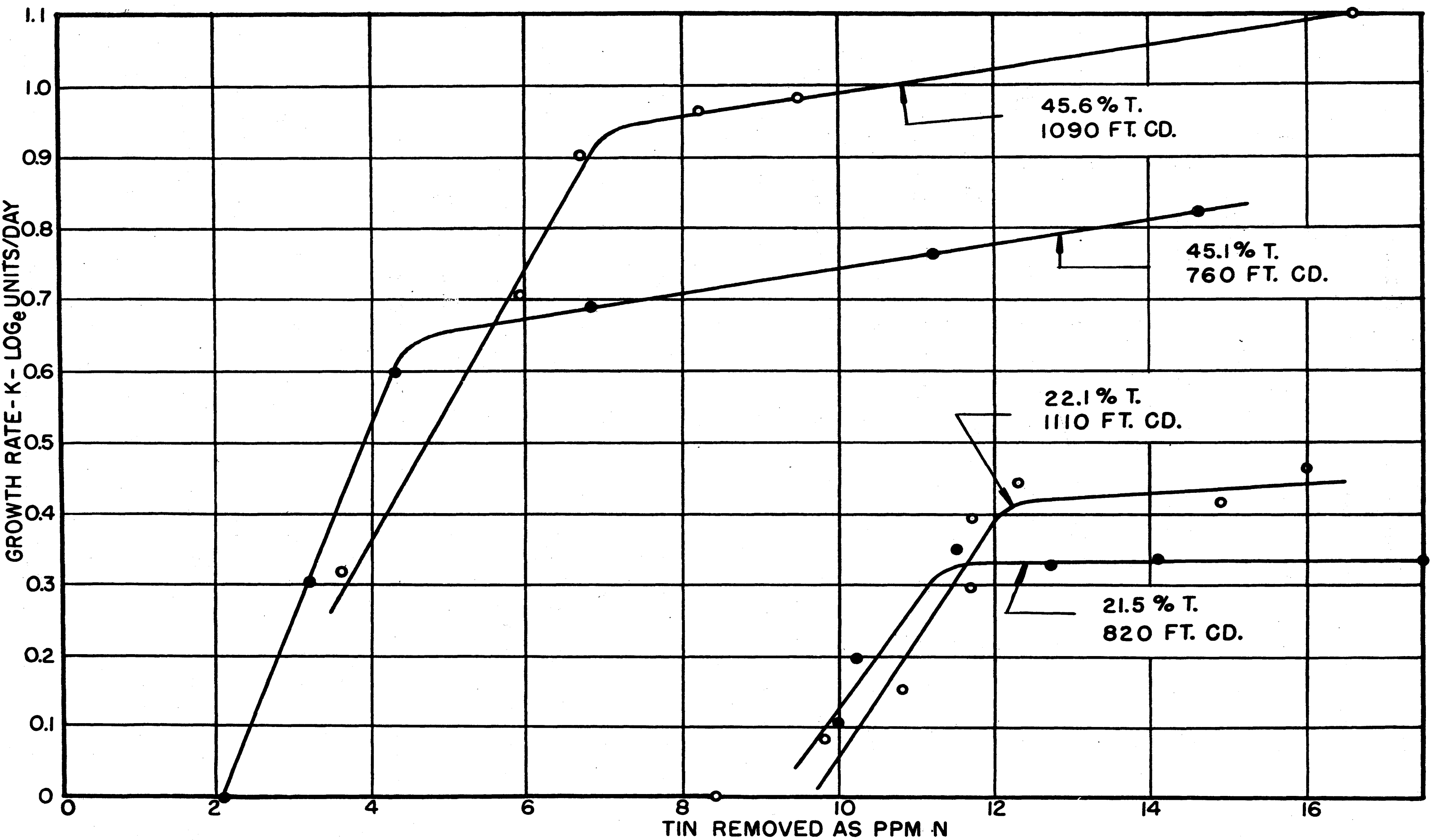


FIG.12 GROWTH RATE AS A FUNCTION OF TIN REMOVED FOR OSCILLATORIA CULTURES.

growth rate expressed as a function of influent-TIN.

In the critical region (the zone of high dependency of growth rate on TIN removed) the extent of the proportionality (slope of the curve) between growth rate and TIN removed is independent of light intensity for the high density cultures and dependent on light intensity for the low density cultures. The span of the critical region for TIN removed, as for the span of the critical region for influent-TIN, was a function of the photosynthetic activity of the culture. Also, in the critical region for both culture densities the lower light intensity produced the greater growth rate for a given value of TIN removed. The amount of TIN removal required to produce a given growth rate in the critical region was about four times higher for the high density cultures than for the low density cultures.

In an attempt to determine the reasons for the differences in the relation of growth rate and TIN removed for the various cultures the relationships between growth rate, influent-TIN and substrate-effluent-TIN were determined. Figures 13, 14, 15, and 16 show these relationships for the 45.1%T-760 ft.cd., 45.6%T-1090 ft.cd., 21.5%T-820 ft.cd. and the 22.1%T-1110 ft. cd. cultures respectively.

Consideration of these four figures with respect to conditions existing in the critical region for each culture shows: (1) that the substrate-effluent TIN for the high density cultures reached a minimum value and remained constant at this value throughout the critical region of nutrient influence, (2) the TIN in the substrate-effluents of the low

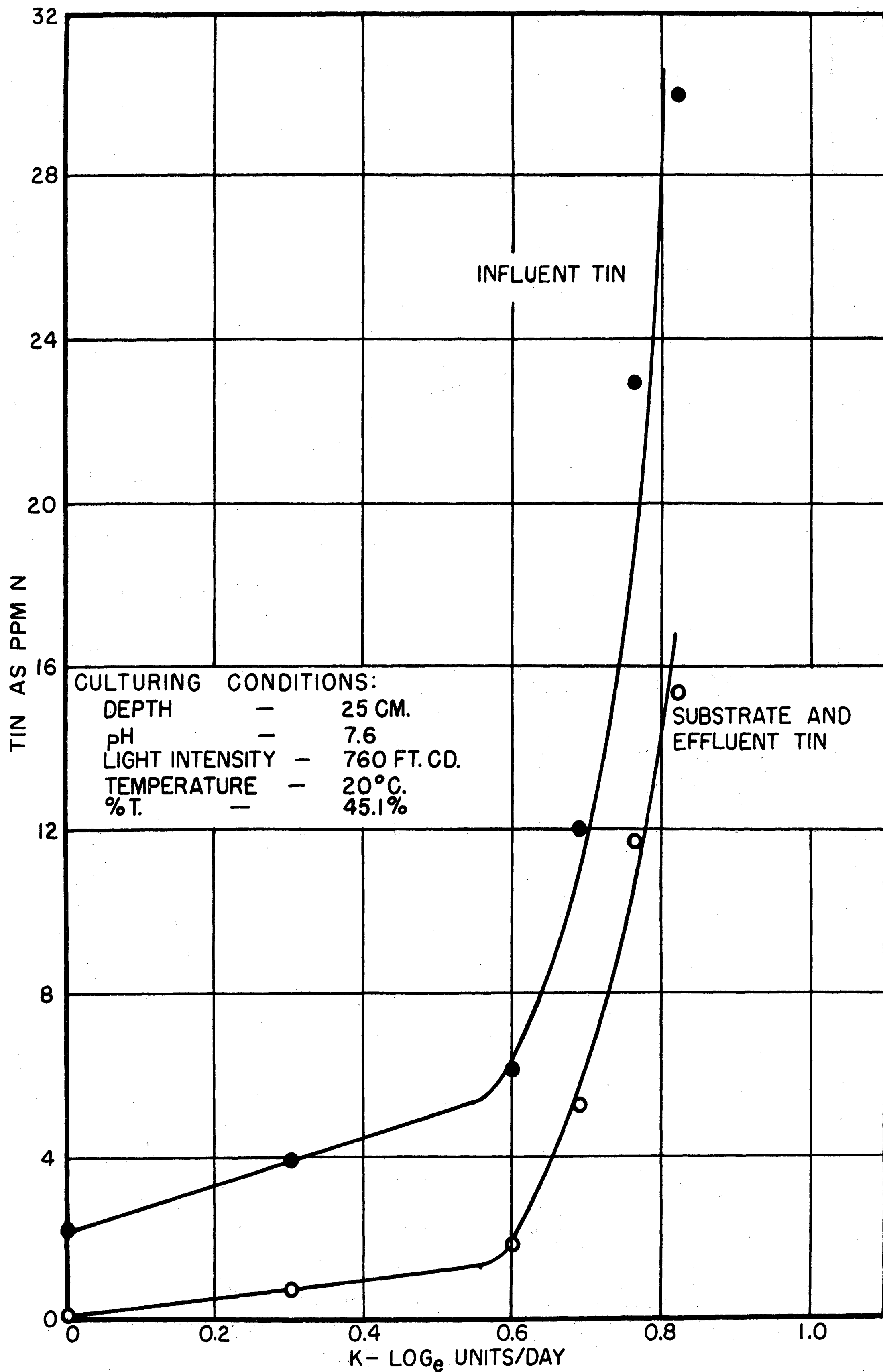


FIG. 13 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE TIN AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

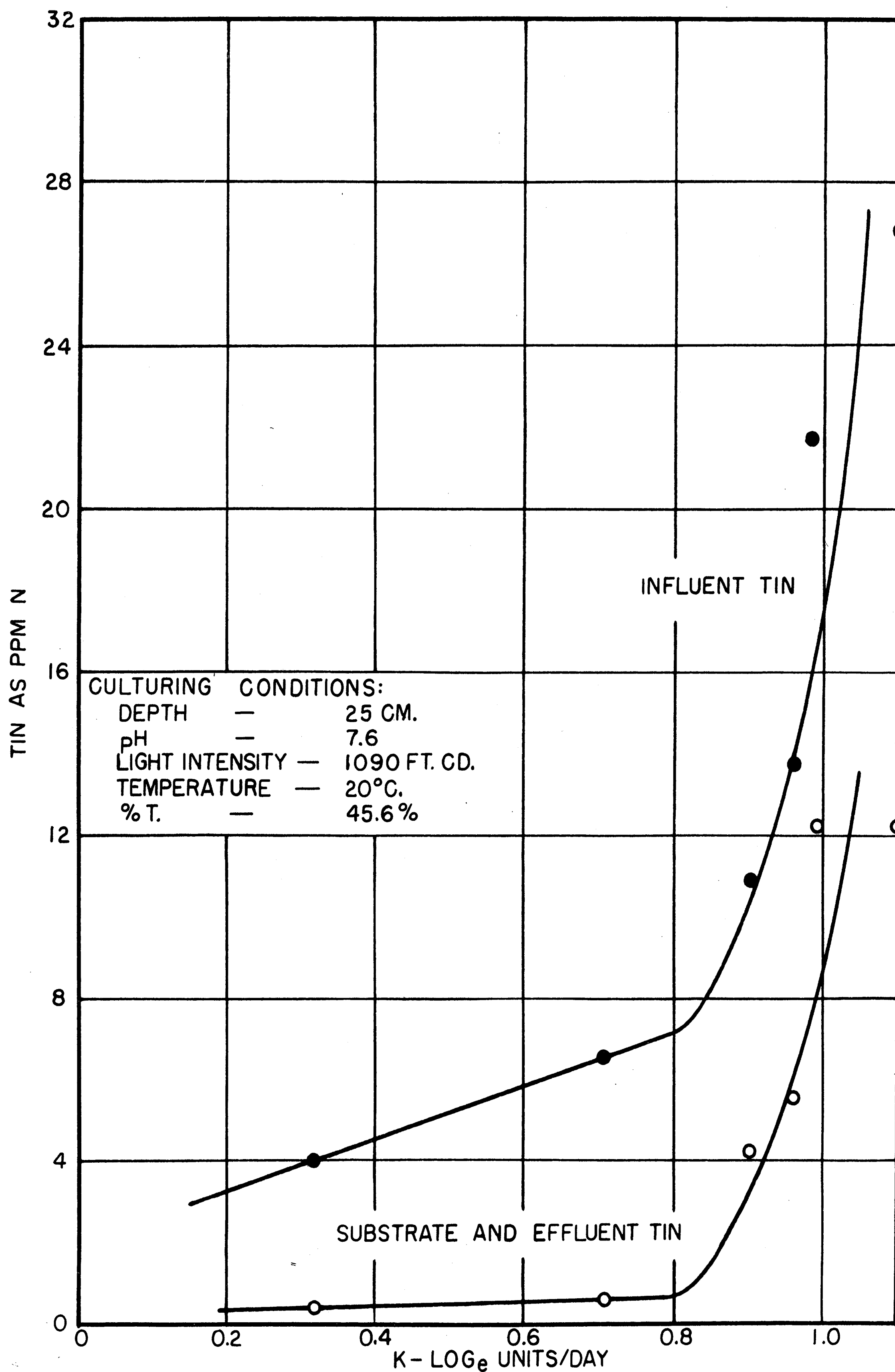


FIG. 14 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE TIN AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

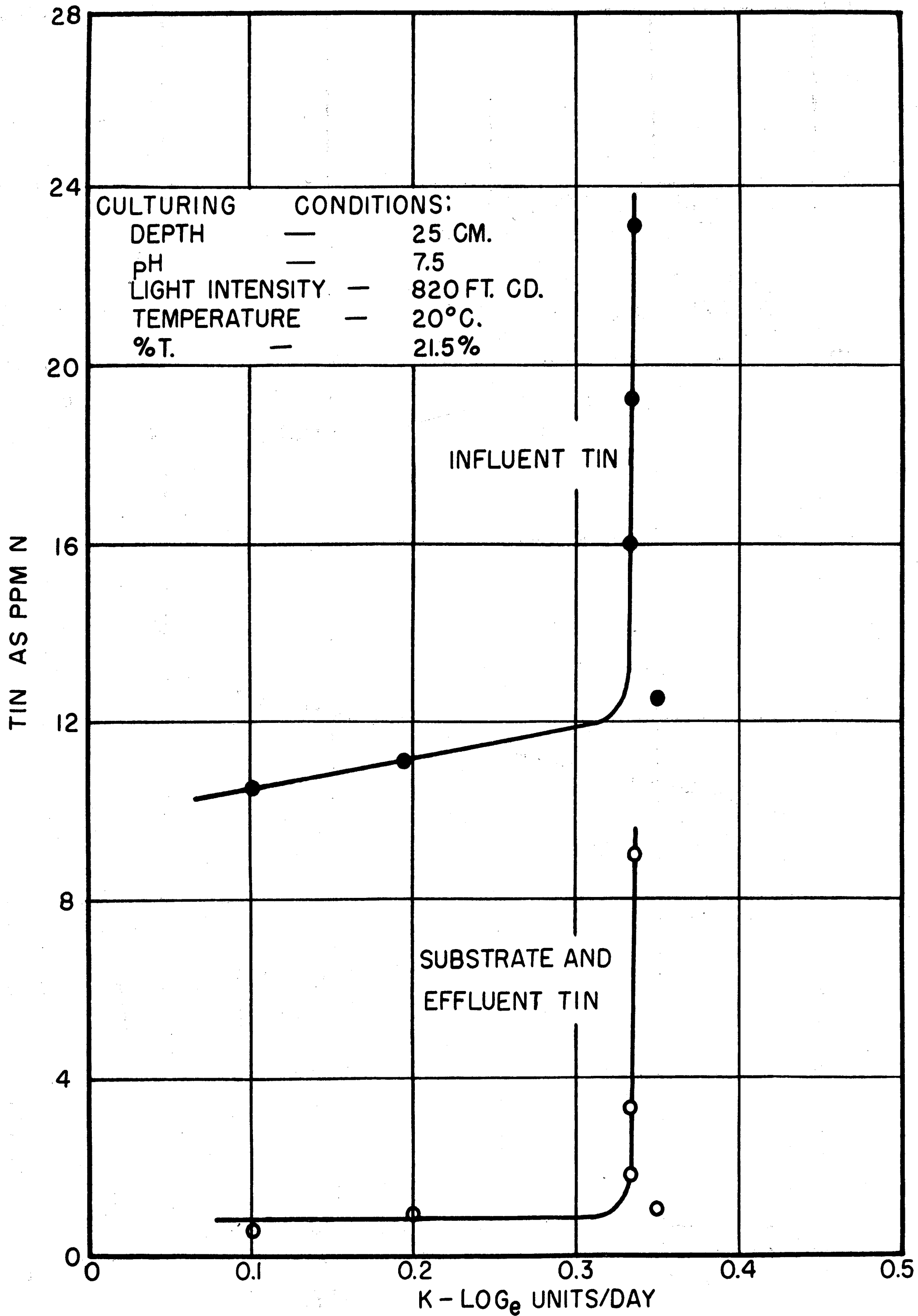


FIG. 15 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE TIN AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

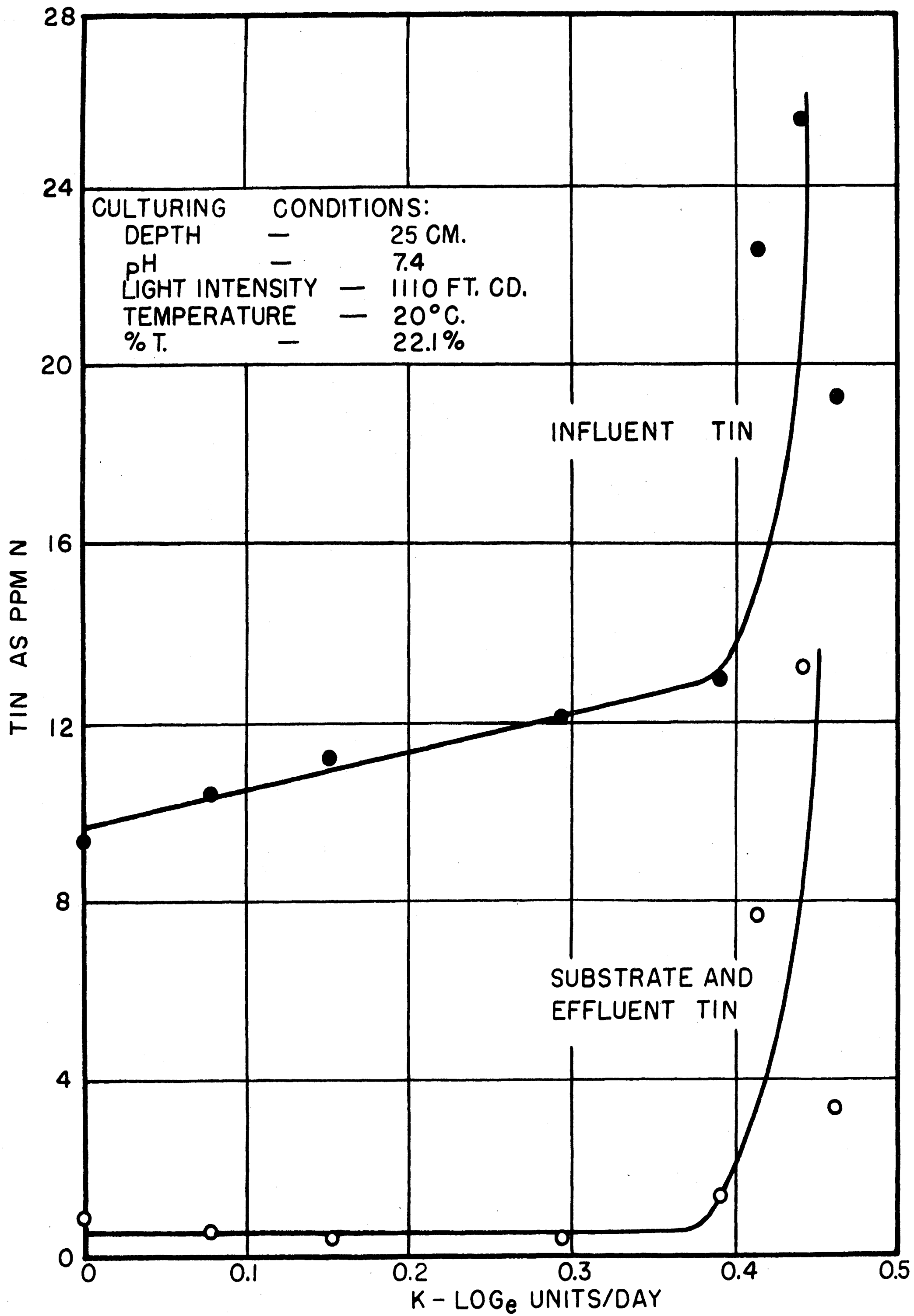


FIG. 16 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE TIN AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

density cultures although reaching low concentrations did not attain a constant minimum value in the critical region, (3) the TIN in the substrate-effluent for the two cultures exposed to the low light intensity was higher than that for the same respective cultures exposed to the high light intensity, (4) the culture with the highest photosynthetic activity (45.6%T-1090 ft.cd.) produced the lowest TIN concentration in the substrate-effluent, (5) TIN removal for the two high density cultures was independent of the substrate-effluent-TIN, being dependent only on the influent-TIN, (6) the TIN removed by both the low density cultures was dependent on both the influent and substrate-effluent TIN, but to different degrees.

From the observational conclusions of the previous paragraph some relationships between the conditions existing in the critical regions of the curves of Figure 12 and the quality changes in the substrate-effluent can be determined. The 21.5%T-820 ft.cd. and 22.1%T-1110 ft.cd. cultures experienced minimum constant TIN values in the substrate-effluent throughout the critical region. The slopes of the curves relating growth rate and TIN removed are equal. These slopes are also the lowest of the four culturing conditions. For these two cultures the TIN removed in the critical region was only a function of the influent-TIN. For the 45.6%T-1090 ft.cd., the TIN in the substrate-effluent was not constant but decreased slightly with decreasing influent-TIN. This culture had a slightly steeper slope in the critical region of growth rate versus TIN removed than that of the two previous cultures.

The TIN removed by this culture was, therefore, mostly dependent on the influent-TIN but the TIN in the substrate-effluent did exert some influence. The 45.1%T-760 ft.cd. culture, experienced the greatest change in substrate-effluent TIN with decreasing influent-TIN in the critical region. TIN removal for this culture was about equally dependent on the TIN in the substrate-effluent and in the influent. The slope of the curve of growth rate as a function of TIN removed for this culture was the steepest of four comparable slopes.

The higher growth rates of the low light intensity cultures in the critical region, as compared to the growth rates of the respective high light intensity cultures can be correlated to the quantities of substrate-effluent TIN for the appropriate growth rates of TIN removed values. Both the TIN in the substrate-effluent and the growth rate of the 45.1%T-760 ft.cd. culture exceeded those values of the 45.6%T-1090 ft.cd. culture for a given TIN removed value in the critical region. For the two high density cultures, the growth rate and substrate-effluent-TIN concentration of the 820 ft.cd. culture exceeded those of the 1110 ft.cd. culture for any selected TIN removed value in the critical region. These aforementioned conditions apply only up to the point where the growth rates of the high light intensity cultures became greater than the growth rates of the low light intensity cultures. At this point the difference in growth rates is due to the greater growth potential of the high light intensity cultures.

From the previous paragraphs then, the rate of change in

grwoth rate with a change of TIN removed in the critical region decreases as the substrate-effluent TIN becomes increasingly independent of influent-TIN. The rate of change being a minimum, but not zero, when the substrate-effluent TIN is completely independent of the influent-TIN. The absolute values of TIN removed defining the critical region depend on the organism concentration and light intensity. As culture density increases so does the TIN removed value associated with zero growth rate.

The greatest growth rate for a given culture density in the critical region, will be experienced under the lighting conditions which provide the greatest TIN concentration in the substrate-effluent. Whether or not TIN removal and growth rate become independent of substrate-effluent TIN will be determined by the culture density. At high densities independence is established, at low densities it is not. The actual quantity of TIN in the effluent of a culture is a function of the photosynthetic activity of the culture. As the photosynthetic activity of a culture increases the effluent-TIN decreases. This applies even when the substrate-effluent reaches a constant value due to culture density.

Investigations to determine if increased photosynthetic activity could substantially reduce the substrate-effluent-TIN of the high density cultures and make the effluent-TIN of low density cultures independent of influent-TIN would be worthy endeavors.

Turning attention to those portions of the curves in Figure 12, beyond the critical region, the portion of the

curves with the flatter slopes, it is observed that the conditions are different for the high and low density cultures. When growth rate was expressed as a function of influent-TIN (Fig. 10) the rate of increase in growth rate with increasing influent-TIN increased as photosynthetic activity increased, and decreased as influent-TIN increased. For the same range of growth rate values the rate of increase in growth rate with increasing TIN removed, for the low density cultures, was independent of light intensity and TIN removed. The rate of increase in growth rate with increasing TIN removed for the 22.1%T-1110 ft.cd. culture was also constant. The magnitude of the rate was lower than that for the low density cultures. For the 21.5%T-820 ft.cd. culture, growth rate was independent of TIN removed above the critical region.

The 21.5%T-820 ft.cd. culture experienced light limited growth at TIN removed values above the critical region of the culture. However, the fact that the 22.1%T-1110 ft.cd. culture experienced increased growth rate with increased TIN removed indicates that light intensity does have an effect on the growth rate-TIN removed relationship, at least at high culture densities. Light does not appear to have been a factor in this relationship for the low density cultures, once the initial difference in growth rates due to light intensity had been established at the upper limit of the respective critical regions.

Comparing the data of the two low density cultures it is found that for a given TIN removal the substrate-effluent TIN is essentially the same for both cultures. This TIN

value is three to four times that of the TIN in the substrate-effluent of the 22.1%T-1110 ft.cd. culture. The slope of the curves for the low density cultures is 2.7 times the slope for the 22.1%T-1110 ft.cd. culture. For a given TIN removal the influent-TIN concentrations of the three cultures are almost equal. It is possible that the explanation for the relationship between growth rate and TIN removed, above the critical regions, may be related to the substrate-effluent TIN. The nitrogen content of the algae might also be a factor. The nitrogen content of the sewage was not sufficiently high to determine at what TIN removed values the growth rates of the three most photosynthetically active cultures would be independent of TIN removal.

In summary Figures 12, 13, 14, 15 and 16 show, (1) when nitrogen is highly limiting, growth rate is a function of the quantity of TIN in the culture substrate and some function of the ratio of organism concentration to nitrogen concentration, (2) the rate of increase in growth rate with an incremental increase in TIN removed in the nutrient limiting region increases as the dependency of TIN removed on substrate TIN increases, the minimum rate occurs at complete independency, (3) in the region where TIN is only slightly limiting, the rate of growth rate increase with increasing TIN removed appears to be a function of the substrate TIN concentration, (4) for the same region as in (3) the actual growth rate for a given TIN removal depends on light intensity and culture density, (5) high density cultures attain a minimum constant nitrogen content in their substrates when nitrogen is limiting,

low density cultures do not, (6) in the nitrogen limiting region the culture with the highest photosynthetic activity produces the lowest TIN concentration in the effluent.

To further consider the problem of nitrogen removal by algal cultures the relationships shown in Figure 17 were established. This figure shows the concentration of TIN removal as a function of productivity. Culture productivity is the product of growth rate times the culture density. The productivity used here is the surface productivity which is the weight of algal material produced per unit time per unit of culture surface area.

That nitrogen removal should be a function of productivity appears reasonable in as much as the nitrogen removed from the substrate is assumed to have been assimilated by the algae. The curves of Figure 17 substantiate that TIN removal is a function of productivity. The extent to which TIN removal was a function of productivity, however, varied with culturing conditions. The rate of increase in TIN removal with productivity was greatest for the 45.1%T-760 ft.cd. culture. The 45.6%T-1090 ft.cd. culture experienced the next greatest rate of increase. Both of the high density cultures had the same rate of increase in TIN removal with increasing productivity. This rate was the lowest exhibited by the four culturing conditions.

The differences in the rates of change in TIN removal with increasing productivity arose from several different sources. For the 21.5%T-820 ft.cd. culture the low dependency of TIN removal on productivity stems principally from

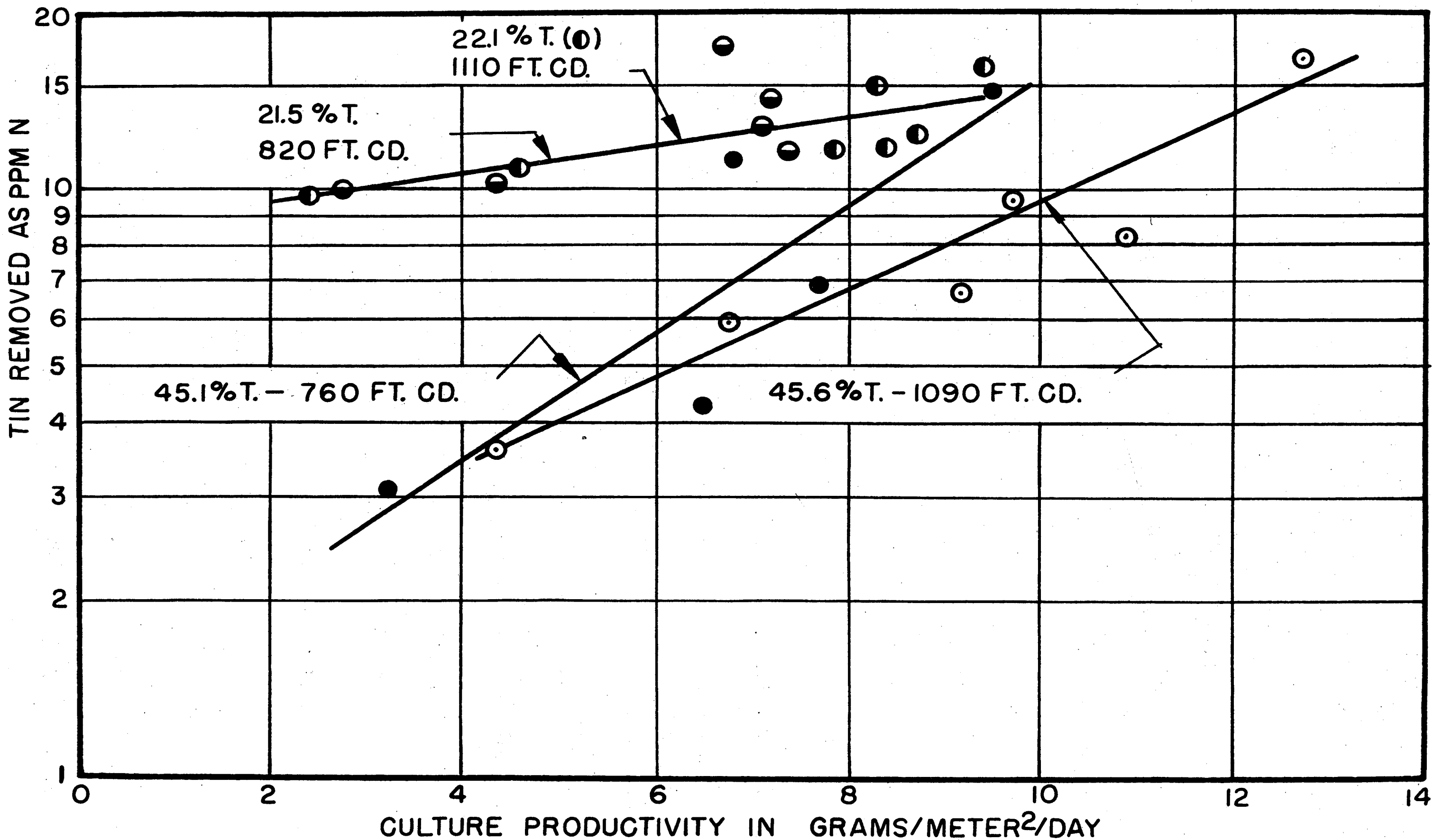


FIG. 17 TIN REMOVAL AS A FUNCTION OF CULTURE PRODUCTIVITY FOR FOUR DIFFERERENT OSCILLATORIA CULTURES.

the fact that only 2 ppm TIN-N removed separates zero growth and maximum growth rate. Within this 2 ppm range the growth rate goes from zero to about 0.3 $\log_e$ units per day, while the ppm TIN-N removed increases from about 9 to 11 ppm. The large change in productivity is accompanied by only a small change in TIN removed. Also, for this culture, growth rate was independent of TIN removed above the critical region. Above about 12 ppm TIN-N removed, no correlation between TIN removed and productivity would, therefore be anticipated. That this situation did exist is demonstrated by the cluster of points at about 7 grams per meter^2 per day of productivity in Figure 17.

The same general conditions that pertained to the 21.5%T-820 ft.cd. culture also apply to the 22.1%T-1110 ft.cd. culture. The one exception for the latter culture is that growth rate did not become independent of TIN removal (Fig. 12). The 22.1%T-1110 ft.cd. culture initially experienced a large increase in productivity accompanied by a small increase in TIN removed. In the second phase a large increase in TIN removal was accompanied by a small increase in productivity. This latter condition accounts for the scatter of the points at the upper end of the curve for the 22.1%T-1110 ft.cd. culture in Figure 17.

The difference in the slopes of the curves in Figure 17 for the two low density cultures is due, at least in part, to the great difference in growth rates of the two cultures for the same amount of TIN removed. The 45.6%T-1090 ft.cd. culture has the flatter slope because for the greater portion of

the TIN removed values its productivity was about 40 per cent greater for any given value of TIN removed.

Although not clearly determinable from Figures 12 or 17 there is expressed in the relationships of the two figures the fact that the nitrogen content of the algae varied with the culturing conditions. The productivity of the cultures per ppm of nitrogen removed is expressed as a function of influent-TIN in Figure 18. The curves of Figure 18 show that the quantity of algae produced per unit of nitrogen removed was highly dependent on both light intensity and culture density. Light intensity however, was a significant factor only at influent-TIN concentrations greater than that associated with the upper limit of the critical region.

For all the cultures the quantity of algae produced per ppm of nitrogen removed increased at a decreasing rate with increasing influent-TIN up to a maximum value. The maximum values occurring at the influent-TIN values defining the upper limit of the critical region for the different culturing conditions. As the influent-TIN was increased above the maximum point the production of algae per ppm of nitrogen removed decreased at an increasing rate for the 45.6%T-1090 ft.cd., 221.%T-1110 ft.cd. and 21.5%T-820 ft.cd. cultures. The 45.1%T-760 ft.cd. culture, however, decreased at a decreasing rate.

The condition of changing quantities of nitrogen required to produce a quantity of algae obviously influences the relationship of TIN removed and productivity. The greater the overall change in the value of productivity per ppm TIN-N

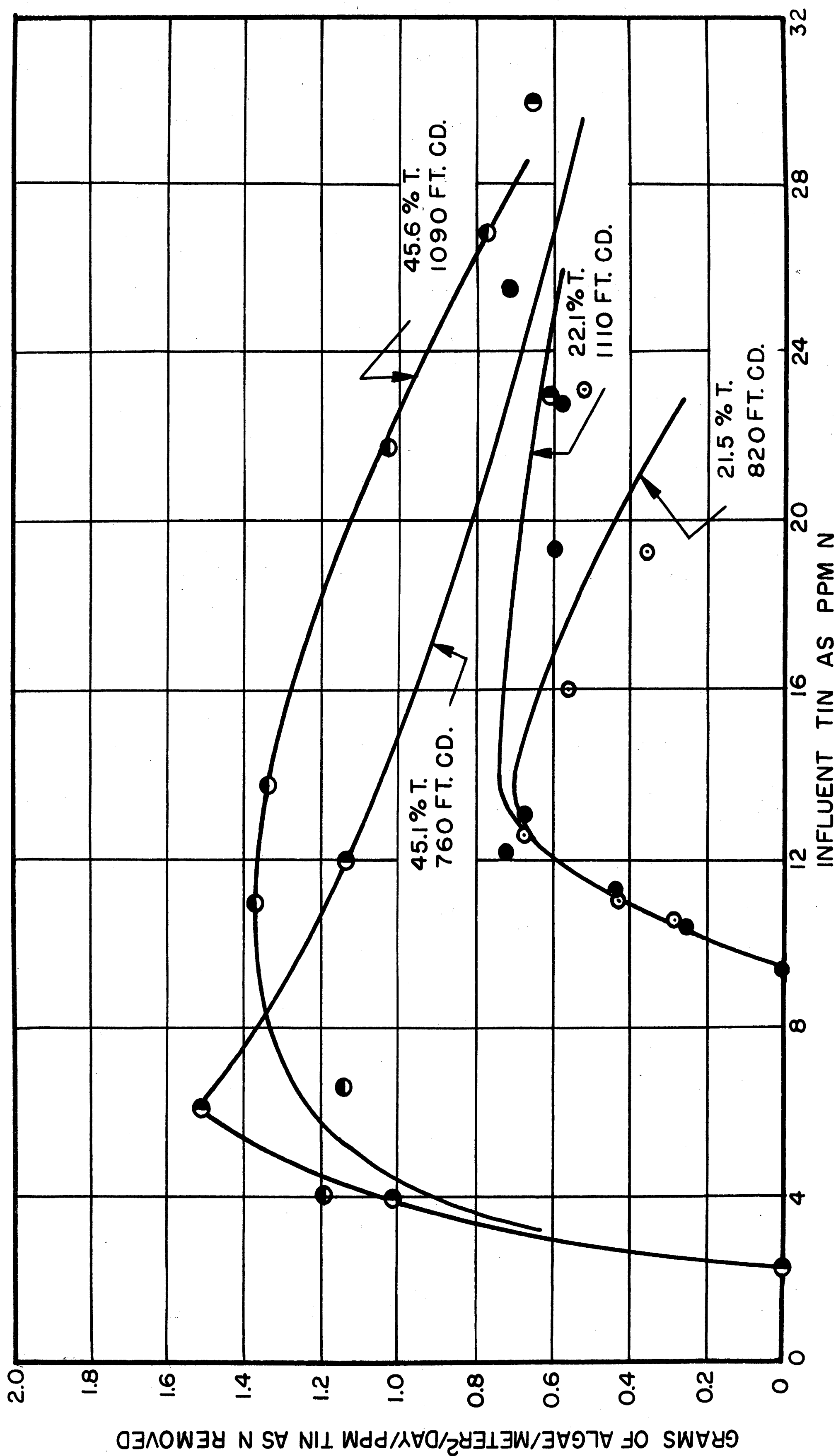


FIG. 18 EFFICIENCY OF USE OF TIN AS A FUNCTION OF INFLUENT TIN FOR FOUR DIFFERENT OSCILLATORIA CULTURES.

removed the lower will be the correlation between productivity and TIN removal. It is apparent that TIN removal is no simple function of productivity. The relationship is dependent on light intensity, growth rate, culture density and the quantity of TIN being supplied the culture.

Figure 18 shows that there was a definite influent-TIN value for each culturing condition which resulted in the maximum production of algal material per ppm of TIN nitrogen removed. To obtain the most effective use of the nitrogen supplied to a culture, the influent-TIN concentration should be slightly in excess of that indicated by the upper limit of the critical region. For tertiary sewage treatment, however it is the removal of nutrients, and not the production of algal material that is important. If, therefore, the conditions of algal density and influent-TIN concentration of a culture being used for tertiary sewage treatment, were such that the culture were operating just at the upper limit of the critical region the efficiency of the process would be at a minimum.

In order to better evaluate the cultures in terms of their ability to remove nutrients, particularly TIN, the quantity of nitrogen removed per unit surface area per day per ppm algae, by weight, in the culture was expressed as a function of growth rate. This relationship is shown in Figure 19. The slopes of the curves in Figure 19 are as would be expected from consideration of Figure 12. In this latter figure, for any given quantity of TIN removed the growth rate of the cultures increased as culture density

decreased and light intensity increased. In the region of comparable growth rates the ability of the algae to consume nitrogen increased as culture density increased and light intensity decreased. For the conditions investigated, therefore, the less favorable the environment for algal growth, the greater was the ability of the algae to remove TIN from the substrate. This condition prevails when an adverse growth climate is produced by reduced light intensity or increased culture density or by both factors.

The previous comparison was made on the basis of equal growth rate for different culturing conditions. For a given culture condition, however, the quantity of nitrogen removed per ppm algae increased as the growth rate increased. It should be noted that for a given culture density the relationships of Figure 19 apply to total TIN removed per day as well as to the removal per ppm of algae. The differences, however, in the two culture densities will be amplified by a factor of two if total nitrogen removal per day is considered.

It should be remembered when referring to Figure 19 that both the growth rate and TIN removal were controlled by the influent-TIN of the culture. Whether the same relationships would apply if growth rate had been controlled basically by light intensity and culture density is not known.

The relationships of Figure 19 are directly applicable to process design. Knowing the growth rate, which can be determined from Figure 10 by using the TIN concentration of the sewage, and the volume of sewage it is possible to determine from Figure 19 the factors necessary for the design of the pro-

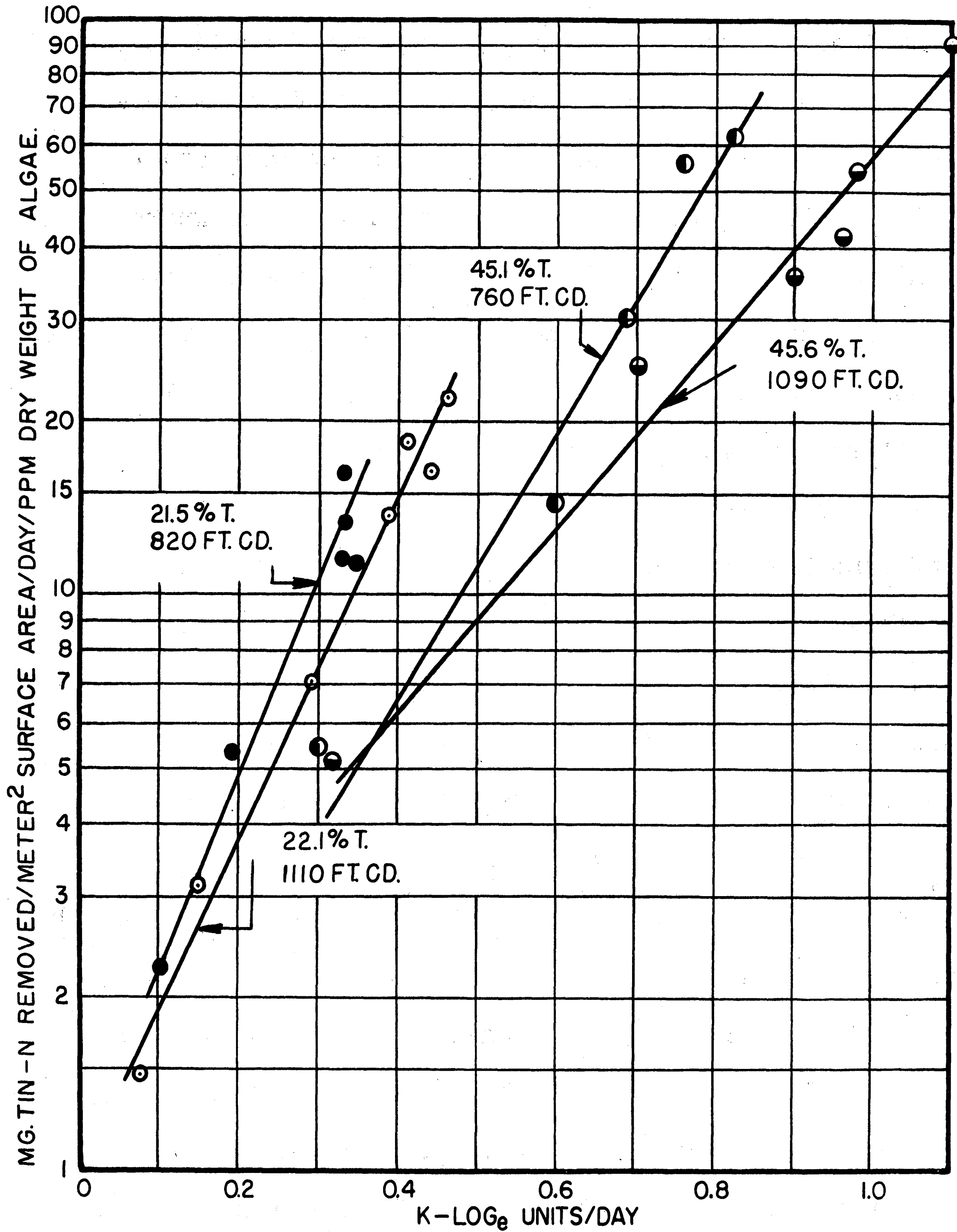


FIG. 19 RATE OF TIN REMOVAL PER UNIT OF SURFACE AREA PER UNIT CONCENTRATION OF ALGAE AS A FUNCTION OF GROWTH RATE FOR DIFFERENT CULTURES OF OSCILLATORIA.

cess. The volume, surface area and degree of treatment can all be determined. The factors so defined would apply only to those situations when the physical conditions were similar to those under which the present cultures were studied. The relationships determined in this study are not known at this time to be generally suitable for projection or interpolation.

To consolidate that portion of the data on TIN concentrations and algal growth which pertains most directly to sewage tertiary treatment, Figures 20 and 21 were constructed. Figure 20 shows the per cent TIN removal as a function of influent-TIN or growth rate for low density cultures. Figure 21 shows the same relationships for the high density cultures. The growth rate of the cultures across the tops of these figures are expressed in relation to the influent-TIN scale. These growth rate scales were determined from Figure 10.

The per cent removal at high influent-TIN values was independent of growth rate for influent-TIN values for the low density cultures. At low influent-TIN values the per cent removal increased at an increasing rate with decreasing influent-TIN values or growth rate. For the 45.6%T-1090 ft.cd. culture the per cent removal leveled off at 90 per cent at the low influent-TIN concentrations. The difference in the two curves is attributable to the substrate-effluent-TIN values of the two cultures at low influent-TIN values. The 45.6%T-1090 ft.cd. culture experienced only a slight change in substrate-effluent-TIN over the low range of influent-TIN values. This combination of decreasing influent-TIN and nearly constant substrate-TIN, results in a decreasing per cent

removal as influent-TIN decreased.

The shapes of the curves for the high density culture are opposite in character to those for the low density cultures. For both high density cultures the per cent removal increased at a decreasing rate with decreasing influent-TIN values. The 22.1%T-1110 ft.cd. culture experienced a point of maximum per cent removal. As influent-TIN was decreased below the maximum point the per cent removal decreased slightly. At no influent-TIN value did the per cent TIN removal become independent of the influent-TIN for the high density cultures. The shapes of the curves are the result of two factors: (1) the TIN removed at high influent-TIN values increased as influent-TIN values decreased (Fig. 11), (2) the substrate-effluent-TIN became independent of influent-TIN concentration at low influent-TIN values.

The effect of light intensity on the per cent of TIN removed was only slight. The greatest influence of light was noted for the low density cultures at low influent-TIN values.

It can be deduced from Figures 20 and 21 that more than one culture would be required to remove the major portion of TIN contained in a sewage. If the TIN concentration, and volume of the sewage were known, it would be possible using the curves of Figures 20 and 21 to select the optimum combination of culture densities and culture influent-TIN for the treatment of the sewage. Knowing the volume and determining the growth rates of the various cultures, the detention time and surface area of the culturing chambers could be determined.

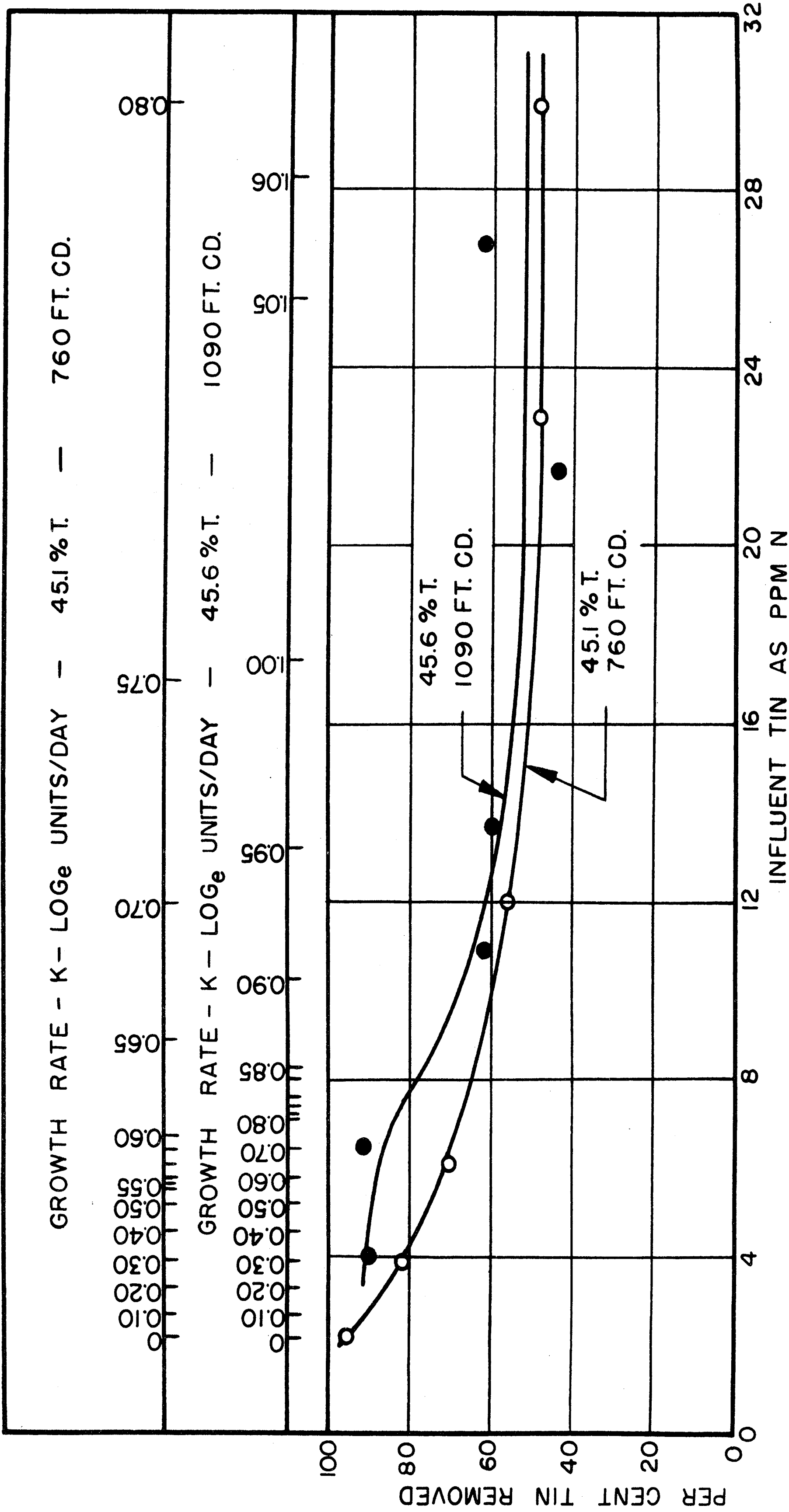


FIG. 20 PER CENT TIN REMOVED AS A FUNCTION OF INFLUENT TIN OR GROWTH RATE FOR TWO LOW DENSITY CULTURES OF OSCILLATORIA.

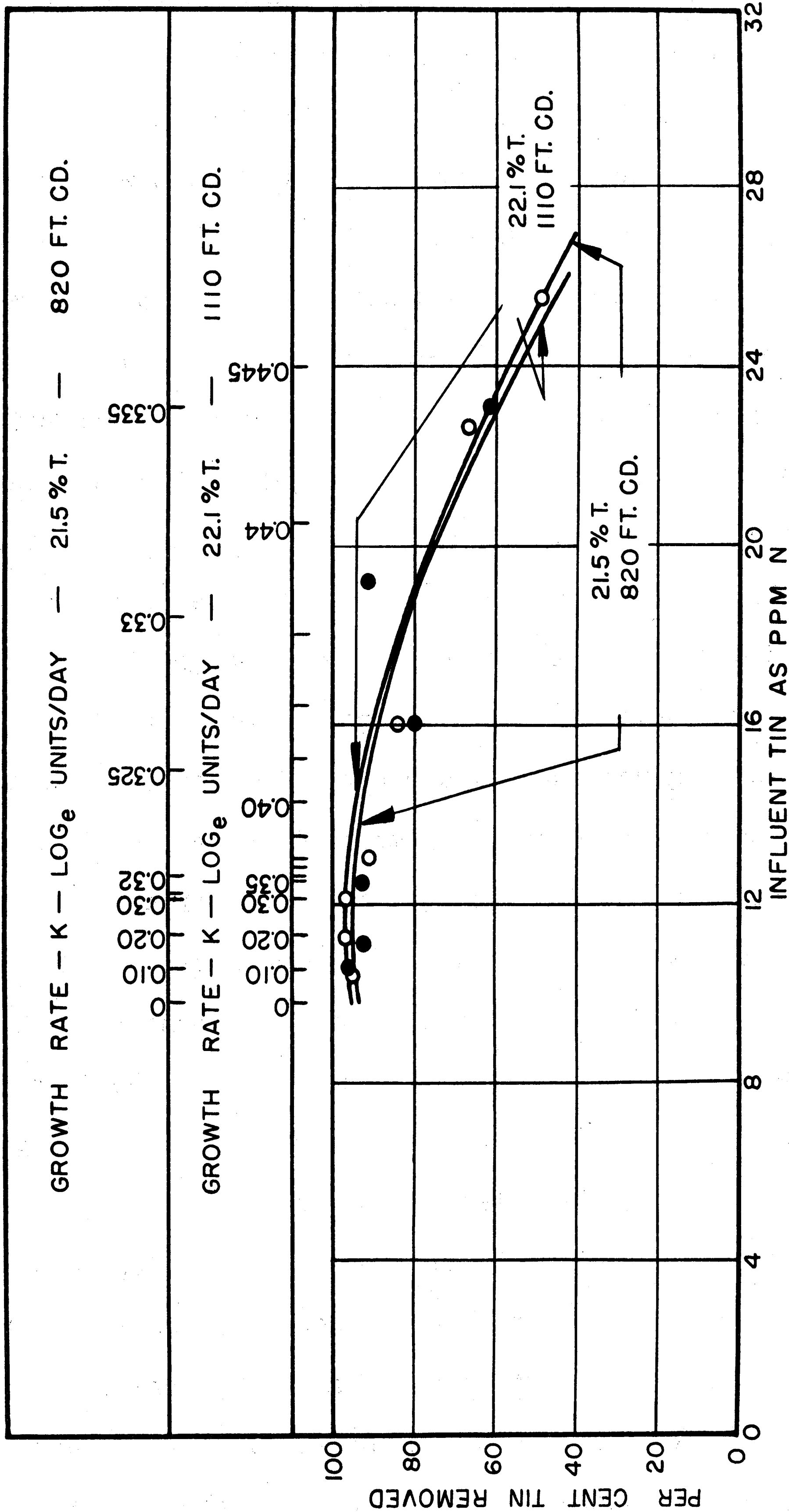


FIG. 21 PER CENT TIN REMOVED AS A FUNCTION OF INFLUENT TIN OR GROWTH RATE FOR TWO HIGH DENSITY CULTURES OF OSCILLATORIA.

The curves of Figures 20 and 21, for example, show that it would be possible to reduce a sewage TIN concentration of 24 ppm N by as much as 98 per cent if both densities of culture were utilized. The total detention time for such a removal would be about four days. These values were obtained as follows: (1) Figure 20 was entered at the 24 ppm value, this shows a potential removal of 50 per cent or 12 ppm TIN-N in the culture effluent and a detention time of about one day, (2) entering Figure 21 at the 12 ppm value indicates a possible reduction of 96 per cent or 0.5 ppm TIN-N in the culture effluent and a detention time of about 3.3 days, (3) the net reduction of TIN through the two cultures would be 98 per cent with a required detention time of 4.3 days. The greater number of curves available such as those presented in Figures 20 and 21, representing various culture conditions the more optimum could be the selection of the components of a treatment process.

The selection of the total inorganic nitrogen as the parameter representing the nutrient concentration made the orthophosphate values, to a major extent, dependent variables. Had the sewage plant effluent always contained the same nitrogen to phosphorus ratio it would not have been necessary to make a choice between the two macronutrients. This ratio, however, varied considerably as can be seen in Appendix III and, therefore, a choice had to be made. Making the orthophosphate a dependent variable does allow an evaluation of the value of TIN as representing the total nutrient conditions. Also the change in the nitrogen to phosphorus ratio made it possible, as will be shown later, to determine what

nutrient was growth limiting. Only the phosphorus in the orthophosphate form was determined on a daily basis.

Figures 22 and 23 show orthophosphate removal as a function of influent-TIN. The nitrogen to orthophosphate ratio in the portions of sewage effluent used for the two low density cultures was relatively constant in each case and the correlation in Figure 22 is therefore, reasonably good. For the high density cultures the nitrogen to orthophosphate ratio in the influent varied rather widely resulting in a much poorer correlation between orthophosphate removal and influent-TIN in Figure 23 as compared to Figure 22. Orthophosphate removal was more successfully accomplished at the high culture densities than at the low ones for a given influent-TIN value. The light intensity exerted a greater influence on the orthophosphate removal at the low culture densities than at the high culture densities. From Figures 22 and 23, it is apparent that the degree to which one nutrient can be used to represent the general nutrient conditions in a culture is highly dependent on the consistency of the ratio of the nutrients in the culture influent.

Figures 24, 25, 26 and 27 show the relationships between growth rate, influent and substrate-effluent orthophosphate for the 45.1%T-760 ft.cd., 45.6%T-1090 ft.cd., 21.5%T-820 ft.cd. and 22.1%T-1110 ft.cd. cultures respectively. The general relationships depicted in these four figures are very similar to the same types of relationships considered for TIN and growth rate (Figs. 13, 14, 15, and 16).

For the 45.1%T-760 ft.cd. culture both the influent and

substrate-effluent orthophosphate decreased as growth rate decreased. In the critical region of nutrient influence the quantity of orthophosphate removed remained constant at about 1.9 ppm while growth rate increased from 0.2 to 0.7 $\log_e$ units per day. Growth was, therefore, independent of the orthophosphate removal in this region. In the same growth range the TIN removal increased 1.8 times. The nitrogen to phosphorus removal ratio varied from 5.5 to 9.2. The higher ratio occurred at the higher growth rate.

The influent of the 45.6%T-1090 ft.cd. culture contained less orthophosphate for a given influent-TIN value than did the influent of the 45.1%T-760 ft.cd. culture. In the critical region of nutrient control, for the 45.6%T-1090 ft.cd. culture, the substrate-effluent orthophosphate changed only slightly with decreasing influent orthophosphate or growth rate. As the growth rate increased from 0.3 to 0.7 $\log_e$ units per day the substrate-effluent orthophosphate increased 0.3 ppm. In this same range of growth rates the orthophosphate removal increased from 0.8 to 1.7 ppm PO_4. The nitrogen to phophorus removal ratio varied from 15.3 at the low growth rate to 10.7 at the high growth rate in the critical region. For the same growth rate range the TIN removal increased 1.7 times.

With orthophosphate, as with TIN, the two high density cultures experienced conditions entirely different from those of the low density cultures. In both high density cultures the substrate-effluent orthophosphate reached a constant minimum value in the critical region.

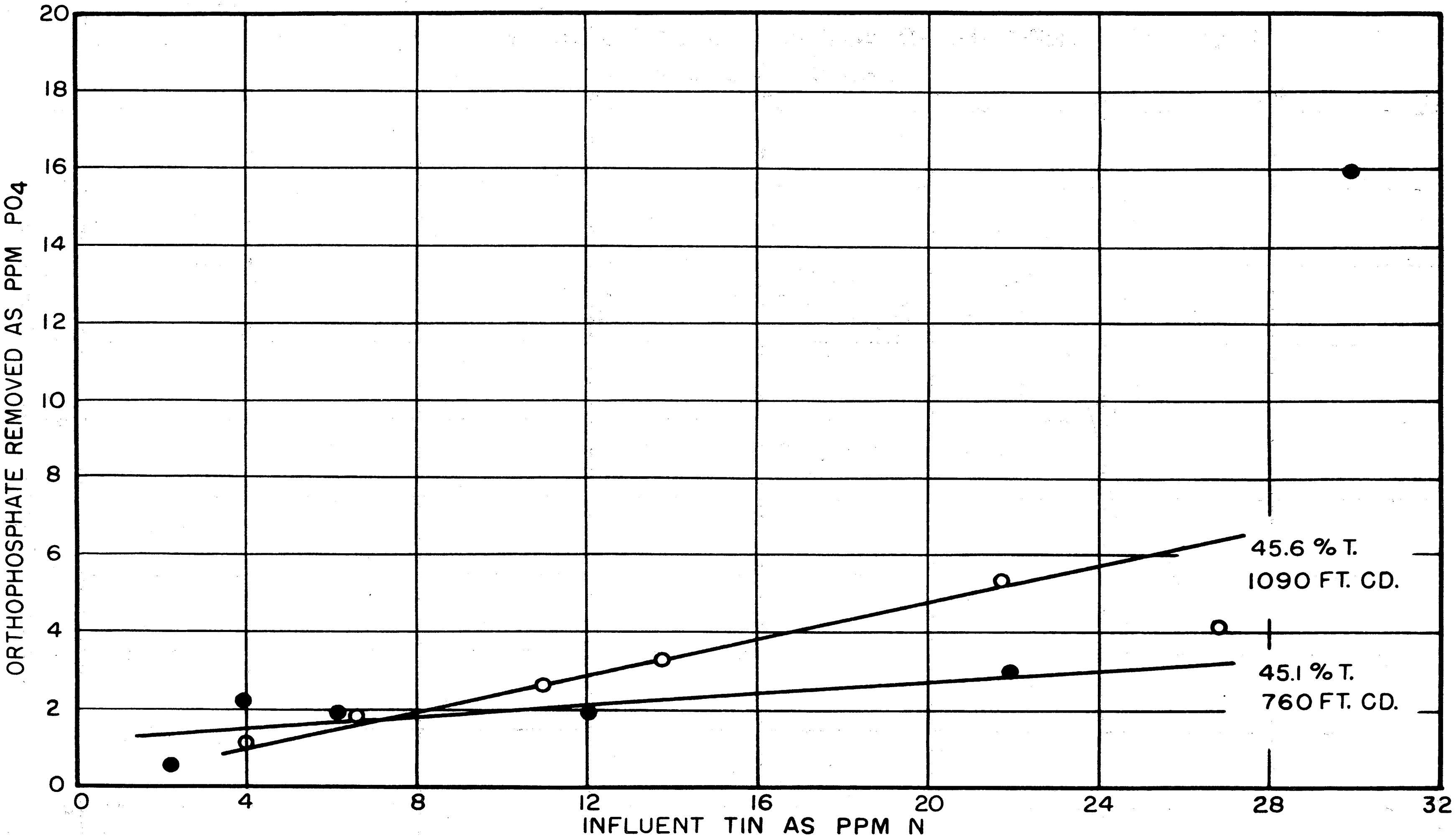


FIG. 22 ORTHOPHOSPHATE REMOVAL AS A FUNCTION OF INFLUENT TIN FOR TWO OSCILLATORIA CULTURES.

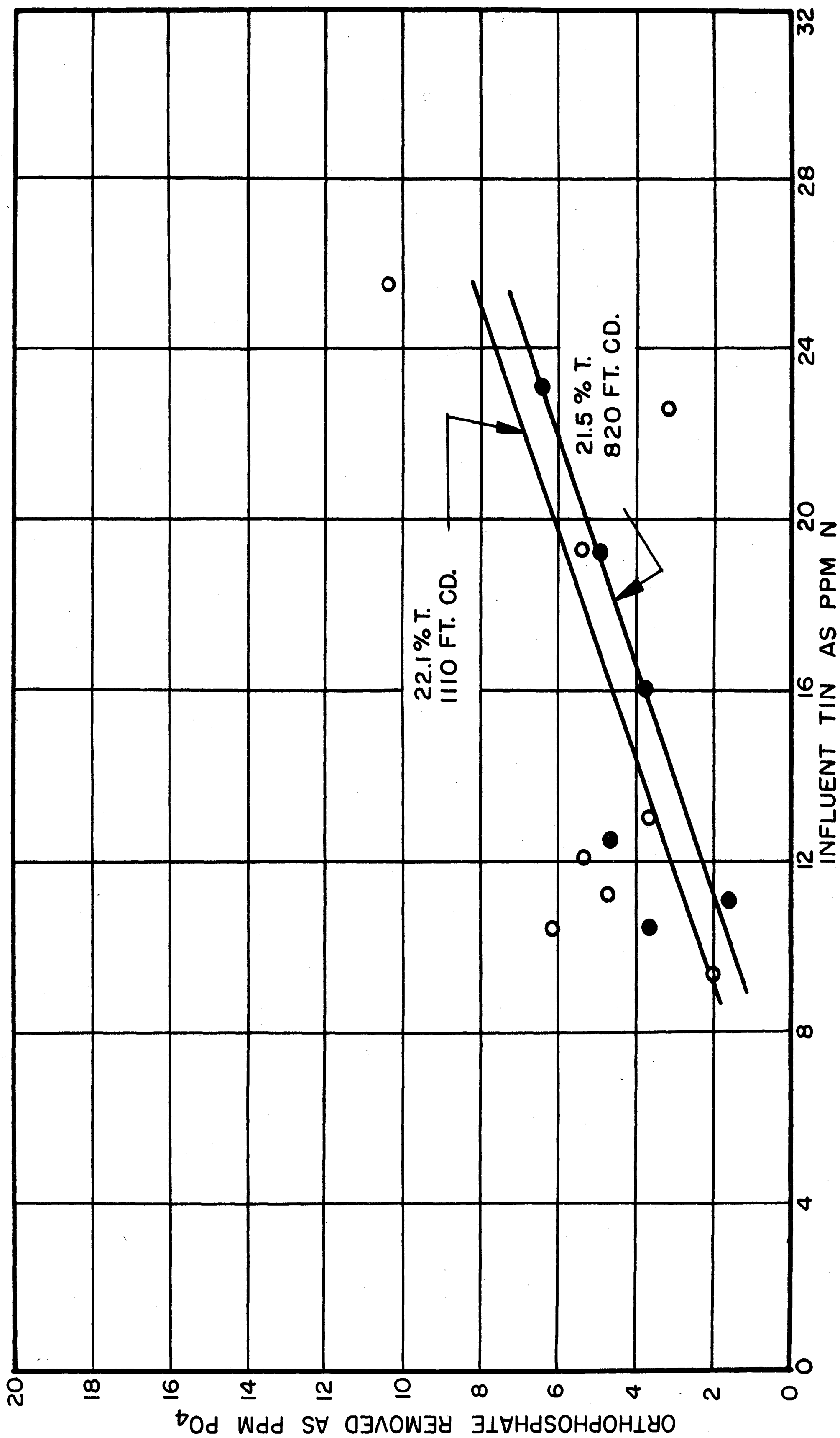


FIG. 23 ORTHOPHOSPHATE REMOVAL AS A FUNCTION OF INFLUENT TIN FOR TWO OSCILLATORIA CULTURES.

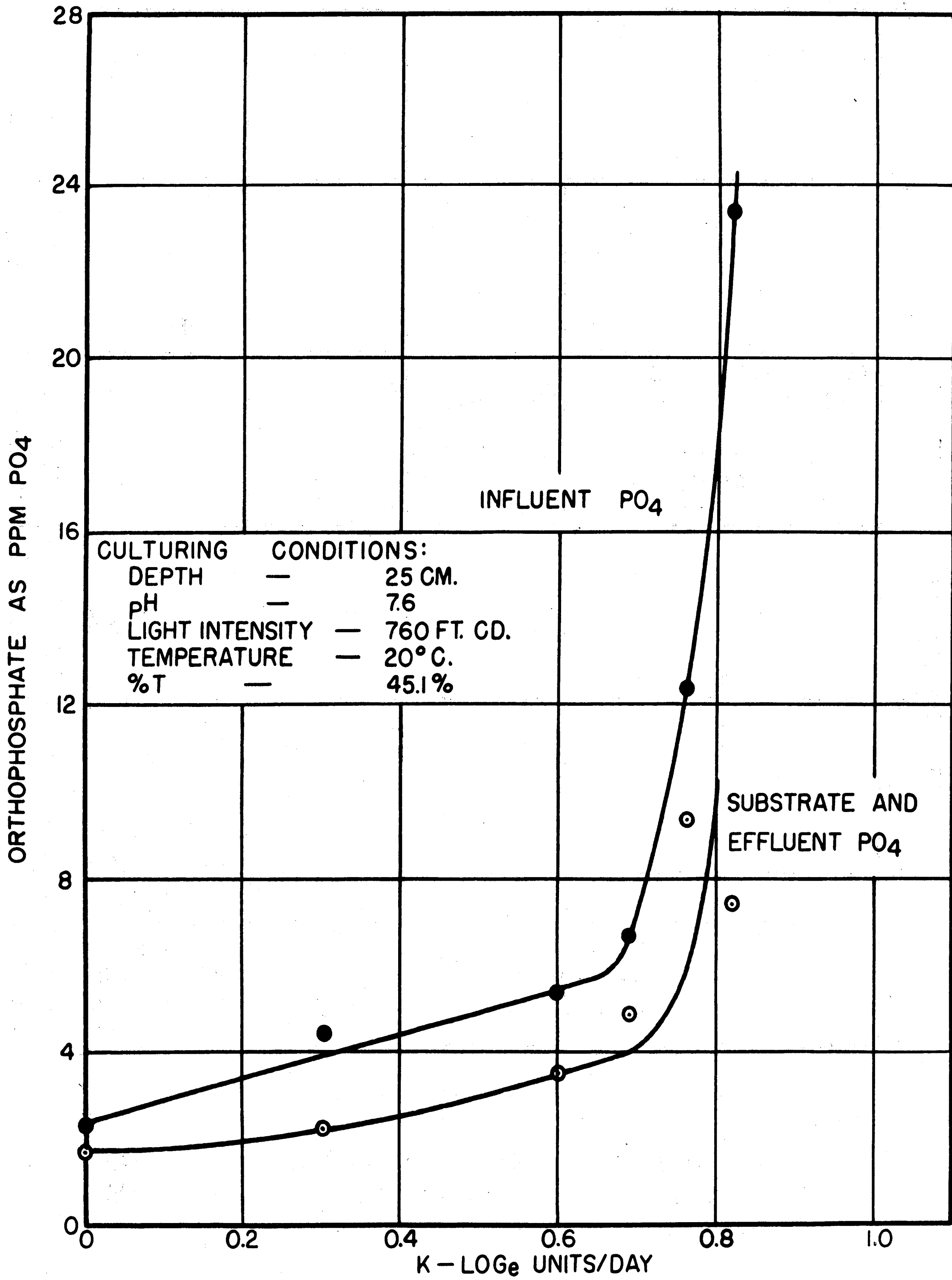


FIG. 24 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUSBSTRATE ORTHOPHOSPHATE AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

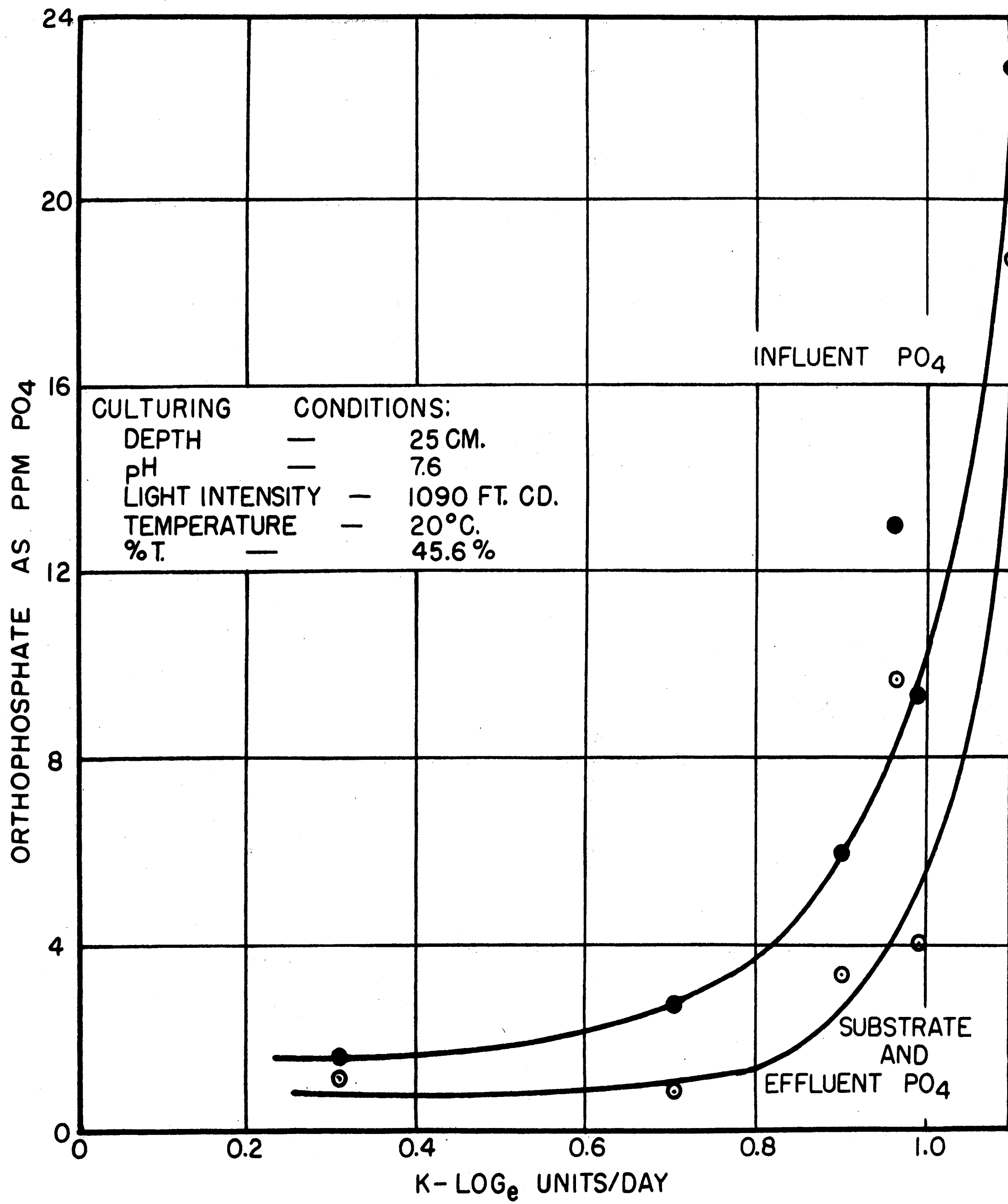


FIG. 25 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE ORTHOPHOSPHATE AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

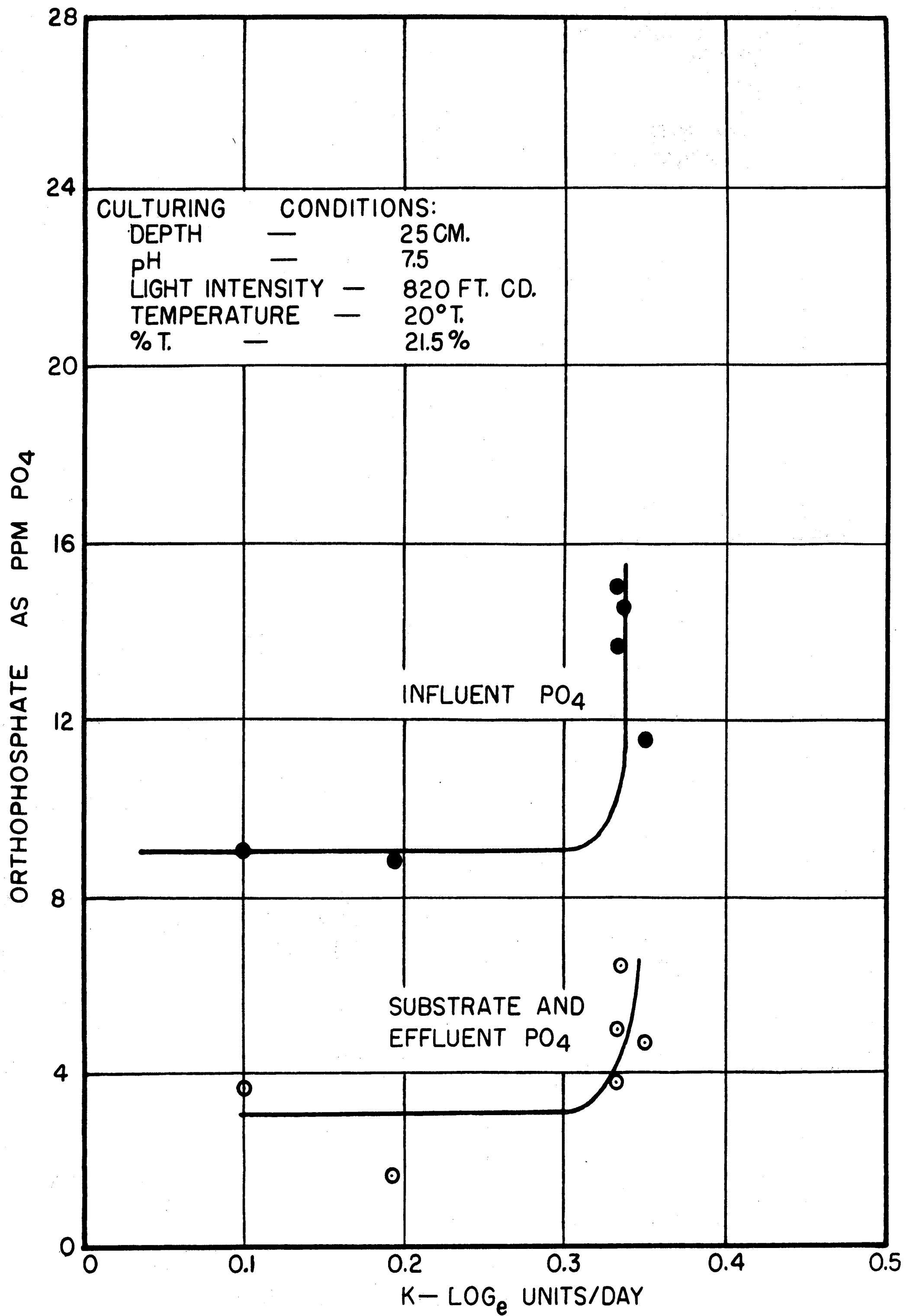


FIG. 26 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE ORTHOPHOSPHATE AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

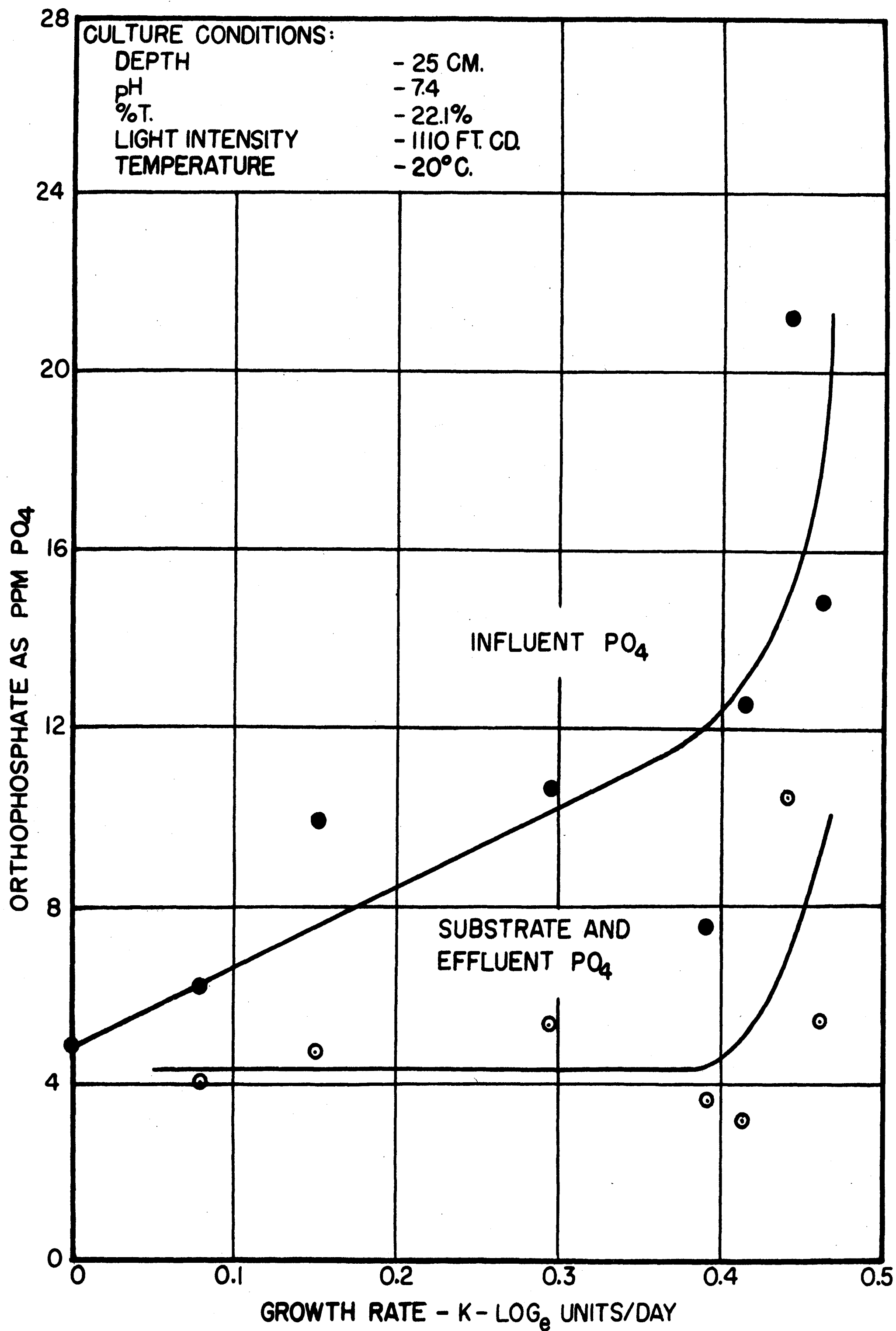


FIG. 27 RELATIONSHIP BETWEEN INFLUENT, EFFLUENT AND SUBSTRATE ORTHOPHOSPHATE AND GROWTH RATE FOR AN OSCILLATORIA CULTURE.

The orthophosphate removal in the 22.1%T-1110 ft.cd. culture was dependent only on the influent concentration. As growth rate increased from 0.1 to 0.35 $\log_e$ units per day the orthophosphate removal increased from 2.5 to 6.8 ppm PO_4. In this same interval the TIN removal increased 1.2 times. The nitrogen to phosphorus removal ratio went from 12.3 at the low growth rate to 5.5 at the high growth rate value. The minimum orthophosphate attained in the substrate-effluent was 4.2 ppm PO_4.

The nitrogen to phosphorus ratio in the sewage plant effluent changed very radically during the series of runs for the 21.5%T-820 ft.cd. culture. The change was so great that although the influent-TIN was reduced in the critical region the influent orthophosphate remained constant at 9 ppm PO_4 . For this culture then growth rate was independent of influent, substrate-effluent orthophosphate and the orthophosphate removed. That is, as the growth rate increased from 0.1 to 0.3 $\log_e$ units per day all the orthophosphate concentrations remained constant. The orthophosphate removal was constant at 6 ppm PO_4. The TIN removal increased 1.1 times within the indicated growth range. The nitrogen to phosphorus removed ratio increased from 5.0 at the low growth rate to 5.7 at the high growth rate.

In addition to the discussion presented in the previous paragraphs, some general comments can be made about the various orthophosphate to growth relationships in the critical regions. These are: (1) the amount of orthophosphate in a culture effluent decreases as photosynthesic activity increases for

low density cultures, (2) high density cultures will attain minimum constant values of orthophosphate in the culture effluent for a wide range of growth rates, (3) the quantity of orthophosphate removed will increase with the increasing culture density, (4) in high density cultures the orthophosphate removal is essentially independent of light intenstiy, (5) the quantity of orthophosphate removed by high density cultures is a function of influent and substrate-effluent concentrations rather than growth rate, (6) the minimum concentration of effluent orthophosphate in a culture will develop when the photosynthetic activity is at a maximum.

The lack of dependency of the high density culture growth rates on the substrate-effluent orthophosphate seems to indicate that at low photosynthetic activity the ratio of organisms to orthophosphate governs the uptake of the nutrient.

Although this study was not intended to determine whether nitrogen or phosphorus first limits growth, it is possible to make such a determination from the data. The variation of the nitrogen to phosphorus ratio in the various sewage effluents makes this possible. In the four culture conditions studied the growth rates at some influent-TIN value became directly proportional to the influent-TIN. This critical TIN value was 7.2 ppm N for the 45.6%T-1090 ft.cd. culture, 5.5 ppm N for the 45.1%T-760 ft.cd. culture, 12.6 ppm N for the 22.1%T-1110 ft.cd. culture and 12.1 ppm N for the 21.5%T-820 ft.cd. culture. At influent-TIN values of 5.5 ppm N and less the growth rates of the two low density cultures were identical. The growth rates of the two high density cultures were the

same at influent-TIN values of 12.1 ppm or less. Growth rate increase with increased influent-TIN was the same for the 45.6%T-1090 ft.cd. below 5.5 ppm N as it was from 5.5 ppm N to 7.2 ppm N. The growth rate increase for the 22.1%T-1110 ft.cd. culture with increased influent-TIN was the same below 12.1 ppm N as it was between 12.1 ppm N and 12.6 ppm N. The data, therefore, clearly indicates that in the region where nutrients are severely limiting, the growth rate of a culture growing in secondary sewage is highly dependent on the nitrogen in the sewage.

Considering the relationship between influent orthophosphate and growth rate, the situation is quite different from that of growth rate and influent-TIN. For the low density cultures in the growth range where both light intensity cultures were nutrient limited, the 760 ft.cd. culture had a much higher influent orthophosphate concentration. This concentration was 1.8 ppm PO_4 greater than that of the 1090 ft.cd. culture at a growth rate of 0.3 $\log_e$ units per day. The difference increased to 3.0 ppm PO_4 at a growth rate of 0.6 $\log_e$ units per day. The same general magnitude of differences exist in the same order for both substrate-effluent orthophosphate and orthophosphate removed for the two low density cultures. Because of these differences in orthophosphate values it would have been impossible for both the low density cultures to have the same growth rate at influent-TIN values below 5.5 ppm N if phosphorus had been limiting growth.

The same overall relationships apply to the two different light intensity cultures having high organism concentrations.

In the region of equal growth rates (0.1 to 0.3 $\log_e$ units per day) the orthophosphate removed by the 820 ft.cd. culture exceeded that of the 1110 ft.cd. culture by 0 to 3.7 ppm PO_4. The substrate-effluent orthophosphate concentration in the 820 ft.cd. was 3.0 ppm PO_4 compared to 4.3 ppm PO_4 in the 1110 ft.cd. culture. The influent orthophosphate of the 820 ft.cd. culture was constant at 9.0 ppm PO_4 in the critical region while it varied from 6.6 to 10.2 ppm PO_4 in the 1110 ft.cd. culture.

The two high density cultures could not have experienced the same growth rates at influent-TIN values below 12.1 ppm N, if phosphorus had been limiting growth because the wide differences in orthophosphate values at equal influent-TIN values would have prevented equal growth rates.

It can be concluded, therefore, that for the four different culturing conditions studied, the nitrogen and not the phosphate in the secondary sewage treatment plant effluent was limiting growth. The only exception to this conclusion would be that there was some micronutrient component in the sewage which always occurred in the same relative proportions as the nitrogen. That such a condition could have existed is possible but it does not seem very probable.

The algae did not exhibit an ability to remove organic nitrogen from the substrate. In every case the total organic nitrogen in the filter effluent was essentially equal to the total organic nitrogen in the influent. The influent total organic nitrogen ranged from 0.01 to 1.60 ppm N. The filtered effluent ranged from 0 to 1.65 ppm N. No significant changes

were noted in the non-orthophosphate phosphorus between the influent and filtered effluent.

The correlation between influent nitrogen content and algal growth agrees to some extent with the data of other investigators using batch cultures. Chu (65) studying various green algae in batch cultures found the lower limit of optimum nitrogen concentration to be between a 3 and 5.3 ppm N. If the initial nitrogen contents were below these values growth rate was dependent on the nitrogen content. He also found that the upper concentration was in the range of 5.3 to 13 ppm of nitrogen for optimum growth. Gerloff et al (68) studying the blue green alga <u>Coccochloris peniocystis</u> indicate that an initial concentration of 13.6 ppm N in a batch culture will provide optimum growth. A plot of their data for growth as a function of initial concentration produces a curve very similar to that of the 45.6T-1090 ft.cd. culture. The plot shows that the upper limit of the nutrient critical region was at about 7.0 ppm N. With an initial nitrogen concentration of 20.4 ppm, however, the <u>Coccochloris peniocystis</u> had not reached a maximum growth.

Studying the blue-green alga <u>Microcystis aeruginosa</u> in batch cultures Gerloff et al (99) found an initial nitrogen concentration of 8 ppm produced maximum yield while 17 ppm N were required to produce the maximum nitrogen content in the algae. An initial phosphorus concentration of 0.25 ppm was required to give maximum yield for this algae. Chu (65) indicates the lower limit of phosphorus concentration to be between 0.081 and 0.9 ppm. Gerloff et al (68) found phosphorus limited

the growth of Coccochloris peniocystis at a concentration of 0.45 ppm.

The data from the batch cultures agrees reasonably well with the data collected in the present investigation for the 45.6%T-1095 ft.cd. culture. The upper limit of the nutrient critical region occurred at 7.2 ppm N. The 5.5 ppm N for the upper limit of the critical region of the 45.1%T-760 ft.cd. culture is also very close to the values found using batch cultures. That Gerloff et al (68) found the same general type relationship between nitrogen supplied and growth rate using artificial substrate as was found using sewage indicates that algae react in the same way to nutrient concentration irrespective of the nutrient source. The upper limits of optimum concentrations as indicated by Chu (65) were not substantiated by the present investigation. The failure of the batch cultures to substantiate the present findings for high culture densities is probably due to differences inherent in batch culture studies and constant density culture studies. It was not possible to check the existing data on phosphorus limitations because the cultures in the present study became limited by nitrogen concentration before critical values of phosphorus were reached.

Some of the problems encountered while conducting this investigation are worthy of mention. The continuous culturing of the blue-green alga Oscillatoria presented some difficulties that had not been encountered by the author while working with the green algae Scenedesmus, Chlorella and Ankistrodemus. The color of the culture was dependent on the influent-TIN.

At high influent-TIN values the color of the culture varied from a dark brownish-yellow to a dark brown. With low influent-TIN values the color ranged from a light yellowish-green to a light greenish-yellow. High light intensities and low culture densities amplified the color changes while low light intensities and high culture densities tended to reduce the color changes. These changes in color were accompanied by changes in transmittance for a given value of culture density expressed in ppm. To evaluate the effect of these spectural changes the specific extinction coefficient of the cultures was determined at the various influent-TIN values. Figure 28 shows the specific extinction coefficient as a function of influent-TIN. The data are somewhat dispersed but definite trends are indicated.

The specific extinction coefficient values of the 45.1%T-760 ft.cd. culture were only slightly influenced by the influent-TIN concentration. While the values of the other three cultures were about equally affected. The specific extinction coefficient decreased rapidly in the critical region of nutrient influence for the 45.6%T-1090 ft.cd., 22.1%T-1110 ft.cd. and 21.5%T-820 ft.cd. cultures. For influent-TIN values above the critical region the specific extinction coefficient values of all the cultures are essentially equal. It should be noted that to keep light transmittance constant the algal concentration had to increase in direct proportion to the specific extinction coefficient decrease.

To further consider the possible changes which took place in the cultures, the dry weight per organism is expressed as a

function of the influent-TIN in Figure 29. Again the data are rather spread but trends are definitely outlined. The individual organism weight in the 45.1%T-760 ft.cd. culture was independent of the influent-TIN except in the critical range where the weight dropped slightly. The individual organism weight in the 45.6%T-1090 ft.cd. culture was a linear function of influent-TIN, increasing as the TIN values increased.

The change in weight per organism with changing influent-TIN was very small for the 22.1%T-1110 ft.cd. culture. The weight, as for the 45.1%T-760 ft.cd. culture, decreased in the critical region with decreasing influent-TIN. The relationship between organism weight and influent-TIN for the 21.5%T-820 ft.cd. culture is quite different from the relationships of the other cultures. For this culture the function was very curvilinear expressing a maximum weight at an influent-TIN of 17 ppm. As the TIN was increased or decreased from this value the organism weight dropped rapidly.

All the cultures to some extent, as indicated in the preceeding paragraphs, experienced change in color, specific extinction coefficient and organism dry weight. These changes produced variations in the culture light transmittance, ppm dry weight and organisms per milliliter.

At the start of the investigation it was anticipated that the previously discussed factors might vary. A decision had to be made, therefore, as to which factor should be maintained constant at all times. Two facts were used as guide lines: (1) photometric techniques are the only means at

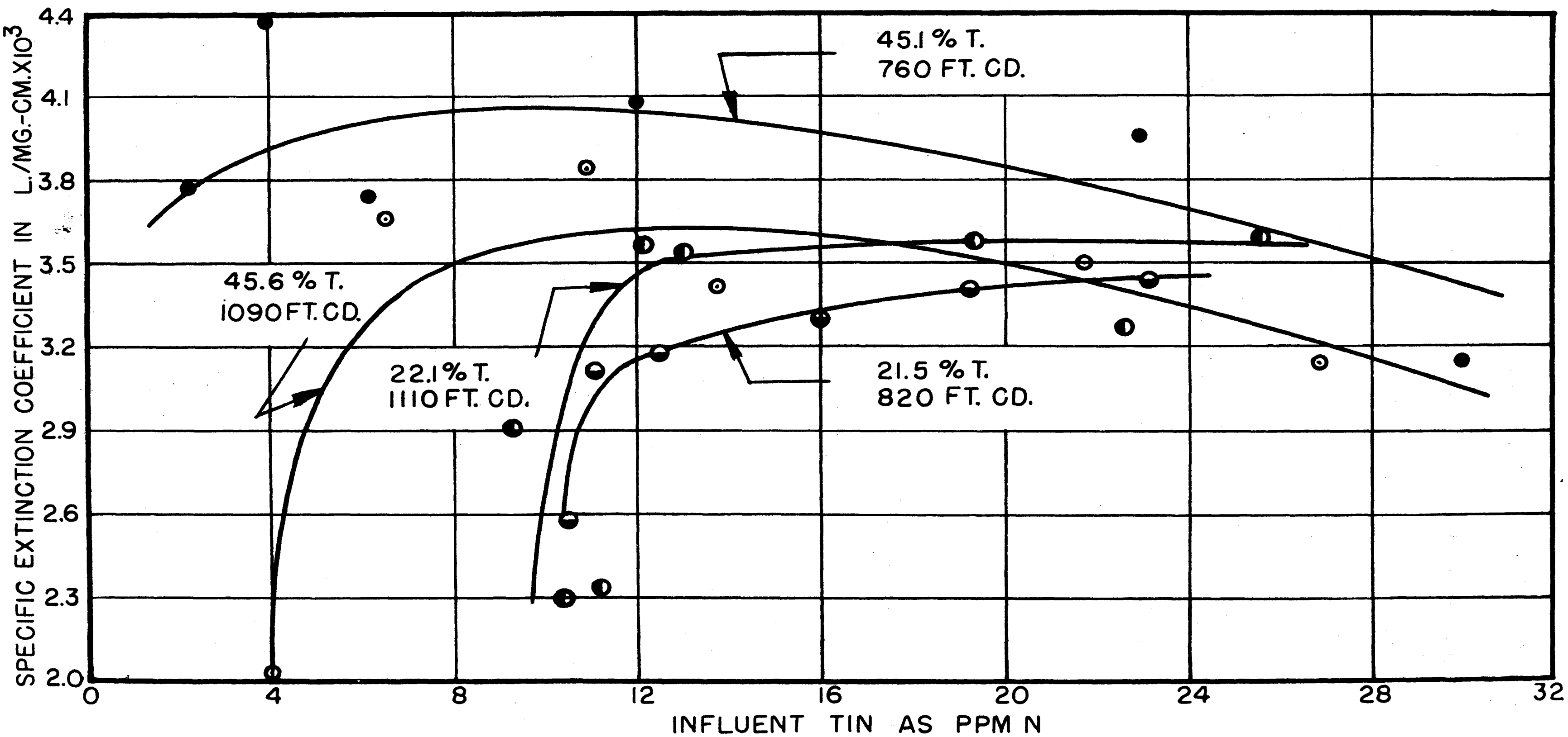


FIG. 28 SPECIFIC EXTINCTION COEFFICIENT AS A FUNCTION OF INFLUENT TIN FOR OSCILLATORIA CULTURES.

present of controlling constant density, semi-continuous dilution cultures, (2) that algal culture reactions to changing external forces could only be validly compared when the light absorption was kept reasonably constant.

It was decided, therefore, to keep the culture transmittance as measured at a wave length of 425 millimicrons and a 20 millimeter light path constant letting the other factors vary as necessary. The validity of this decision is supported by the compatibility of the data.

The Effect of Induced Turbulence on Algal Culture Growth

The influence of induced turbulence on algal culture growth was evaluated by making six experimental runs using batch culturing techniques. The turbulence was created by stirring the culture. Initially only one culture depth was to be used, however, it became necessary to employ two culture depths in order to evaluate more properly the results of the study. The culture depths were 12.5 and 25 cm. The culture volumes for these two depths were 7.5 and 15 liters. The maximum light intensity of the light source was used for each of the six runs.

Chlorella pyrenoidosa was used as the test organism. This organism was obtained from Dr. Starr's collection at the University of Indiana. The algae were cultured on proteose agar for about one year before this study was started. During the study the algae were carried in a liquid synthetic media of the same composition as that used for the investiga-

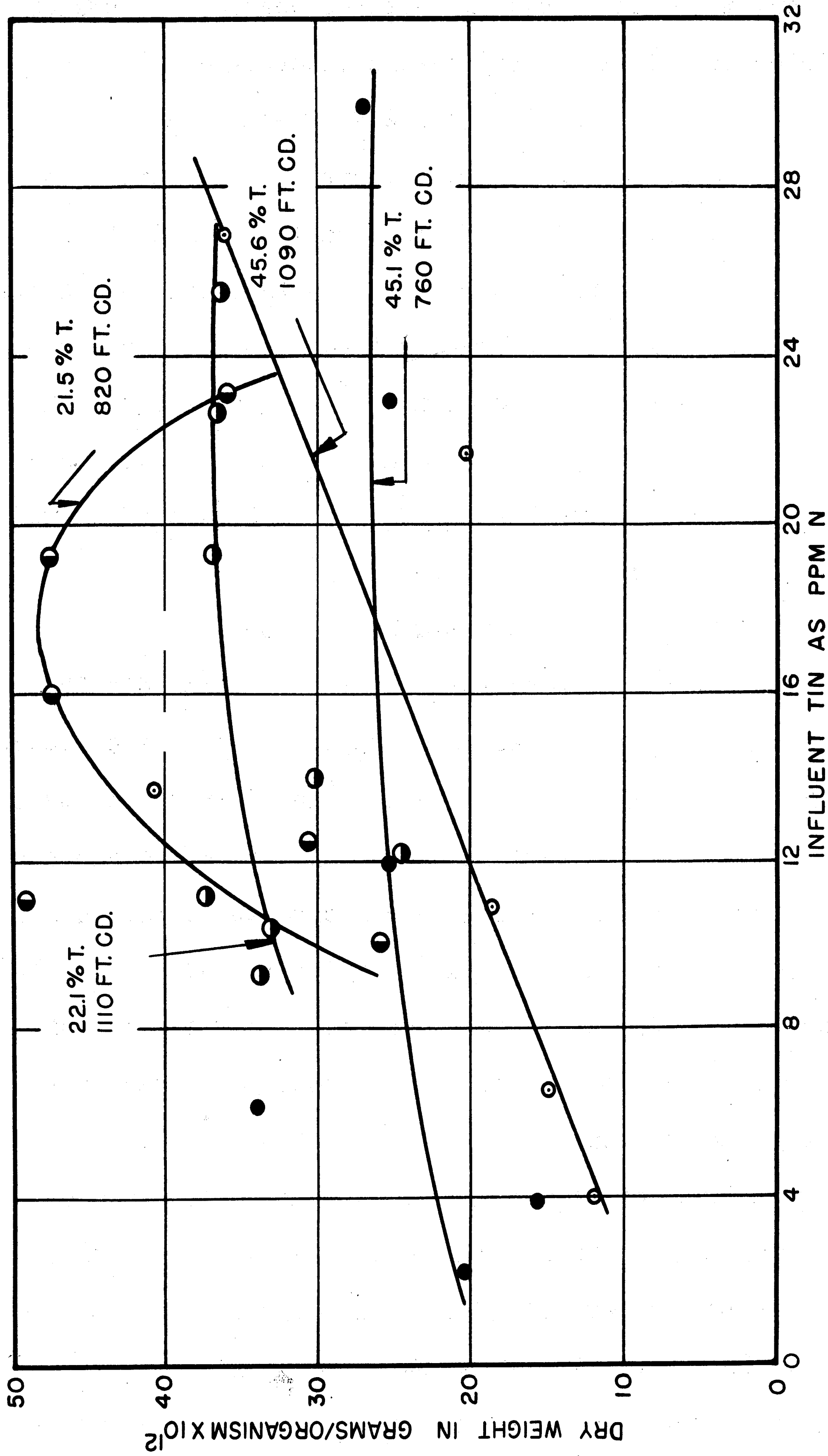


FIG. 29 DRY WEIGHT PER ORGANISM AS A FUNCTION OF INFLUENT TIN FOR CULTURES OF OSCILLATORIA.

tion. The algae carried in the liquid media were used for inoculum. These inoculum cultures were grown in a one liter florence flask containing one liter of media. The inoculum cultures were kept in a 20°C water bath and exposed to 200 to 300 ft.cd. of light intensity. To maintain the algae in good condition the inoculum cultures were transferred every one or two weeks. If the cultures were not transferred frequently enough the cells would clump and then the culture was not suitable for an inoculum. The clumping could, however, be eliminated by making several rapid transfers. To serve as an inoculum the cultures had to have a density of 30×10^6 to 50×10^6 organisms per milliliter.

A synthetic substrate was used for the study. The composition of the synthetic substrate is given in Appendix IV. The required volume of media was made up, then sterilized before use. For sterilizing, the media was placed in stainless steel buckets. After sterilization the media was cooled and placed in the culturing chambers. If the media was not sterilized a large bacterial population would develop rapidly and persist for two or three days. Presumably the bacteria were using the citrate contained in the media as an energy source. The use of EDTA prevented the growth of the bacteria without sterilization but unfortunately the algae would not grow either. The substrate used for the inoculum cultures was also sterilized.

The cultures were unialgal but not pure cultures. Except for the initial sterilization, no attempt was made to exclude bacteria. After placing the media in the culturing chambers sufficient inoculum was added to produce an initial concentra-

tion of about 10^6 organisms per milliliter. The time of the addition of the algae represented time zero. Immediately after the algae were added a sample was taken. The cultures were sampled about every 12 hours for the first 4 to 6 days, after which sampling was done on a daily basis.

Each time a sample was taken the mixing speed, temperature and light intensity were measured. The samples were tested for pH, light transmittance, dry weight and organisms per milliliter. The latter was accomplished using the Coulter Counter. The per cent light transmission was determined at a wave length of 425 millimicrons with a 13 millimeter light path. Before a sample was taken the volume loss due to evaporation was compensated for by adding distilled water. Any sample left after the tests were made was returned to the cultures.

The culture concentration measured in organisms per milliliter was used as the controlling operational parameter, as the other methods of concentration determination had serious limitations. The per cent light transmittance was an acceptable control parameter up to a concentration of about 300 ppm. At higher concentrations the per cent transmittance dropped below 10 per cent. At this transmittance value and less the determination was of questionable value. When the concentration was 500 to 600 ppm the per cent transmission was one or less. Dry weight would have served as an acceptable parameter except for the time involved in the determination. To complete the dry weight determination required 24 hours. In a 24 hour period it was possible for a culture to deterior-

ate to the point where recovery would not take place. The principal type of deterioration was the clumping of the algal cells which rendered the organisms per milliliter determination worthless. The clumping would not, however, affect growth as determined by dry weight. Basically, the organisms per milliliter was selected as the controlling parameter because it was the most readily available test.

Culture deterioration in the form of clumping was much more prevalent in the deeper culture. Microscopic examination of the cultures was routinely carried out to determine if clumping was taking place. Visual examination was also used to evaluate the general condition of the cultures.

The amount of turbulence created in the cultures was determined by the mixing speed. The turbulence increased as mixing speed increased. Mixing speeds of 500, 1000 and 2000 RPM were used. The maximum usable speeds were 1000 and 2000 RPM in the 12.5 and 25 cm. deep cultures respectively. If speeds in excess of these indicated maximums were used the temperature of the cultures increased to a level which killed the algae.

The normal procedure was to initially use the low mixing speed until growth reached either a plateau or exhibited a maximum point, as determined by the organisms per ml. After a plateau or maximum point had been reached the speed was increased to the next higher mixing speed. This procedure was repeated until the maximum allowable mixing speed was attained.

Figures 30 and 31 show the culture concentration measured in terms of organisms per ml. and ppm as a function of time

for two different test runs with a 25 cm. culture depth. Figure 30 shows an initial drop in the number of organisms per ml. while the dry weight was increasing rapidly. This was due, at least in part, to the settling and sticking of the algal cells to the bottom and sides of the culturing chamber. The gain in weight might also be attributed to the accumulation of photosynthate by the cells that did not settle or stick. Whether the algal cells settled and stuck to the bottom and sides of the chamber was dependent on the condition of the inoculum. None of the other cultures tested showed this tendency.

At the point of maximum growth for the two cultures for the 500 RPM mixing speed a maximum point was reached in the organisms per ml. concentration while a plateau was experienced in the dry weight. The culture of Figure 30 experienced a plateau in organisms per ml. and only a discontinuity point in the dry weight for the maximum point of the 1000 RPM mixing speed. At the same maximum point, for the culture of Figure 31, both measures of concentration reached a plateau.

The maximum population developed in both cultures was 70×10^6 organisms per ml. The dry weight was 800 and 900 ppm for the cultures of Figures 30 and 31 respectively.

As measured by organisms per ml. a rapid growth took place after each increase in mixing speed. In some instances the same rapid increase was noticed in the dry weight measurement also. The initial growth rates for the culture of Figure 31 were significantly higher than those of Figure 30. For the 1000 and 2000 RPM mixing speeds, however, the growth

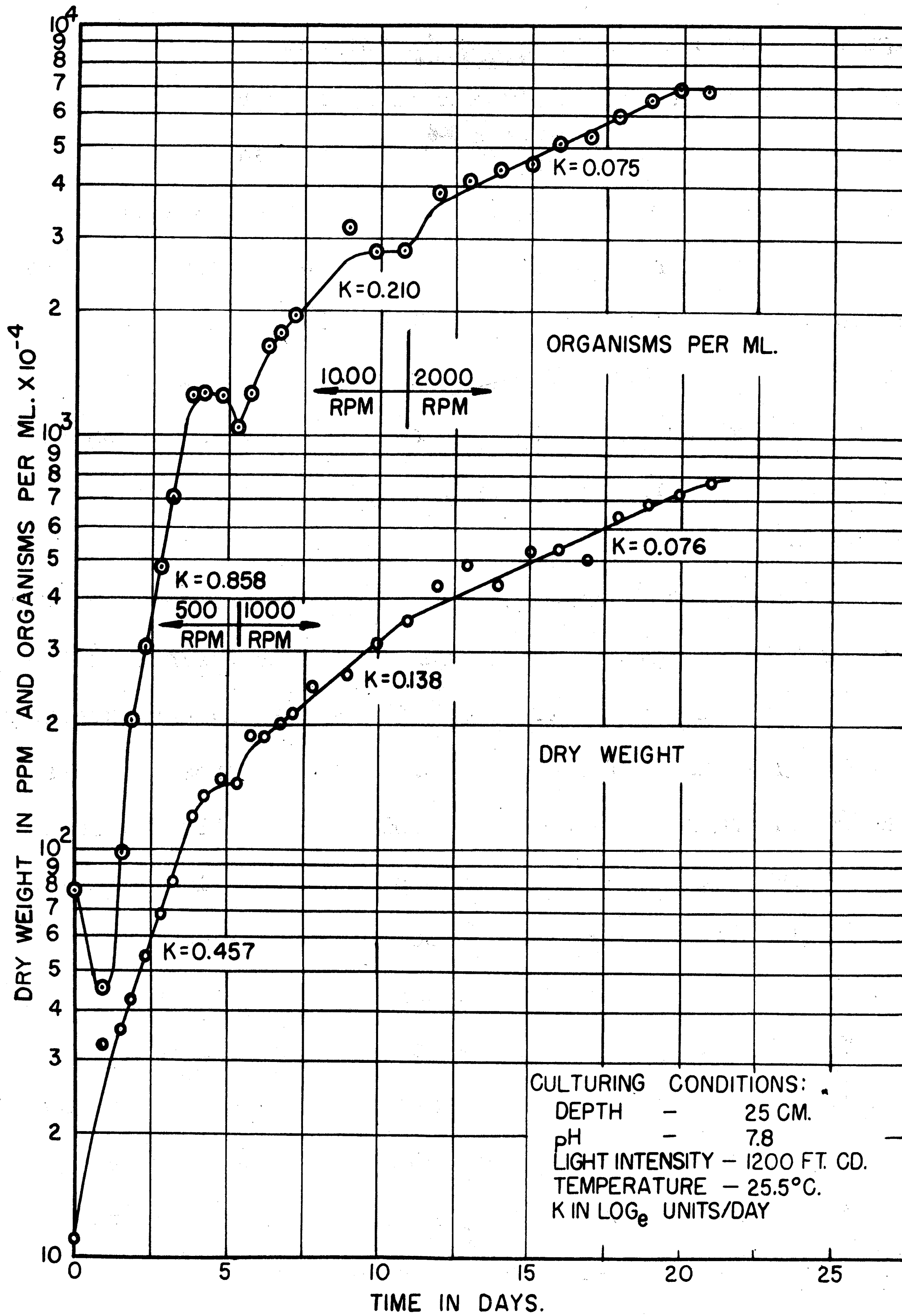


FIG. 30 INCREASE IN ORGANISMS PER ML. AND DRY WEIGHT AS A FUNCTION OF TIME FOR A BATCH CULTURE OF CHLORELLA.

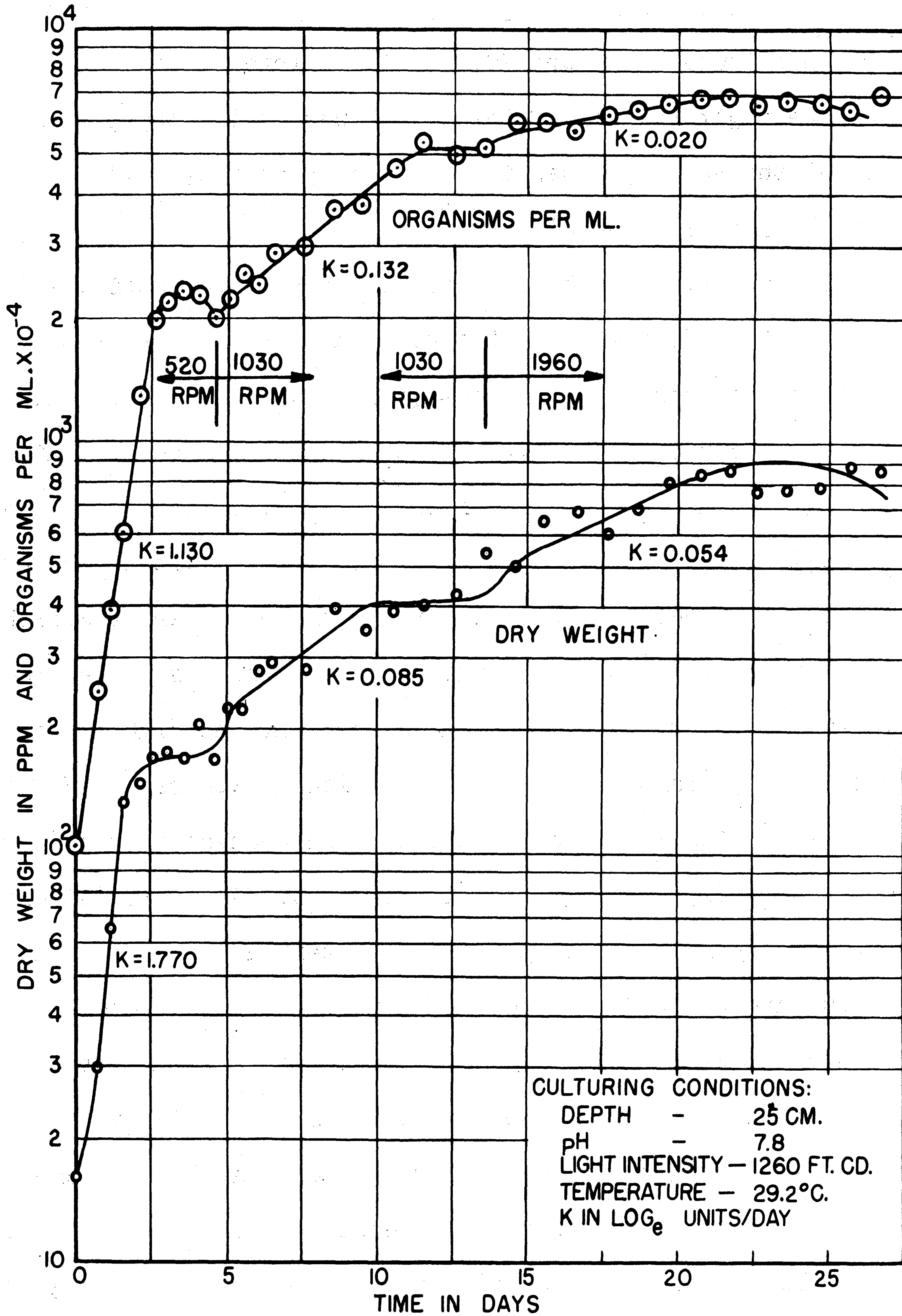


FIG. 31 INCREASE IN ORGANISMS PER ML. AND DRY WEIGHT AS A FUNCTION OF TIME FOR A BATCH CULTURE OF CHLORELLA.

rates of the culture of Figure 31 were 1/3 to 2/3 of those for the culture of Figure 30. It is of interest to note that the major portion of the growth was in the log phase, even at the very high densities. Such a condition is not normally anticipated(40).

Productivity is the product of the culture density and growth rate. If the productivity of a culture is to remain constant with changing growth rate and culture density the product of density and growth rate must be constant. That is, the rate of change in growth rate and culture density must be inversely proportional. For the culture of Figure 30 the productivity was nearly constant for the three different growth rates at the respective maximum culture densities. The productivity ranged from 17.5 grams/meter^2/day at the high growth rate to 15.7 grams/meter^2/day at the low growth rate. For the culture of Figure 31 the balance between changes in growth rate and culture density was not quite as good. For this culture productivity ranged from 52.3 grams/meter^2/day at the high growth rate to 11.7 grams/meter^2/day at the low growth rate. The productivity values were calculated using the growth rates as determined by the dry weight concentration of algae.

The productivities of the two cultures as they reached the maximum culture density were nearly equal. At the higher growth rates, however, the culture of Figure 31 had a higher growth rate for approximately the same culture density. Its productivity values were, therefore, somewhat greater.

The fact that both of the cultures (Figs. 30 and 31) reached essentially the same maximum culture density in terms

of both dry weight and organisms per ml. raised the question as to what was limiting growth. Nutrient limitation had been eliminated since the nutrients in the culture of Figure 30 had been replenished at 12 days after the start of the culture. The other two possible factors that could limit the total maximum population were light and toxic metabolic products. To determine which of these two factors were limiting growth the culturing conditions had to be changed.

It was not possible to change any factor connected with the possible production of metabolic products, therefore, the light conditions had to be changed. Lowering the light intensity would not have resolved the problem and since the light source was already at its maximum it was impossible to increase the intensity. The only way left to change the light conditions was to change the culture depth. The culturing depth was reduced by one-half giving a depth of 12.5 cm. If light were limiting then it was anticipated that the maximum culture density at 12.5 cm. should be double that at the 25 cm. depth. If toxic metabolic products were limiting then the culture depth should have no influence on the maximum culture density.

The data for the 12.5 cm. deep culture is presented in Figure 32. The initial growth rate is much higher than for the two 25 cm. cultures (Figs. 30 and 31). The maximum population density was 1800 ppm and 185×10^6 organisms per ml. In terms of numbers then the 12.5 cm. culture supported 2.5 times the populations of the 25 cm. cultures. As determined by weight, however, the density of the 12.5 cm. culture

was only double that of the 25 cm. cultures. It is also significant to note that: (1) the growth rates of the 12.5 cm. culture and the 25 cm. culture in Figure 30 are the same in the last log growth phase before the maximum culture density was attained and (2) the productivity of the 12.5 cm. culture at the maximum density of the last log growth phase was 15.9 grams/meter^2/day as compared to 15.7 and 11.7 grams/meter^2/day for the two 25 cm. cultures.

The occurrence of the anticipated difference in maximum density and the compatability of the productivity and growth rates of the two different depth cultures leads to the conclusion that light and not toxic metabolic products was limiting growth. The difference in the ratios of numbers of organisms and dry weights for the two different depth cultures indicates a difference in organism size. A difference in size was readily noticeable when the organisms were viewed with a microscope. It was also noticeable that the organisms of the 12.5 cm. culture were much more uniform in size than those of the 25 cm. cultures.

Figures 30, 31 and 32 all demonstrate that the growth of an algal culture is affected by mixing speed. In each instance the mixing was initially at the lowest speed investigated. Growth was allowed to proceed at the low mixing speed until the growth rate reached zero. At the point of zero growth rate the mixing speed was increased and culture growth was again initiated. This procedure was repeated until the maximum mixing speed was attained. It has, therefore, been shown that higher mixing speeds will stimulate

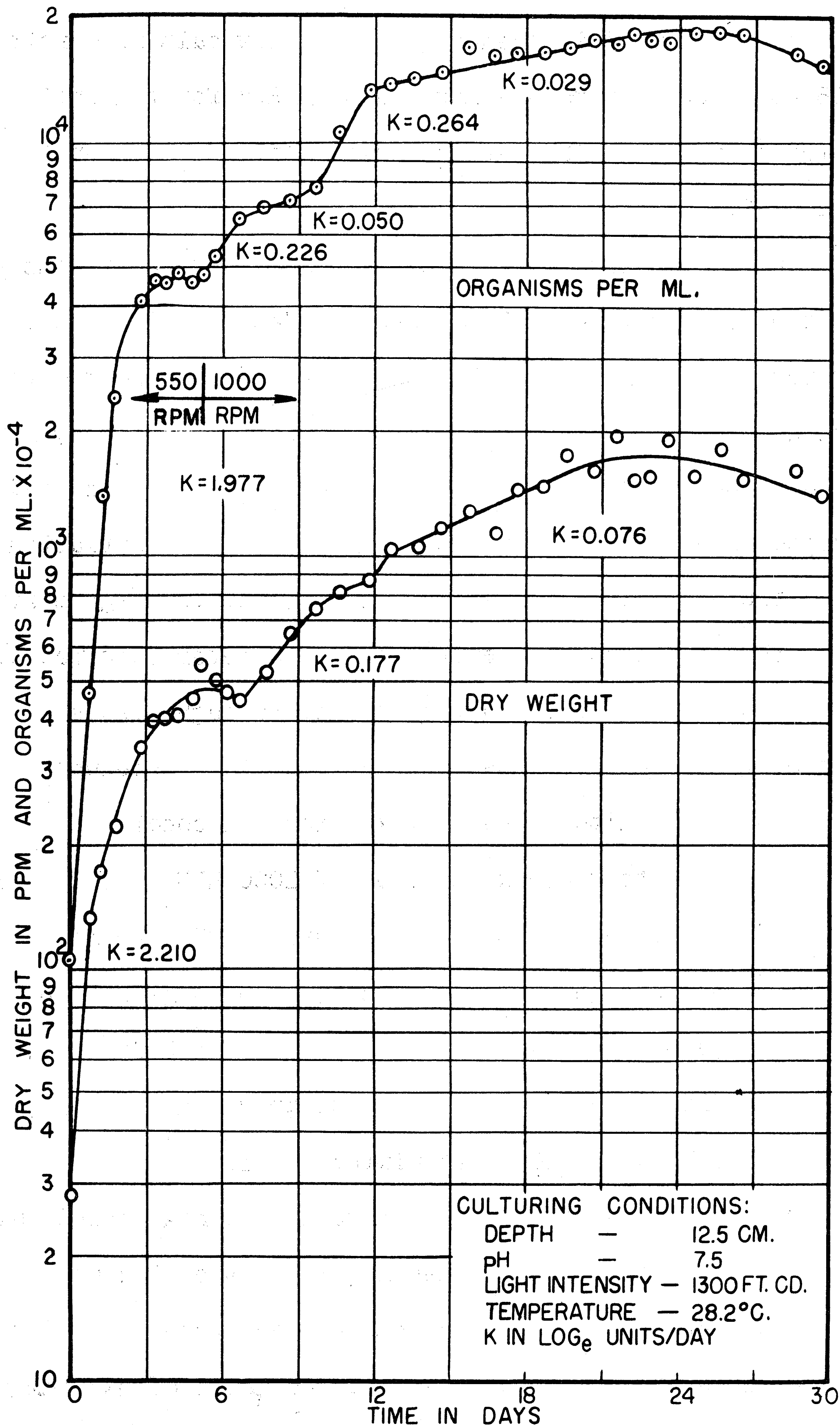


FIG. 32 INCREASE IN ORGANISMS PER ML. AND DRY WEIGHT AS A FUNCTION OF TIME FOR A 12.5 CM. DEEP BATCH CULTURE OF CHLORELLA.

more growth than will low mixing speeds. To determine if mixing speed would influence growth at low culture densities a 25 cm. deep culture was mixed at 2000 RPM for the entire culturing period. The data of this culture are shown in Figure 33.

Up to a culture density of about 28 x 10^6 organisms per ml. the data in Figure 33 are nearly identical to that of Figures 30 and 31. This indicates that growth and growth rate at the low culture densities is not influenced by mixing speed. The organisms per ml. reached a maximum point and dropped slightly before increasing again. This situation being the same as in Figures 30 and 31. At the high mixing speed the dry weight concentration curve never experienced a plateau or maximum point but only went through points of changes in slope. The most significant difference was that the high speed culture did not experience a condition that was comparable to the mixing speed of 1000 RPM for the cultures of Figures 30 and 31. The constant 2000 RPM mixing speed did not change the growth in terms of organisms per ml., in the range previously associated with the 500 RPM mixing speed; it did, however, eliminate the growth characteristics asssociated with the 1000 RPM mixing speed.

The dry weight of the organisms in the high speed culture (Fig. 33) was about three times higher at the time when the organisms per ml. reached the initial peak than in the other two cultures (Figs. 30 and 31). At the point of maximum density, however, the organisms per ml. reached 68 x 10^6 while the dry weight was only 580 ppm. The algal cells in this

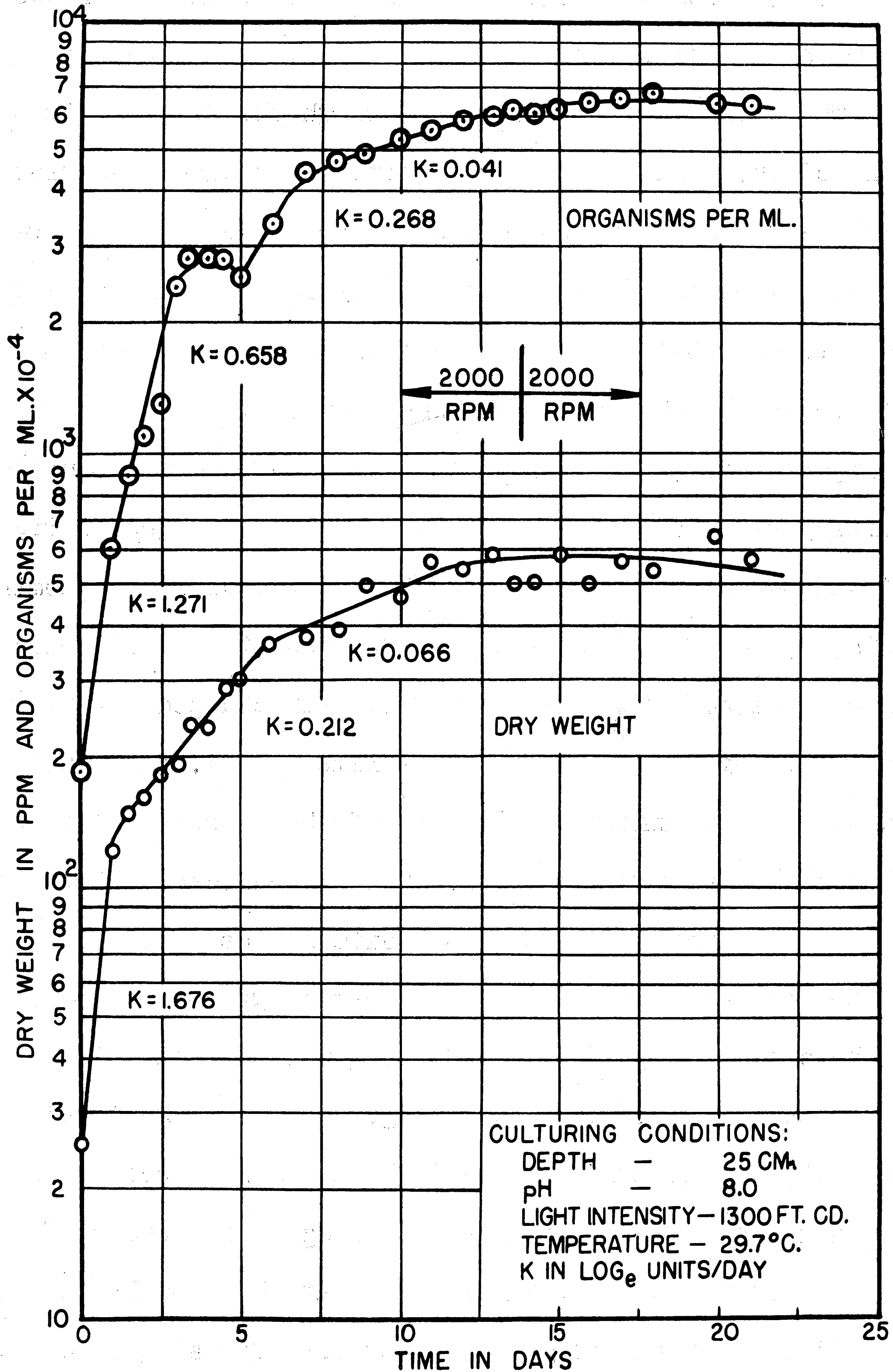


FIG.33 INCREASE IN DRY WEIGHT AND ORGANISMS PER ML. AS A FUNCTION OF TIME FOR A 25 CM. DEEP BATCH CULTURE OF CHLORELLA.

culture were somewhat smaller than those of the other two 25 cm. deep cultures.

The growth of the cultures through the first maximum point of the organisms per ml. seems to be a characteristic of the algae and not of the mixing speed. Growth in terms of weight can be influenced to some extent by mixing speed during the same portion of the culturing time.

To establish that the maximum culture densities indicated in Figures 30, 31, 32 and 33 could not have been produced with a mixing speed of 500 RPM the culturing data shown in Figures 34A and 34B were obtained. For these two cultures a nominal mixing speed of 500 RPM was used throughout the culturing period. Because the algae were slightly clumped for these two culturing conditions the organisms per ml. could not be evaluated. The maximum densities of these two cultures were 400 and 830 ppm for the 25 and 12.5 cm. culture depths respectively. The ratio of these two maximum densities represents further evidence that light was limiting growth. Comparsion of the data of Figures 34A and 34B shows that the 500 RPM mixing speed was able to support growth up to the level associated with the maximum growth of the 1000 RPM speed in Figures 30 and 31 and the end of the second log growth phase of Figure 32. It would seem, therefore, that the 1000 RPM mixing speed had no effect on growth. However, for all the cultures if the mixing speed was not increased at the maximum point of the organisms per ml. curves severe clumping took place. This clumping disappeared when the mixing speed was increased from 500 to 1000 RPM. Even for the cultures of Figures 34A and 34B

at the maximum density the amount of clumping increased as culturing continued beyond the maximum.

The effects of induced turbulence on algal culture growth were: (1) the maximum density of the cultures was increased as the mixing speed was increased, (2) the increased mixing speeds were not successful in producing growth rates at high culture densities equal to the growth rates at low culture densities, (3) growth as measured in terms of organisms per ml. was more dependent on mixing speed than growth determined by dry weight density, (4) growth rates were not the same when determined by organisms per ml. and dry weight in ppm and (5) the maximum density was determined by the quantity of available light.

Since no high growth rates were observed at the higher culture densities it is believed that no light integration as described by Phillips and Myers (41) took place. The lack of light integration or the flashing light effect was probably due to an insufficient light intensity or to an inability to obtain the required light and dark times. In the absence of light integration the productivities generally decreased as the culture density increased. Productivity was a minimum when there was essentially complete light absorption.

The increase in the culture density with increased induced turbulence apparently was due to the exposure of more organisms to the light. Even though the growth rate decreased as density increased, it is significant that sufficient algae were exposed to the light often enough to maintain the cultures in a log growth phase at the higher mixing speeds. The occurrence of a

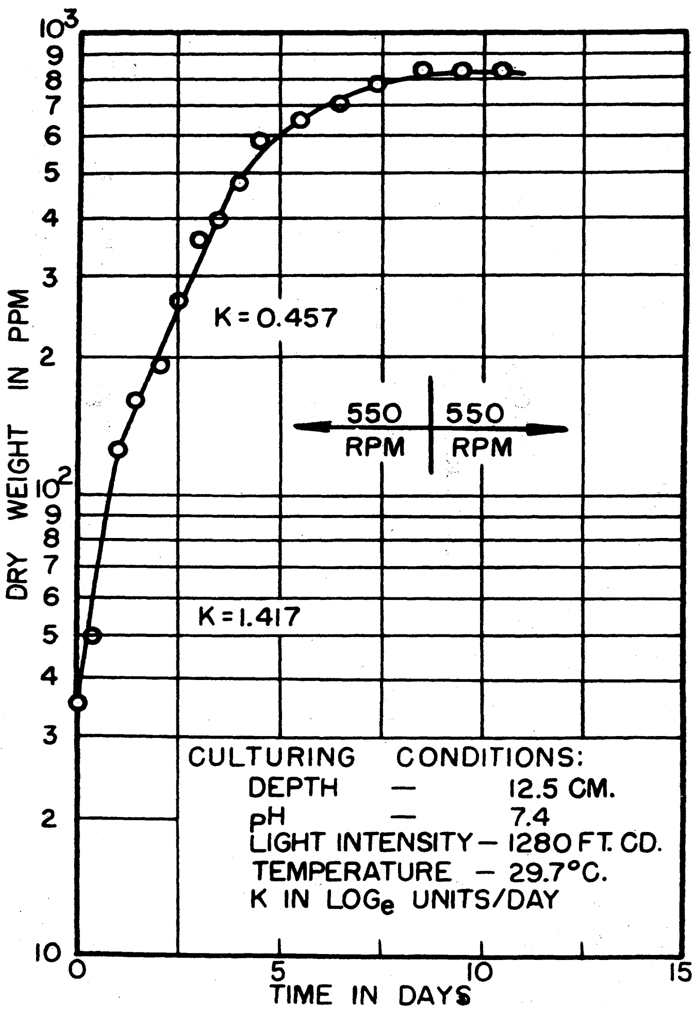


FIG. 34A

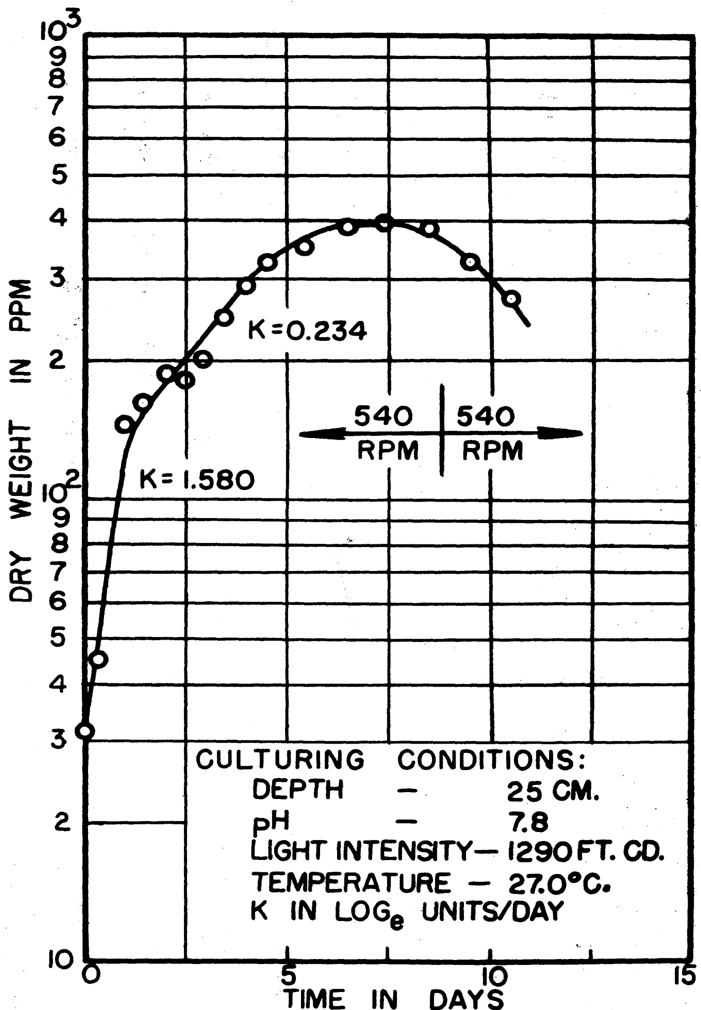


FIG. 34B

INCREASE IN DRY WEIGHT AS A FUNCTION OF TIME FOR TWO BATCH CULTURES OF CHLORELLA HAVING EQUAL MIXING SPEEDS BUT DIFFERENT DEPTHS.

constant growth rate over a wide range of culture densities can be interpreted to mean that for each culturing condition there was an optimum culture density for that productivity associated with each growth rate.

Since there was no increase in productivity at high culture densities the benefits derived from induced turbulence within the limits of this study appear to have no direct value in the tertiary treatment of sewage using algae. This study does, however, point out that at high culture densities mixing as well as light intensity and temperature will affect culture growth rate.

SUMMARY

A study to determine the influence of nutrient concentrations and induced turbulence on algal growth was conducted. The nutrient study was also concerned with the removal of nutrients from the secondary sewage plant effluent when used as a substrate. The influence of turbulence was studied using synthetic media and was concerned only with algal growth.

The Influence of Nutrient Concentration on Algal Cultures

The purpose of this study was to determine what effect changing the concentrations of nutrients supplied to an algal culture would have on such characteristics as growth rate, nutrient removal ability and resulting nutrient residual in the culture effluent. Constant density-semi-continuous dilution culturing techniques were used for this study. Carbon was supplied by addition of 100 per cent CO_2. The pH control was automatic, introducing CO_2 whenever the pH rose above 8.3.

The blue-green alga *Oscillatoria* was employed as the test organism. This alga was isolated from the secondary effluent of the Ann Arbor Sewage Treatment Plant. The algal cultures were dominant but not pure cultures. The principal contaminant was *Chlorella*. The contaminants never exceeded 10 per cent of the total algal particles present. The contribution of the contaminants to dry weight and light attenuation was probably less, due to the difference in size between *Chlorella cells* and *Oscillatoria* particles.

The nutrient supplied was the effluent of the Ann Arbor activated sludge sewage treatment plant. The effluent was sterilized to prevent chemical and biological changes during use. Chemical components lost during sterilization were replaced using reagent grade chemicals. To obtain various nutrient concentrations the sewage plant effluent was diluted with distilled water.

Culture density was determined for operational purposes by per cent light transmittance (%T) at a wave length of 425 millimicrons and a 20 millimeter light path. The total inorganic nitrogen (TIN) was selected to serve as the measurement of the nutrient concentration. The basic independent variable of the investigation was, therefore, the total inorganic nitrogen supplied to the culture.

In addition to varying nutrient concentrations four different culturing conditions were studied. These four different conditions were derived from essentially two different culture densities and two different light intensities. The culturing conditions were 45.1%T-760 ft.cd., 45.6%T-1090 ft.cd., 21.5%T-820 ft.cd. and 22.1%T-1110 ft.cd. The culture densities of 45.1 and 45.6%T were considered equal as were the 21.5 and 22.1%T values. The light intensities of 760 and 820 ft.cd. were considered as equivalent, as were the 1090 and 1110 ft.cd. intensities.

Zero growth rate for the two low density cultures occurred at about 2 ppm influent-TIN-N and for the high density cultures at about 10 ppm N in the influent. For growth rate as a function of influent-TIN there is an initial critical

region where the relationship is linear with growth rate being highly dependent on the influent-TIN. In this critical region the increase in growth rate per incremental increase in influent-TIN was found to be independent of the light intensity and culture density. The span of this critical region, measured in terms of influent-TIN values, was determined by the photosynthetic activity of the culture. The upper limits of the critical regions were 7.2, 5.5, 12.6 and 12.1 ppm influent-TIN-N for the 45.6%T-1090 ft.cd., 45.1%T-760 ft.cd., 22.1%T-1110 ft.cd., and 21.5%T-820 ft.cd. cultures respectively. In the critical region then, actual growth rate was dependent on the culture density while an increase in growth rate was dependent only on the incremental increase in TIN supplied.

At influent-TIN values above the upper limit of the critical regions the growth rates approached, but did not reach, a zero rate of increase with increasing influent-TIN. The maximum influent-TIN values ranged from 23 to 30 ppm N. The maximum growth rate attained was 1.05 $\log_e$ units per day in the 45.6%T-1090 ft.cd. culture. All the cultures except the 21.5%T-820 ft.cd. culture experienced a curvilinear relationship between growth rate and influent-TIN above the critical region. This relationship was linear for the 21.5%T-820 ft.cd. culture.

The TIN removed by the various cultures was a function of the influent-TIN concentration. For the low density cultures this relationship was linear for all values of influent-TIN. The high density cultures exhibited a linear relationship up to about 18 ppm of influent-TIN-N. At influent-TIN values

greater than this the TIN removed decreased with increasing influent-TIN. In the linear phase for both culture densities, removal increased with increasing influent-TIN. The high density cultures removed 4 to 5 ppm N more than did the low density cultures for a given influent-TIN concentration in the linear phase.

Although influent-TIN was the independent variable, it was found that growth rate could also be expressed as a function of the TIN removed (TIN removed being the difference between the influent and effluent concentrations). For growth rate as a function of TIN removed there was also a critical region where the growth rate was highly dependent on the TIN parameter. In the critical regions the growth rate was influenced by both light intensity and culture density. The rate of increase in growth rate with an incremental increase in TIN removed was also influenced by the light intensity and culture density. The amount of TIN removal required to produce growth, increased as culture density increased. The difference in growth rates for the two different light intensities of the same culture density was related to the amount of TIN in the substrate effluent of the cultures. The low-light intensity cultures had the greater growth rates for the comparable culture densities and they had also the higher TIN concentrations in the substrate-effluent. The rate of change in growth rate with increasing TIN removal was found to be related to whether or not the substrate-effluent-TIN was dependent on the influent-TIN. The two high density cultures had the same, and the minimum rate of increase. In addition

their substrate-effluent-TIN was independent of the influent-TIN. The 45.1%T-760 ft.cd. culture had the greatest rate of increase and its substrate effluent exhibited the greatest dependency on the influent-TIN.

TIN removal in the critical region with comparable growth rates was greatest for the two high density cultures. The TIN in the effluent was lowest for the 45.6%T-1090 ft.cd. culture. This culture also had the highest photosynthetic activity. The TIN in the effluent of the two high density cultures reached a constant minimum value in each of the cultures throughout the whole critical region. For the low density cultures, however, the effluent-TIN decreased as growth rate and influent-TIN decreased.

For the portion of the relationship between growth rate and TIN-removal where the nutrient concentration is not as critical, the function is still linear. In this region the actual growth rate was dependent on both light intensity and culture density. For the 21.5%T-820 ft.cd. culture growth rate was independent of TIN removal above the critical region. Once the difference in growth rate for the different light intensities of the low density cultures had been established at the upper limit of the respective critical regions the rate of increase in growth rate with increasing TIN was constant and independent of light intensity. That light cannot be completely eliminated as a factor, however, is shown by the fact that the 22.1%T-1110 ft.cd. culture growth rate did increase with increasing TIN removed even though the 21.5%T-

820 ft.cd. culture did not. The ratio of the rates of increase in growth rates of the low density cultures to the high density culture is approximately equal to the ratio of their substrate-effluent-TIN concentrations. It is speculated that change in growth rate in this region is controlled by some function of the ratio of organism density to the TIN-concentration in the media, when there is sufficient light to support a change in growth rate.

Expressing the TIN removed as a function of culture productivity shows that TIN removal increased as productivity increased but the rate of increase was dependent on the culturing conditions. The 45.1%T-760 ft.cd. culture exhibited the maximum dependency between the two factors. The dependency decreased in order for the 45.6%T-1090 ft.cd. culture and the two high density cultures which had the same correlation. This seems to indicate that TIN removal as a function of productivity is influenced by light intensity, culture density and possibly the quantity of nitrogen required to produce a given quantity of algal material.

Culture productivity per ppm of nitrogen removed is expressed as a function of influent-TIN. The quantity of algae produced per ppm of TIN-N removed decreased with increasing culture density and decreasing light intensity. Each culture exhibited a maximum production of algal material per ppm nitrogen removed at the influent-TIN value defining the upper limit of the respective critical regions. As the influent-TIN was increased or decreased from that value associated with maximum production the production fell sharply. None of the

cultures reached a point where a minimum value of production per ppm nitrogen removed was experienced. In terms of sewage treatment there is indicated a point of maximum inefficiency but no point of maximum efficiency.

To further consider the ability of the cultures to provide tertiary sewage treatment a relationship between mg. of nitrogen removed per unit surface area per day per ppm algae in the culture and growth rate is shown. TIN removal expressed as indicated is a log linear function, for all the culturing conditions, of growth rate. In the range of comparable growth rates for all cultures, the cultures whose conditions were least suited to algal growth experienced the greatest TIN removal per ppm of algae. For any given culture density, however, the quantity of TIN removed per ppm algae increased as photosynthetic activity increased. It was not possible to determine the limits of this relationship.

To consolidate all the data pertinent to the design of a tertiary sewage treatment process two graphs were constructed, one for the high density cultures and one for the low density cultures, relating per cent TIN removed, influent-TIN and growth rate. Knowing the sewage flow and total inorganic nitrogen in the sewage these graphs can be used to determine the degree of treatment, detention time and size of facility. It can also be determined from these graphs the number of different cultures of the same or different density that would be required to give a desired treatment. It is possible for example, to obtain 98% removal for a sewage containing initially 24 ppm N. The data as presented is not considered

at this time to be suitable for interpolation or projection. Design therefore, could only be applied to the environmental conditions used in the investigation.

Phosphorus, the other macronutrient in secondary sewage treatment plant effluent is considered in relation to the basic variable the influent-TIN. Only the inorganic orthophosphate was considered. Orthophosphate removal is presented as a function of influent-TIN. The function is linear for all the culturing conditions with removal increasing as the influent-TIN was increased. The correlation of orthophosphate removal and influent-TIN decreased as the variation in the nitrogen to phosphorus ratio in the nutrient supply increased for any one culturing condition. The conclusion is that one nutrient can be used to represent the general nutrient conditions only if the nutrient always exist in constant ratios.

A consideration of the relationships between growth rate, influent and substrate-effluent orthophosphate shows the relationship to be very random. For the 45.1%T-760 ft.cd. culture the substrate-effluent and influent orthophosphate decreased while the phosphate removed stayed constant throughout the critical region of nutrient influence. The influent orthophosphate for the 45.6%T-1090 ft.cd. culture was much lower than that of the 45.1%T-760 ft.cd. culture. For the former culturing condition the substrate-effluent decreased only slightly with decreasing growth rate, in the critical region, while the orthophosphate removed was reduced by 50 per cent. The 45.6%T-1090 ft.cd. culture produced the lowest quantity of orthophosphate in the effluent of all the

cultures, with a concentration of 0.8 ppm PO_4.

The two high density cultures experienced a minimum constant orthophosphate concentration in their substrate-effluents in the critical region. For the 21.5%T-820 ft.cd. culture this value was 3.0 ppm PO_4. In the 22.1%T-1110 ft.cd. culture the concentration was 4.3 ppm PO_4. The influent-orthophosphate and orthophosphate removed decreased as growth rate decreased for the 22.1%T-1110 ft.cd. culture. In the 21.5%T-820 ft.cd. culture both the influent-orthophosphate and the orthophosphate removed remain constant as growth rate decreased in the critical region.

Because of the random values of the orthophosphate concentrations in the critical regions where comparable cultures had equal growth rates for a given influent-TIN value, it was determined that the nitrogen and not the phosphorus in the media was controlling growth.

No changes were noted between organic nitrogen in the influents of the cultures and that in the effluents. No apparent change took place in phosphorus forms other than the orthophosphate.

Both the specific extinction coefficient and the dry weight per organisms decreased in general with decreasing influent-TIN. To maintain a constant light transmittance value both the dry weight culture density and the number of organisms per milliliter increased at low influent-TIN values. The per cent transmittance of light was held constant at the expense of the other factors because (1) only photometric techniques are available for constant density, semi-continuous dilution

culture control and (2) it was believed that an algal culture's reaction to varying nutrient concentrations could only be determined if the light attenuation was constant for all nutrient values.

The Effect of Induced Turbulence on Algal Culture Growth

An evaluation of the effect of induced turbulence on algal growth and algal growth rates was made using batch, culturing techniques. The cultures were grown on a synthetic substrate. A light intensity of about 1200 foot-candles and mixing speeds of 500, 1000 and 2000 RPM were used.

The test organism was *Chlorella pyrenoidosa* obtained from Dr. Starr's algal collection at the University of Indiana.

To determine what environmental factor was limiting growth it was necessary to utilize two culture depths. The two culture depths used were 12.5 and 25 cm. Because of temperature increases due to mixing of the culture the maximum mixing speed was 1000 RPM in the 12.5 cm. culture and 2000 RPM in the 25 cm. culture. Growth and growth rate were measured in terms of both organisms per ml. and dry weight in ppm.

Studies were carried out by starting at the lowest mixing speed allowing growth to proceed until the growth rate became zero as measured by the density factor organisms per ml. At this point the mixing speed was increased and additional growth took place. The procedure was repeated until zero growth rate was attained with the maximum allowable

mixing speed.

Studies were also made at a constant mixing speed of 2000 RPM with a 25 cm. deep culture and at 500 RPM for culture depths of 25 and 12.5 cm.

Comparison of the data for the two different culture depths indicates that light intensity was limiting growth rate and total growth. Both culture depths experienced essentially the same productivity of their respective maximum populations. The maximum populations were in inverse relation to the ratio of the culture depths.

The results of the investigation indicate that algal growth can be increased by increasing the amount of induced turbulence. Growth rate and productivity decreased as culture density and mixing speed increased. Even though the growth rate was low at high culture densities and high mixing speeds it was still greater than the zero growth rate which would have been experienced at the same densities with low mixing speed. At low culture density the culture population and growth rate were independent of mixing speed for mixing speeds from 500 to 2000 RPM. Growth and growth rate as measured in terms of organisms per ml. were more affected by mixing speed than the same values determined by dry weight concentration.

The actual growth rate value was dependent on whether numbers or weight were used for the determination. No light integration was observed; therefore, there were no high values of productivity asssociated with high culture densities. The gain in growth with increased mixing speeds was attributable

to the increased number of algae which would be exposed to the light with the greater turbulence.

CONCLUSIONS

The following conclusions are based only on the conditions studied and the apparatus used in the experimental work. Projection or interpolation of the conclusions should only be considered within these limits.

Nutrients and Algal Growth

1. The growth rates of the *Oscillatoria* cultures were a function of the total inorganic nitrogen concentration of the culture influents.

2. There was a critical region where growth rate was a linear function of the total inorganic nitrogen supplied to the culture.

3. In the critical region the nitrogen required to produce a given growth rate was a function of the culture density but independent of light intensity.

4. In the critical region the increase in growth rate with an incremental increase in inorganic nitrogen was independent of culture density and light intensity.

5. For a given influent inorganic nitrogen concentration growth rate was a function of both light intensity and culture density in the non-critical region.

6. For all concentrations of inorganic nitrogen studied the quantity of inorganic nitrogen removed was a function of

the inorganic nitrogen supplied.

7. The high density cultures experienced an optimum influent inorganic nitrogen concentration in terms of inorganic nitrogen removal.

8. Growth rate, when expressed as a function of inorganic nitrogen removed, was dependent on culture density, light intensity and inorganic nitrogen removal.

9. When growth rate was expressed as a function of inorganic nitrogen removal there resulted two distinct linear relationships.

10. The high density algal cultures exhibited a minimum inorganic nitrogen concentration in the effluents over a wide range of growth rates. The low density cultures did not exhibit this characteristic.

11. Total inorganic nitrogen removal was found to be an exponential function of culture productivity for a given condition of culturing.

12. For each culturing condition there was an influent inorganic nitrogen concentration which produced the maximum quantity of algal cells per ppm TIN-N removed.

13. For a given growth rate nitrogen removal per ppm of algae increased as light intensity decreased and decreased as culture density increased.

14. When the nutrient concentration became limiting the

growth rate was controlled by the available inorganic nitrogen. Carbon was supplied continuously.

15. It is predicted that by connecting the two experimental cultures together (one high density culture and one low density culture) a 98 per cent removal of inorganic nitrogen might be attained.

16. The light attenuation characteristics of *Oscillatoria* are highly dependent on the substrate inorganic nitrogen concentration.

The Effect of Induced Turbulence on Batch Algal Culture Growth

1. With the experimental apparatus used no light integration was obtained.

2. Total growth increased with increasing mixing speeds.

3. Growth rate decreased as mixing speed and culture density were increased.

4. Due to the absence of light integration productivities decreased as culture densities increased.

5. With sufficient mixing the growth rate of a culture can be maintained in the exponential growth phase for extended periods of time.

6. Modification of the experimental apparatus might

produce conditions which would permit light integration by the algae.

APPENDIX I

Coulter Counter

The Coulter electronic particle counter is an electrical device capable of determining the number and size of microscopic particles suspended in a solution of electrolyte. The suspended particles are pumped through a small orifice (100 millimicrons in diameter by 75 millimicrons in length) which has a large surface area, non-reactive electrode on either side of it. Both electrodes are immersed in electrolyte. A particle passing through the orifice changes the resistance of the conductor. This resistance change produces a voltage pulse of short duration with a magnitude proportional to the volume of the particle (141).

The suspension is pumped through the orifice by means of a mercury column operating under a differential head. The flow of the mercury starts and stops the instrument by means of contact electrodes. The on-off contacts are so located that the volume of suspension drawn through the orifice is exactly 0.5 ml.

The counter used was a Model B Coulter Counter which has two more operational control devices than the Model A, giving it a total of four basic control parameters. These four controls are: (1) upper threshold control, (2) lower threshold control, (3) amplification control, and (4) apperture (orifice) current control.

The instrument counts particles in relation to their size. That is, to be counted a particle must produce a

voltage pulse when it passes through the orifice; this pulse, as already mentioned, is a function of the particle volume. The pulse produced by a particle, after amplification, is displayed on an oscilloscope screen and, in addition, is fed into a discriminator then on to a digital counter. The lower threshold determines the minimum pulse height that will be counted. The upper threshold determines the maximum pulse height which will register a count. With the upper threshold control in operation, counts are made of particles having a volume equal to or less than the upper threshold setting and equal to or greater than the lower threshold setting. The upper threshold control can be taken out of operation, in which instance all particles having a size equal to or greater than that corresponding to the lower threshold setting are counted. The two threshold controls have scales divided into 100 units. When operated independently the two scales have the same volume value per scale unit. It is possible, however, to lock the two controls together such that a preselected increment of particle size is always counted. For this situation, 10 units of the upper threshold scale are equal to 1 unit of the lower threshold scale. The maximum increment of particles that can be counted with this arrangement is that size range which corresponds to 10 units on the lower threshold scale. When the two threshold controls are locked together the upper control determines the size of the increment while the lower control determines the actual size of the particle. For example, with the upper control unit set at 100 and the lower control unit at 50 all particles whose

volumes lie between the sizes corresponding to 50 and 60 on the lower control scale will be counted; if the lower control is moved to 40, without adjusting the upper control, then all particles having a size that is between the 40 and 50 settings on the lower control dial will be counted.

Both the amplification and aperture current determine the actual particle size associated with a given threshold setting. The volume measure per unit of threshold scales is different for each different combination of amplification and aperture current used. The amplification control has nine positions, each position doubles the pulse height, for the same size particle, of the previous control position. Since pulse height is doubled for each position, the volume measure per threshold scale unit is halved for each increase in amplification control position.

The aperture current control doubles the aperture current with each increased setting with a nominal aperture current of one milliampere forming the base. There are, however, three aperture current values which have a nominal milliampere value equal to $2^{\frac{1}{2}}$ as the base. For all, but the three mentioned settings, a change in the aperture current has essentially the same effect on the threshold scales as does changing the amplification control position.

Judicious selection of amplification and aperture current values permits the counting of small volume particles without including an excessive number of "background pulses" in the count. These background pulses are not necessarily constant. Therefore, it is difficult to correct for them using a blank.

Two different instruments and several different locations and electrical sources all produced about the same type of background pulses. In general as both amplification and aperture current are increased the smaller is the particle that can be counted without background interference. Caution must be used at high aperture currents to prevent boiling in the aperture. Boiling results in the release of air bubbles which produce erroneous counts. The problem of boiling is most critical when debris gets caught in the aperture or when rather long filamentous algae are being counted.

In practice the instrument can be used either as a particle counter or to size particles or it can be used for both tasks at the same time. To use the instrument to determine particle size it is necessary to first calibrate the instrument using particles of known size. Among other things, both rag weed pollen and blood cells have been used for this purpose (142). Data collected at the University of Michigan Sanitary Engineering Laboratory using the algae Scenedesmus and Oscillatoria indicate that the volume measure per scale unit is highly dependent on the particle shape. For the filamentous algae length also appears to be a factor.

Using the instrument to count particles does not necessitate knowing the particle size associated with the control settings as long as the relative relationship between the various control positions is known. To determine the number of particles present in a suspension a plot of numbers versus lower threshold setting is made. The upper threshold control is not used. The plot may be made on either log-log or semi-

log paper. As the threshold setting is decreased, that is, the volume of the particles being counted decreases, the counts will increase until a plateau or inflection point is reached. Decreases in the threshold settings beyond this point result in rapid increases in counts.

After applying a coincidence correction the count at the plateau or inflection point is the number of particles in 0.5 ml. of the suspension. The threshold setting associated with the plateau is the setting which can be used for additional total counting of the same general size particles without having to construct a new curve each time a count is made. It is imperative, however, when determining a threshold setting for counting a particular algae to be sure that the setting used did not occur at a false plateau or inflection point. It is possible to get a plateau or inflection point which does not represent the total particles in the sample counted.

Operational Procedure:

A sample was withdrawn from a culture and diluted with a saline solution such that the final volume had a 0.9 per cent NaCl content. The dilution necessarily depends on the number of particles in the original sample. The general practice was to keep the total count per 0.5 ml. below 8×10^4. Maloney et al (142) found that a range of 10^3 to 40^3 counts per 0.5 ml. was optimum. To expedite the operation a 24 ml. pipetter was used to dispense the saline solution. A normal working volume was 50 ml. Therefore, a 1 ml. sample or suitable aliquot was diluted to 50 ml. using saline solution and distilled

water. A blank containing saline solution and an appropriate quantity of distilled water was also made up.

For the batch culture experiments using Chlorella curves of counts as a function of lower threshold settings (Fig. 35) showed that a setting of 40 on a relative scale would count all the algae in the diluted sample. For the constant density cultures using Oscillatoria daily curves of count as a function of threshold setting (Fig. 36) were determined. This was done in order to pick up any dramatic changes in size with changes in the environmental factors studied. Blank counts were made for the same settings of the threshold control amplification control and operative current as were used to count the diluted sample. The blank counts and the sample counts were corrected for coincidence, if required, using the correction table provided by the Coulter Electronics Company. The difference between the corrected sample and blank count times the dilution factor gave the number of particles per ml. of the original sample.

For the Chlorella the number of particles per ml. represented the number of organisms per ml. For Oscillatoria cultures, however, the number of particles per ml. represented only the number of algal particles per ml. and not the number of algal cells. No correlation was made between haemacytometer counts and Coulter Counts; however, Maloney et al (142) reports that the correlation is very good.

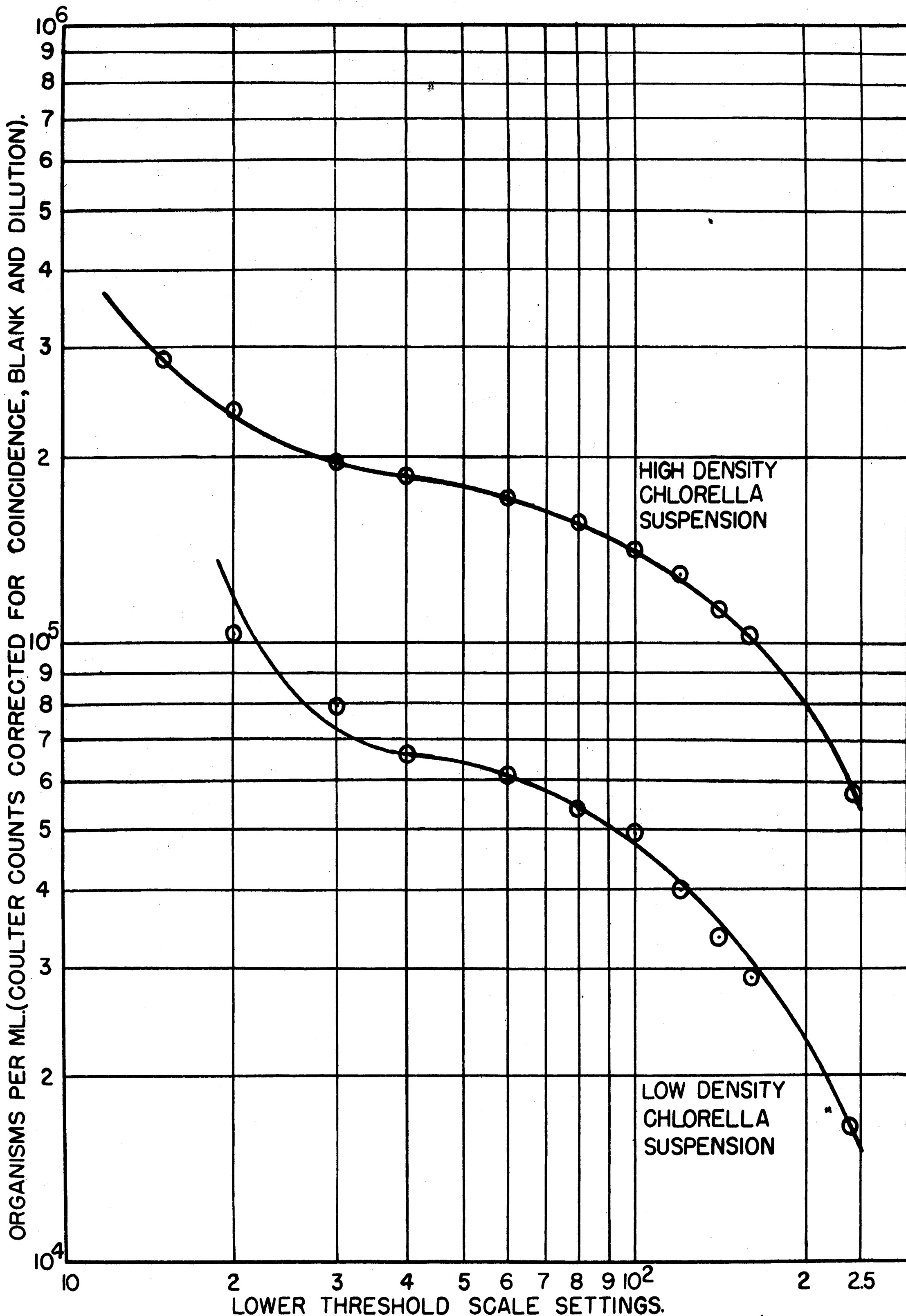


FIG. 35 TOTAL NUMBER OF ORGANISMS PER ML. AS A FUNCTION OF THE LOWER THRESHOLD SETTINGS OF THE COULTER COUNTER FOR TWO SUSPENSION OF CHLORELLA.

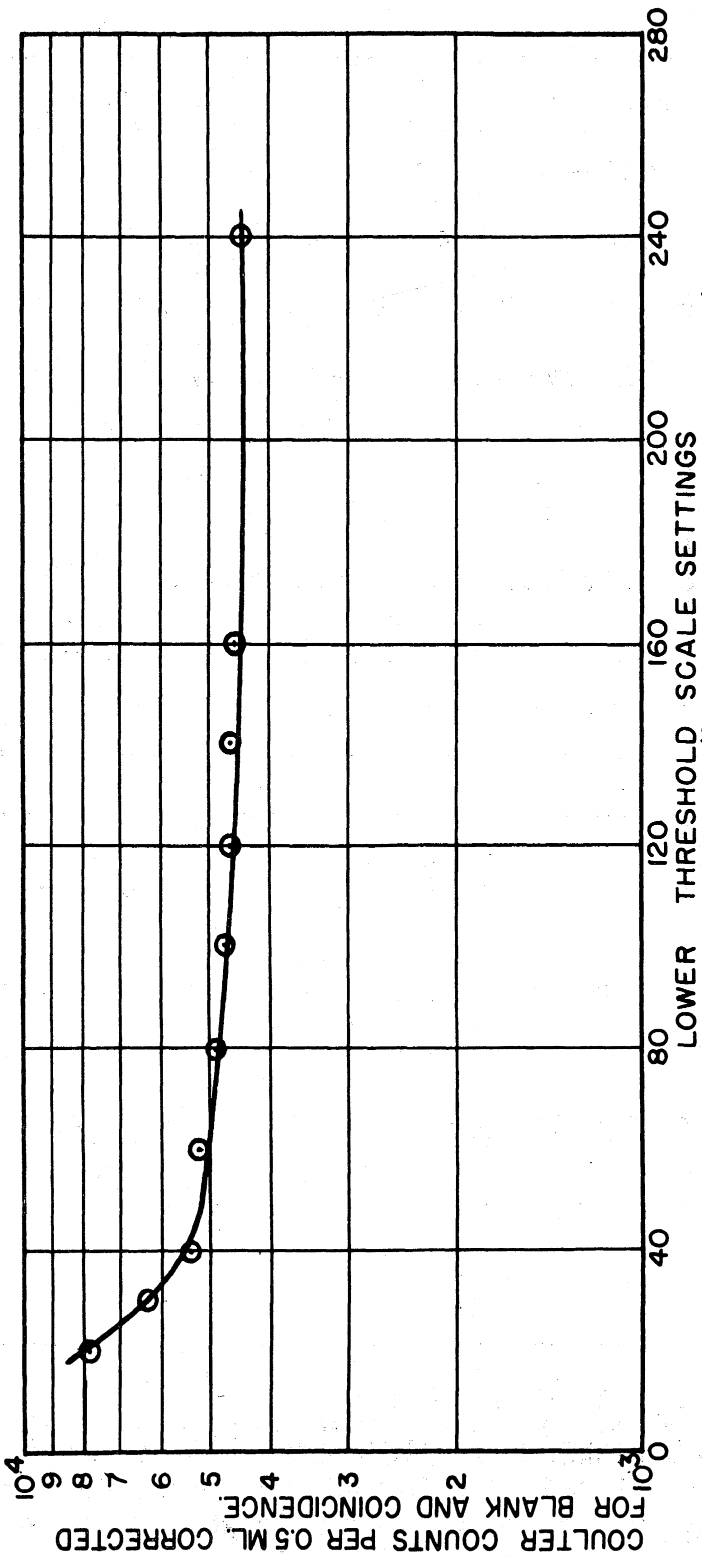


FIG. 36 CORRECTED COULTER COUNT FOR A 1 TO 200 DILUTION OF AN OSCILLATORIA SUSPENSION AS A FUNCTION OF THE LOWER THRESHOLD SETTINGS.

APPENDIX II

Chemical Testing Procedures

Chemical Determination: Ammonia - Nitrogen (143)

Procedure:

1. Use 10 ml. sample or an aliquot diluted to 10 ml. with ammonia-free distilled water.
2. Add 2 drops of Rochelle Salt solution and mix.
3. Add 1 ml. of Modified Nesseler reagent followed immediately by 1 ml. of sodium hydroxide solution and mix.
4. Allow 10 minutes for color development. Measure color density at 410 millimicrons.
5. Use a blank containing ammonia-free distilled water and reagents.

Reagents:

1. Modified Nesslers Reagent - dilute 100 g. HgI_2 and 70 g. KI to 1 liter.
2. Sodium Hydroxide Solution - dilute 160 g. NaOH to 1 liter.
3. Rochelle Salt Solution - dilute 50 g. $KNaC_4H_4O_6\ 4H_2O$ (potassium sodium tartrate) to 100 ml.
4. Use ammonia-free distilled water for reagent preparation.

Chemical Determination: Nitrite-Nitrogen (143)

Procedure:

1. Use 10 ml. sample or an aliquot diluted to 10 ml. Adjust pH to approximately 7.

2. Add 1 ml. sulfanilic acid reagent and mix. Allow to stand 3 to 10 minutes.
3. Add 1 ml. 1-naphthylamine hydrochloride reagent and 1 ml. sodium acetate buffer solution, and mix well.
4. Allow 20 minutes for color development. Measure color density at 520 millimicrons.
5. Use a blank of distilled water and reagents.

Reagents:

1. Sulfanilic Acid Reagent - dissolve 0.60 g. sulfanilic acid in 70 ml. distilled water. Cool, add 20 ml. conc. HCl, dilute to 100 ml. with distilled water, and mix thoroughly.
2. Naphthylamine Hydrochloride Reagent - dissolve 0.60 g. 1-naphthylamine hydrochloride in distilled water containing 1.0 ml. conc. HCl. Dilute to 100 ml. with distilled water and mix thoroughly. Store in refrigerator.
3. Sodium Acetate Buffer Solution - dissolve 16.4 g. $NaC_2H_3O_2$ or 27.2 g. $NaC_2H_3O_2 \cdot 3H_2O$ in distilled water and dilute to 100 ml. Filter if necessary.

Chemical Determination: Nitrate-Nitrogen (143)

Procedure:

1. Use a 5 to 25 ml. sample. Adjust pH to approximately 7.
2. Evaporate to dryness over steam bath in an evaporating dish.
3. Add 2 ml. phenoldisulfonic acid and rub residue

making sure all solids are dissolved.

4. Transfer to a nessler tube, rinse dish 2 or 3 times and add rinsings to nessler tube, bring volume to approximately 20 ml.
5. Add 7 ml. potassium hydroxide solution to the nessler tube. Dilute to 50 ml. and mix.
6. Measure color density immediately at 410 millimicrons.
7. Use a blank of distilled water (not evaporated) plus reagents.

Reagents:

1. Phenoldisulfonic Acid - dissolve 25 g. pure white phenol in 150 ml. concentrated H_2SO_4. Add 75 ml. fuming H_2SO_4 (15% free SO_3); stir well; heat for 2 hrs. on a hot water bath (not boiling).
2. Potassium Hydroxide Solution - dissolve 673 g. KOH in distilled water and dilute to 1 liter.

Chemical Determination: Total Organic Nitrogen (143)(144)

Procedure:

1. Dilute a measured sample to 300 ml. with ammonia-free water and place in an 800 ml. kjeldahl digestion flask. Add 4 glass beads.
2. If necessary, adjust pH to approximately 7.0. Then add 25 ml. of buffer solution.
3. Distill off 200 ml.
4. Add 50 ml. of digestion mixture sample for 30 minutes after fuming (copius white fumes) starts (approximately 20 to 30 minutes after applying heat).

5. Cool residue and add 300 ml. ammonia-free distilled water.
6. Make solution alkaline to phenolphthalein using 50% NaOH solution.
7. Distill off 200 ml. Test for ammonia-nitrogen using 10 ml. of the final distillate.
8. Carry blank, using ammonia-free distilled water, through whole procedure.

Reagents:

1. Buffer Solution - dilute 14.3 g. KH_2PO_4 and 68.8 g. K_2HPO_4 to 1 liter.
2. Digestion mixture - dissolve 125 g. K_2SO_4 in a solution containing 800 ml. of distilled water and 400 ml. of conc. H_2SO_4; add 50 ml. of mercuric sulfate solution (8 g. red mercuric oxide diluted to 100 ml. with 6 N H_2SO_4) and dilute to two liters.
3. 50% NaOH solution - dilute 500 g. NaOH to 1 liter.
4. Use ammonia-free distilled water for reagent preparation.

Chemical Determination: Ortho-Phosphate (143)

Procedure:

1. Dilute a measured sample to 50 ml. in a nessler tube with distilled water.
2. Add 2.5 ml. of ammonium molybdate solution and mix.
3. Add 5 scoops (0.5 g.) of StannaVer Powder and mix.
4. Allow 10 minutes for color development; then measure color density at 700 millimicrons.

5. Use distilled water plus reagent blank.

Reagents:

1. Ammonium Molybdate Solution - dissolve 256 g. $(NH_4)_6Mo_7O_{24}NH_2O$ in 175 ml. distilled water. Add 310 ml. of conc. H_2SO_4 to 400 ml. of distilled water. After acid solution cools mix the two solutions and dilute to 1 liter.
2. StannaVer Powder - Hach Chemical Company Catalogue Number 293.

Chemical Determination: Total Phosphate (139)

Procedure:

1. Place a 5 ml. sample in 30 ml. digestion flasks. Add 3 glass beads.
2. Add 10 ml. conc. HNO_3 and 6 ml. conc. H_2SO_4. Boil to SO_3 fumes (white fumes). Continuous turning of flask is absolutely necessary to prevent bumping and blowing.
3. Follow by two more 10 ml. additions of conc. HNO_3, boiling to SO_3 fumes each time. Addition of HNO_3 to hot flasks must be done carefully.
4. After final boiling cool flasks to room temperature and transfer residue to 150 ml. beaker. Wash flask several times, adding washings to the beaker; boil for approximately 25 minutes (3-5 minutes after fuming starts).
5. Cool beakers to room temperature, add 20 ml. of distilled water and adjust pH to exactly 7.0 with

conc. NH_4OH.

6. Dilute to 50 ml. in nessler tubes and measure orthophosphate concentration.

7. Carry a distilled water blank through all steps.

Reagents:

1. Conc. HNO_3, H_2SO_4 and NH_4OH are all reagent grade commercially available chemicals.

APPENDIX III

Chemical Composition of

Ann Arbor Sewage Treatment Plant Effluent

Sewage Number:	1	2	3	4	5
NH_3 as ppm N	25.8	26.4	24.0	24.2	27.0
NO_2 as ppm N	0.2	0.4	0.4	0.1	0.1
NO_3 as ppm N	0	0.4	2.4	0.5	0.1
PO_4 as ppm PO_4	24.0	23.6	20.2	24.5	14.8
pH	7.2	7.4	7.9	7.4	7.6
Alkalinity as ppm $CaCO_3$	245.5	223.4	199.3	240.0	243.0

Sewage Number:	6	7	8	9
NH_3 as ppm N	25.1	23.7	28.8	30.6
NO_2 as ppm N	0.3	0.7	0.1	0.3
NO_3 as ppm N	0.1	0.1	0.2	0
PO_4 as ppm PO_4	15.5	18.0	18.0	36.8
pH	7.6	7.5	7.7	8.2
Alkalinity as ppm $CaCO_3$	227.8	203.7	234.3	214.6

APPENDIX IV

Synthetic Media Used for Batch Cultures (56)

Basic Media:

Chemical	grams/L
$MgSO_4 \cdot 7H_2O$	0.25
K_2HPO_4	1.00
$Ca(NO_3)_2 \cdot 4H_2O$	0.025
KNO_3	1.000
$FeCl_3 \cdot 6H_2O$	0.035
Na Citrate$\cdot 2H_2O$	0.222
Micronutrients	1 ml./L

Micronutrient Solution:

Chemical	grams/L
H_3PO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
MoO_3(85%)	0.0177
$CuSO_4 \cdot 5H_2O$	0.079

Mix $FeCl_3 \cdot 6H_2O$ and Na citrate$\cdot 2H_2O$ together and add to distilled water. Add other components as listed, thoroughly mixing after each addition. The substrate can be sterilized after all components have been added. Rapid cooling must follow sterilization to prevent precipitation.

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