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Title: THE GROWTH OF CHLORELLA VULGARIS 211/8K IN DILUTED SWINE MANURE AS A FUNCTION OF TEMPERATURE, LIGHT INTENSITY, DEPTH, AND RETENTION TIME

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Abstract approved: _____
Dr. A. Anderson

Experiments were designed to determine the effects of depth, light intensity, temperature, and retention time on the dry weight density of a culture of Chlorella vulgaris 211/8K. The culture medium was diluted swine manure. The indigenous bacterial populations were not examined. Minipond basins with a surface area of 0.1 m^2 were used to culture the algae. Agitation and carbon dioxide were supplied by a single air bubbler running along the basin floor. Lighting was provided by fluorescent and incandescent bulbs at the ceiling of the growth chamber. Temperatures were maintained by submersible aquarium heaters and were monitored continuously.

Analysis of variance was used for the statistical interpretation of the data. This analysis allowed prediction of the effects of depth, light intensity, temperature, and retention time, and interactions of these factors, on the culture density.

The depths of 5, 10, and 15 cm were compared. Of these, 10 cm was determined to be the optimum culture depth giving maximum yield of algae on a grams/day basis. Yield from cultures 5 cm deep was limited by the small harvest volume potential. Growth at culture depths of 15 cm was light-limited. This 15 cm depth resulted in the lowest culture densities at all temperatures and light intensities.

Light intensities of 300, 800, 1200, and 1800 ft. c. were tested. This range includes intensities lower than and greater than the reported saturation intensity of 500 ft. c. for Chlorella. At 300 ft. c. the growth of Chlorella vulgaris 211/8K is limited by the photochemical reaction rate. The maximum culture densities were attained with intensities of 800 and 1200 ft. c. The turbidity of the swine waste probably reduced these levels to near-saturating levels. The culture density was reduced when the light intensity was increased further to 1800 ft. c., suggesting photo-inhibition of growth and/or photorespiration occurred at this higher light intensity.

Over the temperature range of 25, 30, 35, and 40 C, culture density was not influenced in a major way by temperature. Chlorella vulgaris 211/8K grew equally well at all of these temperatures because temperature was not the limiting factor. Of the four factors studied, temperature by itself was the least important in determining the culture density.

Culture density increased as the retention time increased from 1.8 to 11.0 days. As the retention time increases the harvest volume decreases. A compromise must be reached between culture density and harvest volume to optimize yield on a grams/day basis. A 2.0 to 4.0 day retention time resulted in optimum yield under the conditions tested. The fact that culture density increased with retention time implies that with time the diluted swine manure becomes a medium more suitable for algal growth.

The four factors also interacted to affect the culture density. Temperature interacted separately with depth, light intensity, and retention time to influence the culture density. The combination of depth and light intensity was the fourth two-way interaction affecting the intensity. Analysis of variance indicated light, temperature, and depth were the components of the only three-way interaction affecting the culture density.

An equation derived through statistical analysis of variance can be obtained which predicts the culture density (Y) given the light intensity (λ), temperature (τ), depth (δ), and retention time (γ). The subscripts in the following equation refer to the levels of light (i), temperature (j), depth (k), and retention time (ℓ). The experimental error is given by $E_{ijk\ell}$. The sampling error for each sample number (m) is given by $e_{ijk\ell m}$. The mean of all densities is given by μ.

$$Y_{ijk\ell m} = \mu + \lambda_i + \tau_j + \delta_k + \gamma_\ell + (\lambda\tau)_{ij} + (\lambda\delta)_{ik} + (\tau\delta)_{jk} + (\tau\gamma)_{j\ell}$$

$$+ \ldots (\lambda\tau\delta)_{ijk} + E_{ijk\ell} + e_{ijk\ell m} .$$

A more practical equation containing only the four factors shows the general effects of depth (D), temperature (T), light intensity (L), and retention time (R) on the culture density (Y).

$$Y = 0.6911 - 0.03D + 0.013T + 5.4057(10^{-5})L + 0.0613R.$$

The Growth of Chlorella vulgaris 211/8K in Diluted Swine Manure as a Function of Temperature, Light Intensity, Depth, and Retention Time

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE GROWTH OF CHLORELLA VULGARIS 211/8K IN DILUTED SWINE MANURE AS A FUNCTION OF TEMPERATURE, LIGHT INTENSITY, DEPTH, AND RETENTION TIME

INTRODUCTION

Justification

If we can agree that food and water supplies have always been, and are today in short supply for some persons, then we can act to solve many problems leading toward shortages. Improper waste disposal systems are among numerous causes resulting in diminished water supplies. Keller, Mattoni, and Myrick (1965) suggested changing the water resource concept from obtaining, using and disposing of water, to obtaining, using, and reusing water. The United States is slowly reacting to such suggestions.

Consider water supply, food supply, and waste disposal as three separate problems regulating a human eco-system depicted in Figure 1. Waste disposal becomes rate-limiting when feedback from pollutants diminishes water resources. Preventing the collapse of the system dictates converting wastes to useful products (Keller, Mattoni, and Myrick, 1965).

As the popularity of livestock production in confined quarters increases, so do associated difficulties, such as the disposal of vast quantities of manure and the pollution of both surface and ground water

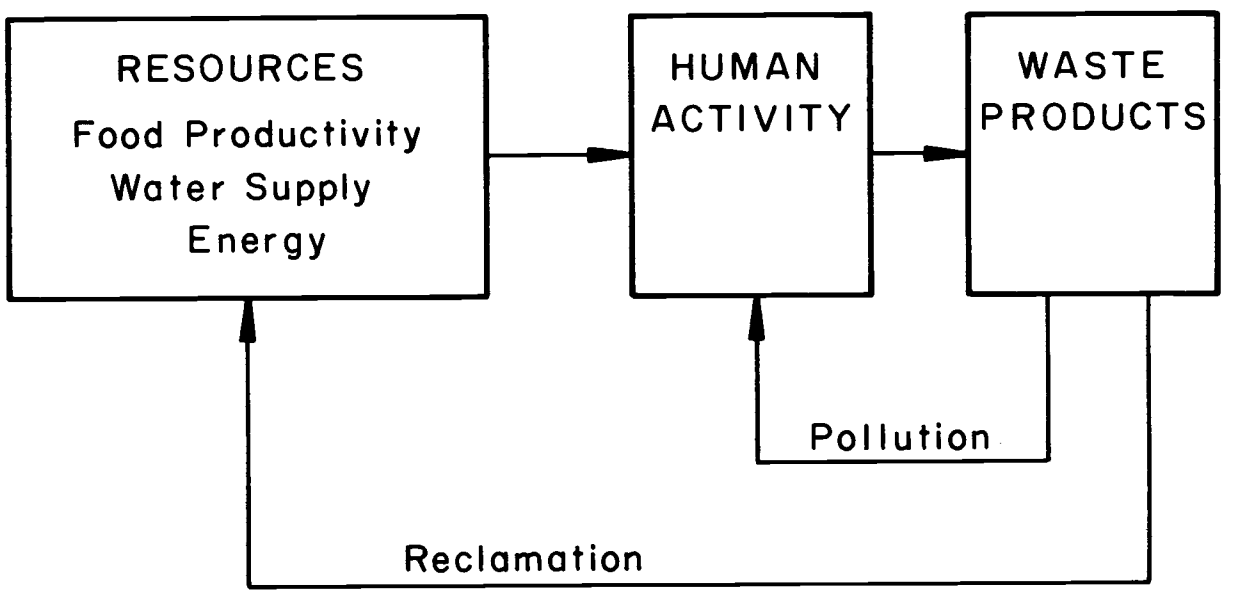

Figure 1. Interrelations of resources and products to total human activity (Mattoni, Keller, and Myrick, 1965).

due to organic carbon, nitrogen, and phosphorus present in the wastes (Jones and Patni, 1974). The quantity of wastes is extraordinary. Cattle, sheep, goats, chickens, turkeys, horses and swine excrete some five million tons of waste daily in the U.S.A., of which 800,000 tons is dry matter, 32,000 tons is nitrogen and 10,000 tons is phosphorus. Benne, Hoglund, Longnecker, and Cook (1961) discussed the worth of animal manure and concluded that where the population includes both young and old animals, it is a safe assumption that about 75 percent of the fertilizing constituents in the feed are excreted in solid manure and urine. The capacity of the local environments to dilute, stabilize, and dissipate this increasing accumulation of wastes is being exceeded in many instances.

Disposal of manure on the land is a desirable practice, however sufficient land is not available. Current manure management programs are expensive in terms of equipment and labor. They are often environmentally detrimental and may tend to aid the proliferation of pathogens. Further development of crop production potential through the use of manufactured fertilizers, irrigation or large scale drainage projects requires capital not presently available. Furthermore, the increasing use of water and fertilizers cannot proceed indefinitely.

Waste treatment by biological activity goes back to the beginning of life itself as an entire spectrum of microbes evolved to handle degradation of excretory and waste products of other microbes. The

efficiency is naturally high and may be maintained in a flexible steady-state environment. Single-celled organisms have numerous advantages supporting their use in biological waste treatment programs including (i) the ability to utilize recycled nutrient elements; (ii) the opportunities for genetic improvement, and (iii) the ability to produce protein on smaller areas than cereal crops.

More specifically, algal growth using waste materials as a substrate looks impressive for several reasons. Unicellular green algae have a high protein content with a favorable amino acid spectrum (Heitman and Hintz, 1967). A pilot-plant study at Lancaster, California, revealed algae to contain 51.2 percent protein as compared to soybeans with 16.25 percent protein, corn with 7.5 percent and sugar cane which had a protein level of 1.78 percent (Keller, Mattoni, and Myrick, 1965). There is a high yield of photosynthetic products per unit time and area of solar illumination (Tamiya, 1957). Algae are 100 times more efficient in utilizing solar energy than are cereal crops (Heitman and Hintz, 1967). The protein yield per acre of Chlorella is more than ten times that of soy-beans (Oswald, 1962). Results of feeding trials demonstrate that algae provide satisfactory protein supplements for swine, comparable to meat and bone meal (Heitman and Hintz, 1967). Extensive reports indicate the high food value of algal protein for man as well as animals (Morimura and Tamiya, 1954; Tamiya, 1956; Krauss, 1962).

Golueke and Oswald (1973) note that algae incorporate external solar energy into the regeneration system, in addition to preserving entrapped energy. In comparison to conventional agriculture, the consequences of waste utilization by algae with regard to ecology are far superior. Despite evaporative losses, much of the water required for algal cultures is recoverable. Nutrients needed for algae production would, in part, represent pollutants in ordinary ecosystems if otherwise discharged (Keller, Mattoni, and Myrick, 1965).

Algal cultures are being employed in three primary systems as reported by Tamiya (1957). First, cultures are being used in which algae proliferate in sewage in symbiosis with bacteria. The purpose here is to accelerate stabilization of organic manure. Caldwell (1946) and Myers (1948) reported that organic matter stabilization effected by bacteria is enhanced by simultaneous algal growth. The culture of nitrogen-fixing algae with the intention of utilizing these to increase soil fertility has been considered.

Thirdly, and pertinent to this study is the use of algal cultures for production of useful organic substances such as food, feed and/or some special organic material. The idea of unicellular algae being cultured for food reportedly originated with Harder and von Witsch (1941) and Spoehr and Milner (1949). The Berkeley group of Ludwig, Oswald, Golueke, and Gotass tried to define methods of maximizing algal biomass production, as well as attempting to devise an

appropriate economical phase separation process. Oswald later termed this algae production process: "Controlled Photosynthesis" (Keller, Mattoni, and Myrick, 1965).

Other applications of algal cultures not described by Tamiya are water purification only, oxygen production, phycocolloid production, e.g., algin, and production of pigments capable of converting solar energy into electrical energy.

The United States is presently catching up with UNICELPE, the European Association of Single-Cell Protein Producers. Sherwood (1974) summarizes evidence "that single-cell protein as an industrial product has finally arrived." He discusses single-cell biomass production in France, Scotland, Italy, and Romania, as well as stressing Japanese and Russian involvement and the relative disinterest shown by the United States.

Not all reports concerning algae production are favorable. Heitman and Hintz (1967) review some problems. Algae have a bitter taste (Morimura and Tamiya, 1954). Diets with high levels of algae, e.g., 40 percent or higher, have actually drecreased feed intake by some animals. If diets are unpelleted, animals may sort out the algae and leave them (Heitman and Hintz, 1967). Algae have proven to be a low-energy feed due to low carbohydrate digestibility and high ash content. According to Hemens and Stander (1969), Wuhrmann (1964) reported that the upper limit for nitrogen-removal from waste

ponds by incorporation into an autotrophic microphyte lies between 65 and 85 percent. This results in inefficient pond production in areas without constant high temperatures and high light intensities.

Obviously, different objectives will determine the species and environmental conditions imposed on the culture. Krauss (1962) lists three possible objectives: (i) the production of large samples of algae from a mixed culture, (ii) the production of an algal product obtainable from any one of a number of species, and (iii) the production of large amounts of one particular organism.

Objectives

Research was undertaken to determine the effect of varying laboratory-controlled combinations of light intensity, temperature, pond depth, and swine waste retention time on the growth of Chlorella vulgaris 211/8K in miniponds. Light intensities used were 300, 800, 1200, and 1800 foot candles. Temperatures maintained in 5, 10, and 15 cm deep cultures were 25, 30, 35, and 40°C. Retention times ranged from 1.8 days to 11.0 days.

The primary objective of this study was to determine the combination of these variables which resulted in optimal algae growth. Other objectives were to determine the effects of aeration on culture density, pH, and nitrogen removal. Algae numbers were compared with millipore dry weight densities. The effects of stirring algal

cultures on densities were observed and recorded. Harvested material from algal cultures was collected by centrifugation and analyzed for elemental composition. Protein content of the harvested material was estimated. Identification of bacteria present in the culture was started.

LITERATURE REVIEW

Swine Waste

Scheltinga (1969) points out that handling of manure has often been improper as well as inadequate. Gases (CO_2, H_2S, CH_4, and NH_3) produced during manure decomposition have been lethal to animals and brought illness to workers in areas of inadequate ventilation. Waste production is not in balance with ordinary demand for wastes. This fact, plus the additional difficulties of transport and odor, limits land waste disposal.

Alternate methods of manure processing have been reviewed. They include dehydration, incineration, and the application of soil as a high capacity treatment system.

Oxidation ditches and lagoons have been studied extensively. Hazen, Hermanson, and Johnson (1969) used effluent from an anaerobic lagoon at the Iowa State University Swine Nutrition Farm as influent waste for activated sludge studies. They concluded that the waste had the essential factors to sustain biological growth and assumed inhibitors were absent. Generally, lagoons effectively store manure but the effluent is not suitable for discharge into streams (Barlow, Boersma, Miner, Phinney, and Oldfield, 1975). The odors have been analyzed and reportedly are objectionable (Clarke, 1965).

Oxidation ditches have been used to handle wastes from large swine populations. Scheltinga (1969) mentions the use of ditches lined with a bituminous material to handle wastes from 1000 pigs. He also speaks of an oxidation tank holding 500 liters of manure produced by 160 pigs with an average weight of 150 pounds. Jones and Patni (1974), in addition to discussing hazards associated with improper waste disposal, report on nitrogen and phosphorus fates during a seven-month study of a full-scale oxidation ditch and a one-acre lagoon system used to treat wastes produced by 410 swine located in Toronto, Ontario. Settling of nitrogenous compounds, ammonification, nitrification, and denitrification resulted in loss of total Kjeldahl nitrogen from the mixed liquor in the ditch. After nearly seven months of operation, the cumulative loss of total Kjeldahl nitrogen from the ditch liquor was about 50 percent of the total nitrogen input. The seven-month study showed that phosphorus accumulated in the system with only 15 percent unaccounted for. The loss was thought to be entrapped by sludge or the soil layer beneath the unlit bottom of the ditch.

Efforts have been made to develop procedures for photosynthetic reclamation of wastes, as exemplified by a pilot scale operation at the University of California (Dugan, Golueke, Oswald, and Rixford, 1970). This operation involves a partially closed system of animal waste management based on the integration of anaerobic and aerobic phases,

the recycling of water, and the recovery of algae. A detailed materials balance indicated the system to be economically favorable. The Bureau of Solid Waste Management of the U.S. Public Health Service has supported efforts with specific objectives such as determining the energy content, organic loadings, balance of water and nutrients, and overall performance of such waste treatment systems.

Algae reportedly served in lowering pollution levels of wastes indirectly by providing oxygen for bacterial oxidation of complex organic pollutants and directly by depleting the substrate of certain dissolved elements, chiefly nitrogen and phosphorus (Keller, Mattoni, and Myrick, 1965). Readers are warned that whereas the mass culture of green algae in synthetic media may yield densities of several grams per liter, there are limitations on attainable algal densities in waste cultures, primarily determined by carbon and nitrogen content (Tamiya, 1957).

The quantity and quality of the waste varies depending on the number of swine, the species involved, manure collection methods, housing design, bedding materials, animal weight, and types of feedstuff used. Scheltinga (1969) provides the following data: a pig produces a gallon of waste daily, of which 1.1 pounds is dry matter and 0.05 pounds is total nitrogen.

There is no question that swine waste is a highly concentrated source of organic matter, plant nutrients, and microorganisms. The

Michigan State University foursome of Benne, Hoglund, Longnecker, and Cook (1961) provided an analysis of hog wastes (Table 1).

Table 1. Composition of swine waste.

Component	Pounds of Component Per Ton of Waste
Water	1440
Total organic matter	399
Total carbohydrates	297
Easily digestible carbohydrates	190
Total mineral matter	161
Hard-to-digest carbohydrates	107
Crude protein	93
Calcium	11.40
Nitrogen	10.00
Crude fat (sol of dry ether)	9.00
Potassium	7.60
Phosphorus	2.80
Sulfur	2.70
Magnesium	1.60
Iron	0.56
Zinc	0.12
Boron	0.08
Manganese	0.04
Copper	0.01
Molybdenum	0.002

On the basis of 1961 prices, Benne, Hoglund, Longnecker, and Cook (1961) estimated the combined value of nitrogen, phosphorus, and potassium in the hog manure to be $2.63 per ton.

Nutrition of Algae

Nutritional requirements for algae vary considerably as they are heterogeneous physiologically and morphologically. O'Kelley (1974) lists the following inorganic (not C, H and O) elements as being required by one or more algal species: N, P, K, Mg, Ca, S, Fe, Cu, Mn, Zn, Mo, Na, Co, V, Si, Cl, B, and I. Of these, N, P, Mg, Fe, Cu, Mn, Zn, and Mo are needed by all algae and are not replaceable.

Elemental roles are exemplified by several macro- and micronutrients. Sulfur is obtained primarily through sulfate reduction, with most of it being proteinaceous, although Chlorella does produce sulfolipids (Benson and Shibuya, 1962; Kennedy and Collier, 1963). Weeding and Block (1960) reported that Chlorella vulgaris absorbed methionine rapidly for a brief time, more slowly for a long time. Using selenium as a sulfur analog, Shirft (1954a, b) showed the necessity of sulfur for cell division. Selenium inhibits sulfate absorption in Chlorella vulgaris (although methionine partially alters this inhibition) and changes morphological growth patterns of this organism. Giant cells reportedly formed as a result of selenomethionine being supplied in the medium. Division was temporarily arrested while cell enlargement continued.

Potassium is an enzyme activator. With deficiencies, green algae pass through an initial stage of high-carbohydrate levels. In later periods, protein levels andprotein turnover rates are affected (Pirson and Badour, 1961). Sodium may replace potassium in part (Emerson and Lewis, 1942; Allen, 1952). Walker's (1953) study of Chlorella indicates strontium may substitute for calcium when the latter is absent from the culture medium.

Because Chlorella has chlorophyll and carries out molecular phosphate transfers, manganese is required. Without manganese, cells become chlorotic, enlarged, and extensively vacuolated (Retovsky and Klasterska, 1961). Manganese is an active component of the oxygen evolving system, apparently associated with a protein. Photoactivation of the oxygen evolving system is an interesting phenomenum. Dark-grown cells have a lag period before evolving oxygen upon shift to illuminated conditions. This lag reflects the activation of oxygen centers and involves the light-driven transformation of inactive, soluble Mn^{+2} to a bound, active form. A photo-oxidation of Mn^{+2} evidently occurs since compounds reducing higher oxidation states of manganese also inhibit photoactivation.

Copper's metabolic role is associated with plastocyanin, which is used in the photosynthetic process. Excess copper may be detrimental with effects ranging from loss of cellular potassium to

inhibition of cell division (Kanazawa and Kanazawa, 1969). Specifically, Chlorella cells were not killed at a concentration of cupric ions which did stop growth. This copper toxicity was greater at pH 8 than at pH 5 (Nielsen and Kamp-Nielsen, 1970). McBrien and Hassall (1967) employed Chlorella vulgaris to show that copper absorbed under anaerobic conditions inhibited respiration, photosynthesis, and growth more severely than if absorbed aerobically. Evidently, more binding sites such as sulfhydryl groups are available for copper attachment under anaerobiosis.

Altman, Fetter, and Kaindl (1968) indicated that the zinc content of Chlorella cells parallels mRNA content. Molybdenum is essential for Chlorella growth (Walker, 1953; Loneragan and Arnon, 1954). The chloride ion is required in the photosynthetic Hill reaction (Arnon, 1955), ATP-formation and FMN-catalyzed phosphorylation (Vernon, 1962). Eyster (1962) reports a doubling of Chlorella growth following the addition of 3.4 (10^{-6}) M NaCl to a suitable growth medium. It should be noted that free chlorine is algistatic at 0.2 ppm, whereas free bromine is algicidal.

Chlorella species evidently do not have a detectable boron requirement (Bowen, Gauch, Krauss, and Galloway, 1965; Gerloff, 1968). McBride et al. (1971) claimed that boron concentrations from 0.001 to 10.0 mg/l did not affect growth of a Chlorella vulgaris strain. Warburg et al. (1955) report that vanadium stimulated carbon dioxide

uptake in photosynthesis at low light intensities, perhaps catalyzing the reduction of carbon dioxide. Eyster (1962) claimed that vanadium accelerated photosynthesis under high light intensity. As reported by O'Kelley (1974), Yamamoto, Fujita, and Ishibushi (1970) showed the vanadium content of algae to range from 0.3 to 10.6 mg/l.

Chu (1943) reports that algae did not grow at concentrations of less than 20 μg of phosphorus per liter. Growth reduction appeared at 50 μg/l. He suggests that 100 to 2000 μg/l is optimal for growth of green algae. Actual cell phosphorus content fluctuates depending on whether or not the medium has limiting phosphorus levels. The phosphorus content of Chlorella ranges from 1.0×10^{-7} to $1.5\ 10^{-6}$ μg P/cell. High-energy phosphates are generated by substrate phosphorylation, oxidative phosphorylation, and photophosphorylation.

Chlorella can also use inorganic polyphosphates (inorganic condensed phosphates), which may be composed of as many as 55 phosphate units, at the same rate potassium phosphate is utilized according to Galloway and Krauss (1963), as reported by O'Kelley (1974). Such polyphosphates were first discovered in algae by Sommer and Booth (1938). Apparently, these are phosphorus reserves which can be utilized to sustain growth during periods of phosphorus deficiency. O'Kelley (1974) cites Wintermans' (1955) report that Chlorella vulgaris synthesizes polyphosphates from external orthophosphates in the light. Also cited was Kuhl (1962) who demonstrated

the influence of light on polyphosphate synthesis in Chlorella.

Algae are separated from other microorganisms by requiring only three vitamins, namely: B_{12}, thiamine, and biotin. Chlorella vulgaris requires only the first two vitamins, with B_{12} being predominately required.

Composition of Swine Manure Compared with Algal Needs

There is substantial analysis of the swine waste used for the research under discussion herein. Table 2 compares the mineral composition of swine waste to that of several nutrient media employed in the mass culture of algae.

Adequate replenishment will prevent macronutrients (P, S, Mg, and K) from becoming limiting in algal ponds employing swine waste as a substrate. Micronutrients are also adequate for growth as Table 3 illustrates.

Barlow et al. (1975) also provide analyses of the same waste utilized in the experiments to be discussed. Table 4 is a summary of the analyses.

A replenishment method (Krauss and Thomas, 1954) determines nutrient requirements of mass cultures. Nutrients are supplied at a rate equal to their removal as harvested algae. Apparently, K, Ca, and to a lesser extent S, are not fully utilized. Any accumulation is

unlikely to inhibit Chlorella and other species with a high salt tolerance.

Table 2. Mineral composition of swine waste compared to that of several nutrient media used in the mass culture of algae (Barlow, Boersma, Miner, Phinney, and Oldfield, 1975).

Element	Swine Waste			Mass Culture Media[2]		
	Pit	Lagoon	Lit.[1]	A	B	C
	- - - - - - - - - mg/liter - - - - - - - - -					
Nitrogen ($NO_3 + NH_4$)	282	443	330	173	139	139
Phosphorus (PO_4)	90	100	84	285	62	171
Potassium	189	276	228	842	543	600
Magnesium	45	37	48	346	25	70
Sulfur (SO_4)	-	-	81	325	33	64
Calcium	96	109	342	30	9	1
Na	88	101	-	-	67	-
Cl	-		-	60	119	-
Fe	-	-	168	10	-	1
Zn	0.11	0.11	-	20	0.05	0.005
Mn	0.28	0.10	-	4	0.50	0.05
Cu	0.01	0.01	-	4	0.02	0.002
B	0.14	0.20	-	20	0.35	0.05
Co	-	-	-	1	0.02	0.001
Mo	-	-	-	4	0.06	0.001

[1] Calculated from E.J. Benne et al., 1961.

[2] A J. Meyer, 1971 (Chlorella media).
B G. Hemerick, 1973.
C R.W. Krauss and W.H. Thomas, 1954 (Scenedesmus obliquus media).

Oswald (1962) published a comprehensive article on what he termed "Controlled Photosynthesis," demonstrated schematically by Figure 2. He claims that over 90 percent of the biologically available dissolved organic matter originally present in the waste can be

Table 3. Mineral composition of swine waste compared to literature values of the mineral composition of Scenedesmus and Chlorella (Barlow, Boersma, Miner, Phinney, and Oldfield, 1975).

Element	Content Based on Dry Weight[1]					Relative Composition	
	A	B	C	D	E	Algae[2]	Waste[3]
	- - - - - - - - - % - - - - - - - - -					- - % of N - -	
N	8.14	7.90	6.10	8.00	6.80	100	100
P	2.22	1.72	1.23	1.10	1.42	21	26
K	0.92	1.60	0.74	1.50	1.41	16	67
Mg	1.60	0.57	0.89	0.50	0.54	11	12
S	--	1.12	0.91	1.10	0.34	9	23
Ca	1.93	--	0.76	--	0.06	12	53

[1] A Scenedesmus and Chlorella (Hintz et al., 1966).
B Chlorella (Gromov, 1968).
C Chlorella (Krauss, 1953).
D C. vulgaris (Groghegan, 1953).
E S. obliquus (Krauss and Thomas, 1954).

[2] Mean of A-E.

[3] Mean of Table 2 values.

Table 4. Nutrient content of the media used in the algal screening experiment (Barlow et al., 1975).

Parameter	Untreated Manhole Waste	$FeCl_3$ PPT Manhole Waste	Inorganic Media: Bishops
pH	7.4	5.6	6.5
Salt mmhos/cm	2.55	2.95	3.27
Inorganic N-ppm	220	194	112
Potassium-ppm	141	121	313
Calcium-ppm	36	42	4
Magnesium-ppm	69	66	24
Sodium-ppm	74	62	299
Boron-ppm	0.08	0.12	--
Copper-ppm	0.03	0.03	--
Manganese-ppm	0.19	1.10	0.11
Phosphate-ppm	40	< 1	124

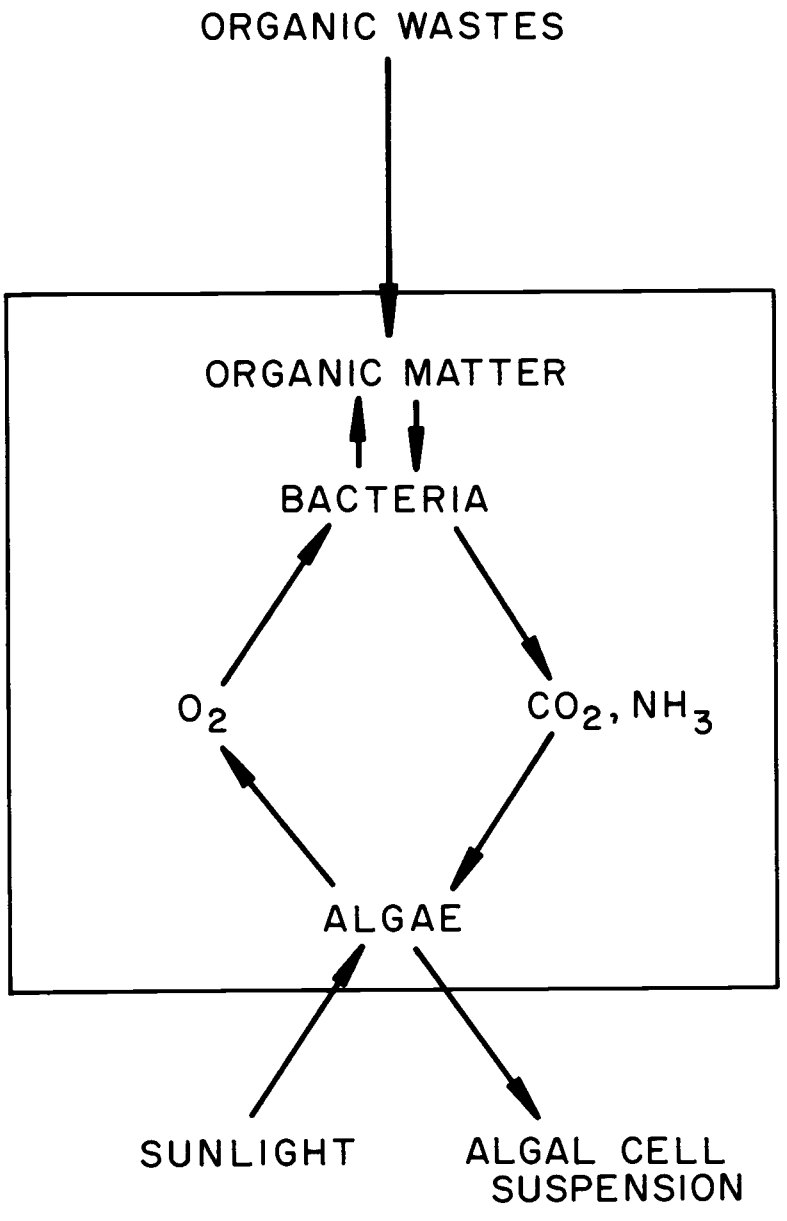

Figure 2. Controlled photosynthesis (Oswald, 1962).

rendered insoluble, with most being incorporated into algal cells. Barlow et al. (1975) cite organic matter for having a promotional effect on algal growth. Not only are the small organic molecules directly utilized by the heterotrophic algae, but the microbes present also attack organic matter, increasing available CO_2 in the process.

There is much concern over pretreatment of the swine waste, whether it be to concentrate the manure, to clarify the manure, or to alter the nutrient availability. Investigators agree that pretreatment is undesirable due to added system complexity, control, and cost. The organic matter levels decrease with many methods of pretreatment. Mechanical filtering with sand, diatomaceous earth, and/or porous cleaners have been reviewed. Filtering generally seems to be both inadequate and non-profitable in terms of time and labor.

Clarification with ferric chloride was examined by Barlow et al. (1975). They determined that such clarification depleted the wastes of needed phosphates and often decreased the pH to about 5.5, both of which inhibit algal growth. The reported advantage was a significant increase in light transmittance, which is otherwise limited by the high turbidity. In addition to the negative features of treated waste, there is the reported advantage of using untreated waste for algal culturing. As Table 5 indicates the highest dry weight densities were obtained using untreated waste.

Table 5. Mean growth of four algal species on swine waste and inorganic culture media in flat bottomed flasks at 30 C, after 3 days, 7 days, and 10 days of inoculation (Barlow et al., 1975).

Culture Media	Dilution	Inorg. N	pH	Algal Growth 3 Days	7 Days	10 Days + CO_2
	%	ppm		- - - -mg/liter- - - -		
Inorganic						
Bishop's	100	112	6.5	87	218	758
Gordon's	100	50	6.5	63	173	503
Untreated waste						
Manhole	100	220	7.6	738	610	1088
Manhole	50	110	7.5	468	463	858
Manhole	25	55	7.4	242	272	730
$FeCl_3$ treated waste						
Manhole	100	194	5.6	98	100	-
Lagoon	100	255	6.9	103	129	523

Waste concentration has also been reviewed by Barlow et al. (1975). Findings indicate that algal production and photosynthetic efficiency increase linearly with increasing concentration. The growth response to waste concentration was probably not due to differences in nitrogen concentrations since algae from low waste-concentration ponds had higher crude protein content. The miniponds effectively reduced the nitrogen content of the waste, while no nitrate accumulation occurred. Most of the nitrogen is lost, probably due to NH_3 volatilization enhanced at the high pH values. The algae absorbed from 20 to 30 percent of the nitrogen.

As Pratt (1942) states, "changes in the pH value immediately bring about a readjustment among numerous other component forces that influence the rate and amount of growth." In a batch culture the pH increase paralleling growth may lead to the precipitation of complexes of Mg, Ca, NH_3 and phosphates, hence having a water-softening effect. Researchers at Concord, California, report that as much as 50 percent of the permanent hardness may be removed from the culture medium (Oswald and Golueke, 1959).

It appears then, that untreated swine waste is a quality substrate for algae and is likely to support cell densities in the order of 0.5 to 3.0 grams per liter.

Retention Time

Harvest volume determines the retention time of the waste and influences the density of the culture. The hope is to obtain maximum yields with minimal costs. Since the cost per unit mass of algae harvested by centrifugation depends on the density of the suspension, there must be economic trade-offs between maximum yield and maximum economic yield according to Barlow et al. (1975).

Oswald (1962) asserts that having produced one crop of algae, effluent water from algal ponds should be sufficiently depleted of nutrients to inhibit secondary algal growth and therefore it should be suitable for discharge to natural waters. On the other hand, the

concentrations of essential nutrients must remain optimal to insure prolific growth. Oswald further recommends maintaining the algae in their most rapid growth stage throughout the culture. This implies utilizing the entire area of the growth unit and completely absorbing available light energy.

Tamiya (1957) also stresses the significance of maintaining an adequate population density in a steady, or quasi-steady state for attaining worthwhile algal yields. According to his report on the Tokyo project, the steady-state population density was more easily maintained during winter months. Tamiya does caution that excessive densities impose limitations on light penetration.

When the nutrient supply is adequate, attainable cell concentrations become a function of input rate, light energy, and suspension depth. Tamiya (1957) claims that carbon and nitrogen are the principal elements determining algal densities. A potential problem is natural precipitation which reportedly increases as the culture density increases.

Hemens and Stander (1969) report on light attenuation by effluent discharged from the South African Pretoria Sewage Works. They concluded that at depths greater than 10 centimeters the intensity in the culture would be less than 100 ft. c. Hemens and Stander adopt the value of 100 ft. c. as the minimum intensity for effective nutrient assimilation in a lagoon. Establishing proper densities includes such

considerations as irradiance limitations.

Graham, Phillips, and Myers (1951) discuss culture yield and chlorophyll content as a function of cell quantity. Sorokin (1971) claims that at the highest population density in a mass culture, growth is never exponential and seldom linear. Yield, he contends, becomes a function of time. Luebbers and Parikh (1966), working at the University of Missouri, studied the effects of cell concentration on oxygen production and respiration rate. They concluded that both are parallel functions of the concentration. Leubbers and Parikh reason the function to be not quite linear.

Myers (1963) contends that the production rates of algal ponds depend largely upon the size and geometry of the culture. The production rate is related to volume or surface area. Tamiya (1957) reports that at high population densities, any increase in the number of algal cells becomes proportional to the area of illuminated surface, hence reaffirming the necessity for a large illuminated surface to volume ratio.

One of the highest recorded population densities was 55 grams of dry weight per liter. This involved Chlorella cultures, 0.5 centimeters thick, growing in a glass annuli. This density is far above that expected from mass cultures where the depth approaches 10 centimeters.

A continuous flow system is one of the two methods ordinarily employed for the culture of algae, the other being the batch system. The first system involves removal and replacement of a selected percentage of each basin on a continuous basis. This method is best suited to the objective of obtaining high rates of protein production. The batch system is represented by a series of basins, with only the outflow from the last basin in the sequence being harvested. A selected percentage of the first basin is passed to the second one and is replaced with fresh influent waste. Complete nitrogen removal would be a typical objective of the batch system.

Myers and Clark (1944), Cook (1951), and Phillips and Myers (1954) report several methods for the automatic control of culture conditions. These authors agree that population density and culture medium composition are only roughly maintained through harvesting methods associated with replenishment of consumed nutrients. Tamiya (1957) discussed the replenishment method proposed by Krauss. Here, elements are added to the solution at the same rate at which they are removed by algae. Analyses of both nutrient media and algal cells determined these removal rates. Tamiya insists that if we are to accept the replenishment method, there must be adjustments made to the composition of the replenishing medium, taking into consideration any possible alterations in the elementary composition of the algal cells.

Harvesting methodology is another subject discussed in a variety of sources. Cell removal by micro-strainers has been considered. McGarry (1970) writes on aluminum sulfate and polyelectrolyte flocculation. Synthetic polymer flocculants have been discussed as well. Continuous centrifugation using a modified dairy cream separator and plastic bagging of algae have been reviewed.

Nitrogen analyses can indicate algal growth. Controlling nitrogen levels may prove to be advantageous in regulating biomass production. Krauss, according to Koch and Matthern (1965), reported that algae can use 100 percent of the available nitrogen from the medium, but never 100 percent of magnesium or phosphorus. Krauss further suggested that a minimum concentration of nutrients is required by algae to develop a concentration gradient for rapid transport of nutrients across a cell wall. The specified standard indicating safe drinking water is 10 milligrams of nitrogen per liter. This is not compatible with the objective of making use of waste to sustain high single-cell protein yields with algal cultures. Spoehr and Milner (1949) claim that nitrogen concentrations below 30 to 40 milligrams per liter limit growth and protein production by Chlorella.

Barlow et al. (1975) concluded that ammonium content of the effluent collected upon centrifugation was related to the retention time as depicted by Table 6. With continuous lighting at the rate of 400 $\mu E/m^2$ sec Chlorella produced maximum yields at the retention

Table 6. Effect of retention time on the NH_4^+N content of the effluent and the nitrogen balance of a 0.1 m^2 minipond containing Chlorella vulgaris 211/8K growing on untreated swine waste at 37 ± 1 C (Barlow et al., 1975).

Retention Time	NH_4^+ Content		Daily Nitrogen Balance of Minipond			
	Waste	Effluent	Waste In	Effl. Out	Algal N Out	NH_4^+N Lost
days	- - - mg/l - - -		- - - - - - - - - - grams N - - - - - - - - - -			(%)
6.7	250.0	18.5	0.375	0.028	0.175	0.218 (58)
5.0	250.0	30.2	0.500	0.060	0.166	0.307 (61)
4.0	250.0	43.8	0.625	0.110	0.161	0.363 (58)
3.3	250.0	49.8	0.750	0.149	0.152	0.440 (59)
2.9	250.0	59.2	0.875	0.207	0.133	0.502 (57)
2.5	250.0	71.3	1.000	0.285	0.129	0.540 (54)

time of two days. They asserted that culture density was little affected by retention time down to a retention time of 1.67 days, at which point the culture density decreased drastically. Crude protein of the harvested algae remained relatively constant despite harvest volume changes.

Under diurnal lighting, maximum densities were attained at a retention time of 2.5 days. Generally, production increased with decreased mean retention time. Tables 7 and 8 summarize research concerning retention time.

Table 7. Effect of retention time on the dry matter and protein production of Chlorella vulgaris 211/8K growing on untreated swine waste at 37 ± 1 C under continuous lighting (Barlow et al., 1975).

Retention Time	Crude Protein Content	Algal Production		
		Dry Matter	Protein[1]	Culture Density
days	%	- - g/m^2/12 hours light[2] - -		
10.0	42.5	10.93	4.65	1.092
6.7	43.1	16.28	7.01	1.085
5.0	39.3	25.52	10.02	1.021
3.3	40.7	22.91	9.33	0.764

[1] Adjusted to 7.5% ash.

[2] Lights ran continuously at 400 μE m^{-2} sec^{-1}.

Table 8. Effect of retention time on the dry matter and protein production of Chlorella vulgaris 211/8K growing on untreated swine waste at 37 ± 1 C under a diurnal regime (Barlow et al., 1975).

Retention Time	Crude Protein Content	Algal Production		
		Dry Matter	Protein[1]	Culture Density
days	%	- -g/m^2/12 hours light[2]- -		
6.7	45.2	17.91	8.09	1.006
5.0	45.6	18.20	8.29	0.984
4.0	45.5	20.93	9.51	0.952
3.3	46.7	21.53	10.05	0.874
2.9	46.1	22.44	10.35	0.796
2.5	46.4	25.10	10.92	0.771

[1] Adjusted to 7.5% ash.

[2] Intensity of 400 $\mu E\ m^{-2}\ sec^{-1}$.

Gotaas and Oswald (1955) arrived at a formulation relating various factors, one of which is retention time of pond waste, affecting algal yield. Gotaas claims that the optimum retention time varies primarily as a function of the season and local climatological conditions.

There is little question as to the importance of proper retention time for the waste. Energy content, competitive and/or parasitic population increases, and metabolic accumulation are all contributing parameters, as are environmental conditions, type of algae cultured and type of operational model employed.

Light Intensity

Govindjee and Braun (1974) remind us that of all electromagnetic radiation only the visible light is absorbed by chlorophylls and used in the photosynthetic process. Absorption spectra measurements and photosynthetic action spectra, based on oxygen production per incident quantum (as a function of the light's wavelength), were used to demonstrate the specific radiation involved in photosynthesis.

Myers (1963) makes two points following studies of absorption of monochromatic light by Chlorella. First, algae have typically high absorption qualities. Secondly, cell concentration and culture depth are functions requiring careful selection of incident light to assure optimal photosynthetic activity.

Table 9 lists the three major pigments absorbing incoming radiation. Chlorophylls absorb blue and red light; carotenoids absorb blue and green light. Green, yellow and orange light can be captured by phycobilins.

Two pigment systems, photosystems I and II, and two primary photoreactions, designated I and II, are mainly responsible for the photosynthetic event. The original concept of two photosystems are photoreactions being involved together stemmed from the Emerson enhancement effect concept. The photosynthetic rate is greater when short wavelengths and long wavelengths are used simultaneously than

Table 9. The photosynthetic pigments (Rabinowitch and Govindjee, 1969).

Chlorophyll	Characteristic Absorption Peaks: In Organic Solvents, nm	Characteristic Absorption Peaks: In Cells, nm	Occurrence
		A. The Chlorophylls	
Chl a	420, 662	435, 670-680 (several forms)	All algae
Chl b	455, 644	480, 650 (two forms?)	Green algae
Chl c	444, 626	Red band at 645	Diatoms and brown algae
Chl d	450, 690	Red band at 740	Reported in some red algae(?)

Types of Carotenoids	Characteristic Absorption Peaks, nm*	Occurrence
	B. The Carotenoids	
I. Carotenes		
α-carotene	In hexane, at 420, 440, 470	In red algae and in siphonaceous green algae it is the major carotene
β-carotene	In hexane, at 425, 450, 480 (the 480 nm band may be shifted to 500 nm *in vivo*)	Main carotene of all other algae
II. The Xanthophylls		
Lutein	In ethanol, at 425, 445, 475	Major carotenoid of green algae and red algae
Fucoxanthin	In hexane, at 425, 450, 475 (*In vivo*, absorption extends to 580 nm)	Major carotenoid of diatoms and brown algae

Types of Phycobilins	Absorption Peaks	Occurrence
	C. The Phycobilins	
Phycoerythrins	In water, and *in vivo*: 490, 546, and 576 nm	Main phycobilin in red algae; also found in some blue-green algae
Phycocyanins	At 618 nm, in water and *in vivo*	Main phycobilin of blue-green algae; also found in red algae
Allophycocyanin	At 654 nm, in phosphate buffer (at pH 6.5) and *in vivo*	Found in blue-green and red algae

*Locating carotenoid bands *in vivo* has been difficult (except in the case of purple bacteria) due to their strong overlapping with blue-violet bands of chlorophylls. The bands *in vivo* are estimated to be shifted by about 20-40 nm to the long wavelength side from their position in solution.

if one sums the individual effects of each wavelength alone (Emerson, 1958; Emerson and Rabinowitch, 1960; French, Myers, and McCleod, 1960; Govindjee and Rabinowitch, 1960; Blinks, 1963; Govindjee, 1963, Govindjee, Govindjee, and Hoch, 1964; Myers, 1971). The initial photoreaction, designated reaction II, results in reduction of a cytochrome and production of molecular oxygen resulting from the oxidation of water. Reaction I involves reoxidation of the cytochrome and NADP reduction.

Other photosynthesis models proposed in the last ten years are those by Franck and Rosenberg (1964), Arnold and Azzi (1968), Knaff and Arnon (1969) and Rurainski (1971), as reported by Stewart (1974). Ruranski's model indicates NADP reduction may occur independently of photosystem I.

Figure 3 shows which pigments exist in each photosystem. Photosystem I is located on the outer side of the thylakoid membrane and photosystem II on the inner side. This makes an excitation energy between them possible. It may be that the two systems lie side by side on the thylakoid membrane, however.

Lynch and Strehler (1955) note that incident light itself can induce changes in the absorption spectra of Chlorella. The previous light exposure, time of exposure to the new radiation, temperature, and time of exposure to darkness following irradiation are a few of the factors influencing spectral changes. Govindjee and Braun (1974)

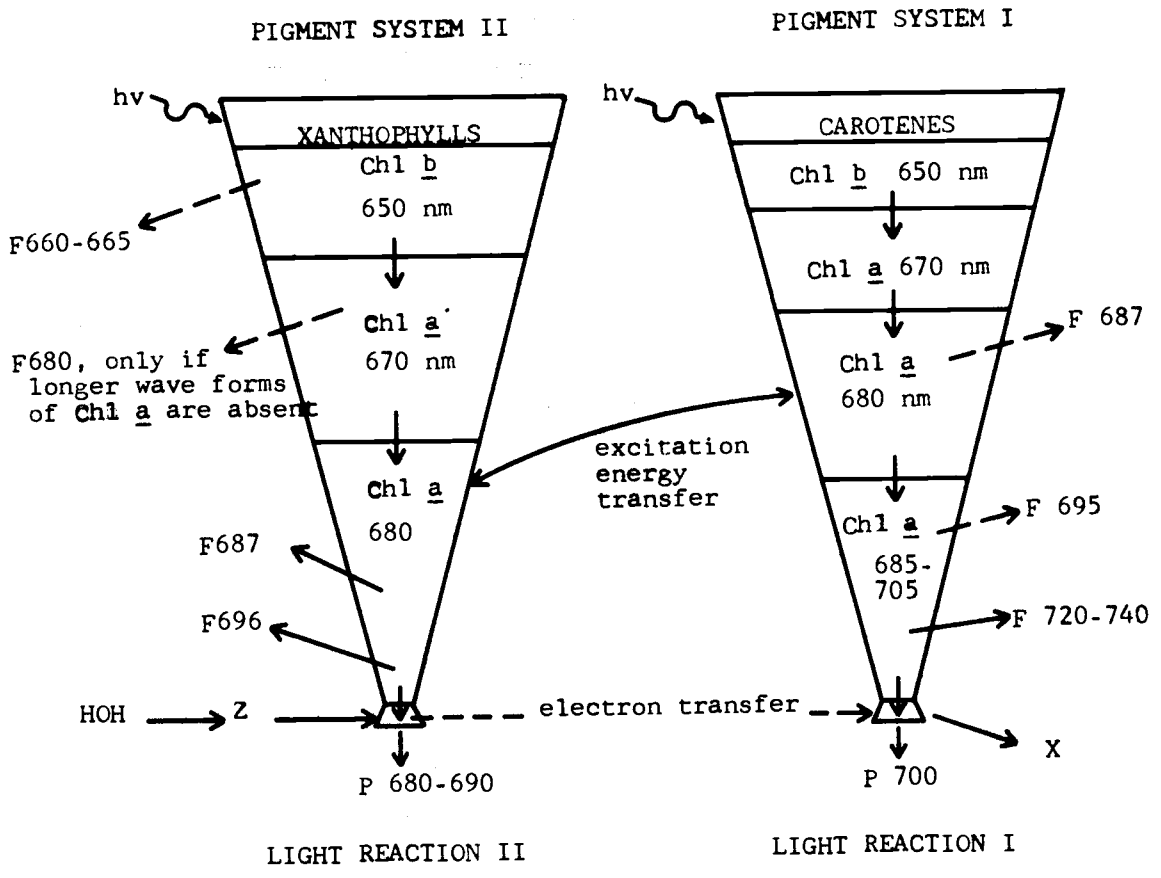

Figure 3. A working hypothesis for the approximate distribution of pigments in the two pigment systems in green algae. The chlorophyll (Chl) is followed by the maximally absorbed wavelength. F refers to fluorescence bands. Z is the primary electron donor of photosystem II. X is the primary electron acceptor of photosystem I. P stands for the pigment trap (Govindjee and Braun, 1974).

claim one can observe spectra differences of algal cells in weak light where photosynthetic rates are low. Differences are almost undetectable in cells cultured under light maximizing photosynthetic rates.

Chlorophyll is the basic pigment associated with light absorption and photochemistry, hence it will be reviewed in detail here. In vitro examinations have led to the conclusion that there are four major chlorophylls: Chl a, found in all algae; Chl b, common to green algae; Chl c, actually subdivided to c_1 and c_2; and Chl d. Table 10 shows the chlorophyll distribution among algae.

Table 10. Distribution of chlorophylls among the algae (Meeks, 1974).

Algal Group	Chlorophyll			
	Chl a	Chl b	Chl c (c_1 and/or c_2)	Chl d
Cyanophyceae	+	-	-	-
Rhodophyceae	+	-	-	+
Cryptophyceae	+	-	+	-
Dinophyceae	+	-	+	-
Rhaphidophyceae	+	-	+	-
Chrysophyceae	+	-	+	-
Haptophyceae	+	-	+	-
Bacillariophyceae	+	-	+	-
Xanthophyceae[1]	+	-	+	-
Phaeophyceae	+	-	+	-
Prasinophyceae	+	+	-	-
Euglenophyceae	+	+	-	-
Chlorophyceae[2]	+	+	-	-

[1] Includes Eustigmatophyceae.

[2] Includes Charophyceae.

+ Means the chlorophyll is present in at least some members.

- Means the chlorophyll has not been recorded in any member.

Chlorella has a typical Chl a to Chl b ratio of 2:1 to 3:1 as reported by Strain, Cope, and Svee (1971). Chlorophyll concentrations generally are determined spectrophotometrically using specific extinction (absorption) coefficients of the appropriate chlorophyll in a certain solvent.

Chlorophyll a, the only primary photosynthetic pigment in oxygen-evolving photosynthetic organisms, the other pigments being referred to as secondary, ranges from 0.3 to 2.0 percent of algal dry weight (Rabinowitch, 1945). This primary pigment has shown two main absorption bands in vitro. One band is in the Soret region corresponding to a 430 nanometer wavelength. The second band is in the red region corresponding to light of two wavelengths, 660 nanometers and 665 nanometers.

Chlorophyll b functions as a light harvesting pigment transferring absorbed light energy to chlorophyll a for primary photochemistry (Meeks, 1974). This secondary pigment has two absorption maxima, one correlating to the 645 nanometer wavelength, the other to the wavelength of 435 nanometers.

A variety of factors influence chlorophyll content in algae; among them are nutritional conditions, light intensity, temperature, and cell age. Deficiencies of essential chlorophyll constituents, namely Fe, N, and Mg, have altered synthesis and cell content of chlorophyll (Kirk and Tilney-Bassett, 1967; O'Kelley, 1968). Within

limits, chlorophyll content of most algae is inversely proportional to the light intensity (Graham, Phillips, and Myers, 1951; Kirk and Tilney-Bassett, 1967; Brown and Richardson, 1968; Sheridan, 1972).

In dense cultures where the average irradiance per cell is low, cells ordinarily produce an excess of chlorophyll. Myers (1955) states that under these conditions cells can absorb between 10 and 20 times more light than they can use efficiently when exposed momentarily to full sunlight. A low saturation intensity usually accompanies these conditions. Below a light intensity of 3 klux the chlorophyll content of Chlorella vulgaris does not increase further, but remains at its maximum level.

Investigating the physiological basis for light regulation of chlorophyll biosynthesis in Chlorella vulgaris, Beale and Appleman (1971) concluded that the degree of light limitation to chlorophyll synthesis is the primary factor controlling dry matter gain. Chlorophyll content increases when light is growth-limiting, otherwise it decreases. These authors hypothesize the regulation as being due to the accumulation of a photosynthetic product under non-limiting conditions which inhibits chlorophyll synthesis.

The amount of chlorophyll per unit of dry algal matter decreased with increasing light intensity. Prolonged exposure to high intensities may cause photodestruction of chlorophyll (Kok, 1956). A similar situation has been reported for a thermophilic algae during exposure

to subminimal temperatures (Castenholz, 1972) according to Meeks (1974).

At the molecular level, several laws describe the absorption of energy. The Grotthus-Draper Law of Photochemistry establishes that only absorbed light can yield chemical changes. That each absorbed photon excites but one molecule is written into the Stark-Einstein Law. Einstein further postulated that a single electron accepts all the incident energy of one quantum, thereby raising the electron to a higher energy state.

Principal electronic states and some transitions of chlorophyll are diagrammed in Figure 4. Chlorophyll is a singlet in its ground state, as are most pigments of biological importance. This signifies its spin multiplicity, which equals $2S+1$, where S equals the magnitude of the net spin for the entire atom or molecule. A spin multiplicity of one implies that the spin projections of all electrons taken along a magnetic field cancel one another. A brief summary of light absorption by chlorophyll follows.

Initially, there is photon absorption by a π electron of rhodospin, a carotenoid-opsin protein complex. A triplet or a singlet chlorophyll state occurs depending on the resulting spin orientation of the electron. Two main excited singlet states are recognized for chlorophyll, varying in energy and therefore in the type of light prompting their formation. The excited state of primary importance

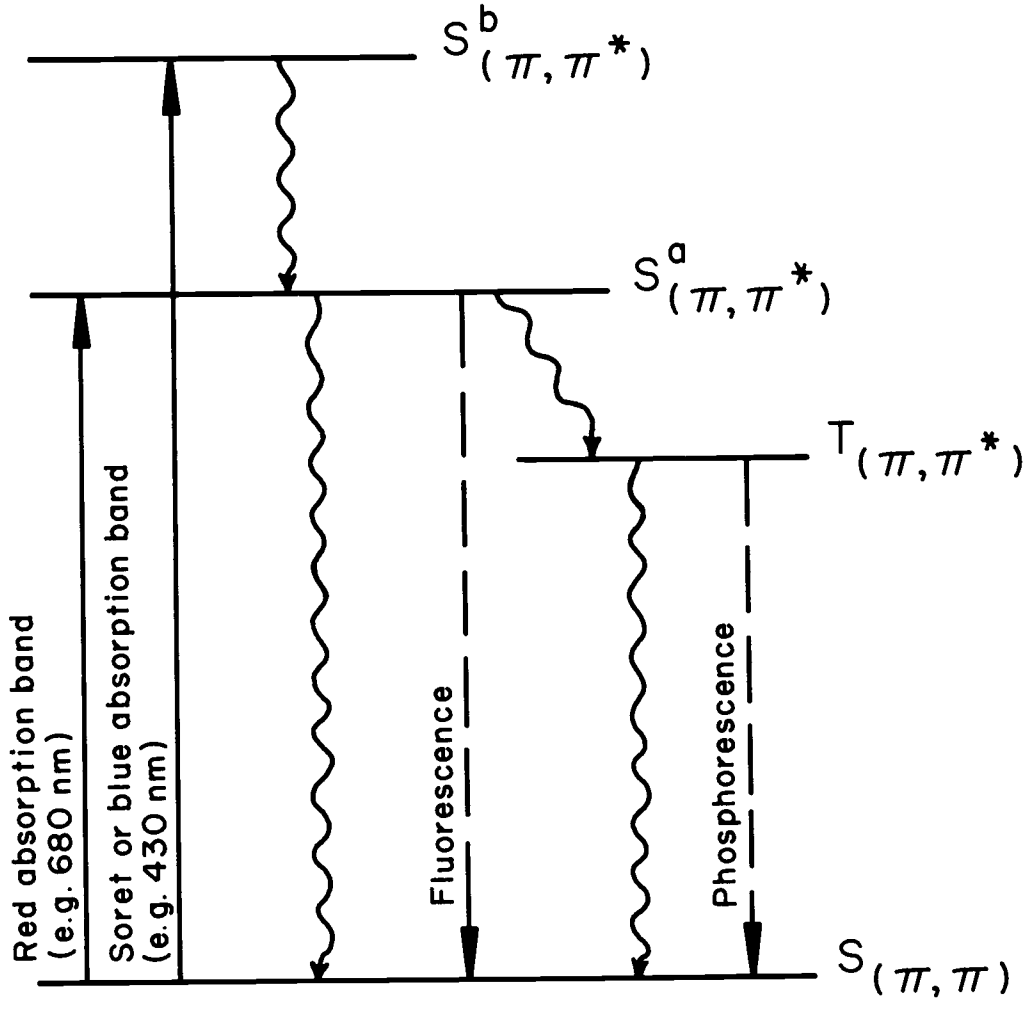

Figure 4. Diagram of energy levels indicating the principal electronic states and some of the transitions of chlorophyll. Straight vertical lines represent the absorption of light. Wavy lines indicate radiationless transitions. Broken lines indicate those de-excitations accompanied by radiation (Nobel, 1974).

in photosynthesis is the lower excited state which results when the incoming light has a wavelength approximating 680 nanometers.

Excitation to the triplet state is only about 10^{-5} times as probable as is prompting to a singlet state for two reasons. There needs to be a substantial increase in the electron's energy while simultaneously reversing the electron spin orientation. The necessity of coincidence for these two events results in the low frequency of occurrence. Note that n electrons may be excited like π electrons. However, the former event has a lower frequency of occurrence since π and π^* orbits may spatially overlap, n and π^* orbits generally do not.

De-excitation follows excitation events, with many possible results. Fluorescence and delayed fluorescence occur with light emission. Heat is generated with radiationless de-excitation. Phosphorescence may result in the case of an excited triplet state, with electromagnetic radiation being emitted. Excitation energy may also be transferred to another molecule. The excited electron may leave the originally excited molecule. Certain chlorophyll molecules involved in photochemical reactions which permit conversion of radiant energy into chemical or electrical energy will show this latter de-excitation pathway.

Tamiya et al. (1953) points out that with optimal carbon dioxide and nutrient salt levels, the rate of Chlorella growth becomes a

function of temperature and light intensity available to each cell. Tamiya formulated the probability of mutual cell shading of algal cells as a function of population density, cell size, and thickness of the culture along the direction of the light beam. Experimental results suggest that exponential growth is associated with negligible shading and linear growth with mutual shading to the extent that the culture totally absorbs incident light. Kok and VanOorschot (1954) claim that if the density is kept high enough to ensure complete light absorption, the growth rate will become independent of cellular concentration.

The optimal culture depth when using inorganic culture media is one where all cells are exposed to some light, but where very little light passes through the culture. The principle is to avoid an unlit dead area on the bottom of the culture where fixed carbon is lost to respiration. Barlow et al. (1975) reported that a light intensity of 1200 ft.c. was extinguished in a four centimeter deep suspension of manure to be used in the present study.

Hemens and Stander (1969) applied the Beer-Lambert Law to predict light-absorption characteristics of an algal culture. The equation, as presented by the authors, states:

$$I = I_0 e^{-Ecd} , \tag{1}$$

where I_0 is the incident intensity (number of quanta striking each

second on 1 cm^2 of the surface normal to the direction of the beam), I is the residual intensity at depth d (cm), c is the dry matter concentration (mg/l), and E is the extinction coefficient (cm^2/mg). Oswald and Gotaas (1955) found E to be between 1.0×10^{-3} and 2.0×10^{-3} cm^2/mg. Tamiya et al. (1953) showed E to equal 3.8×10^{-3} cm^2/mg for Chlorella ellipsoidea. Hemens and Stander (1969) predict 10 centimeters to be the maximal depth for exploiting photosynthetic activity.

Myers (1963) prepared a diagrammatic explanation of illumination decay during passage through a dense algal culture (Figure 5). It illustrates the vast difference between the high illuminance of full sunlight (about 10,000 ft. c.) compared to the much lower light intensity at which Chlorella normally become light-saturated (500 ft. c.). Myers states that the total area beneath the curve is proportional to the total amount of light absorbed by cultured cells. The lower shaded area is proportional to that fraction of light absorption which is used with maximum efficiency by these cells.

As light passes through an algal suspension it becomes spectrally limited to a narrow green band. This fact plus the knowledge that water absorbs red light more than blue, leads to the conclusion that photosynthetic efficiency decreases as the culture depth increases. Luebbers and Parikh (1966) report that 80 percent of the infrared light (wavelengths above 800 nm)

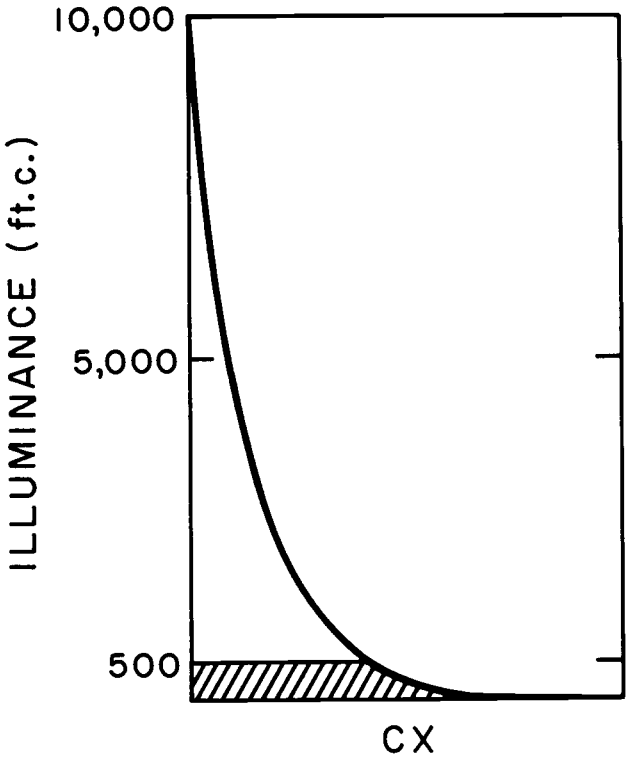

Figure 5. Illuminance in an algal suspension as a function of cx, the quantity of algae per unit area of illuminated surface (Myers, 1963).

is absorbed in about five centimeters of water, whereas the visible spectrum (wavelengths from 400 to 800 nm) has a greater penetrating capacity.

Emerson and Lewis (1943) studied quantum requirements of Chlorella (Figure 6) and concluded requirements for red and yellow light were the lowest and requirements for blue-green light were the highest. Tanada (1951) calculated relative quanta energies as a function of wavelength (Figure 7).

The efficiency of photosynthesis as a function of wavelength can be calculated using Figures 7 and 8. The heat of combustion of one mole of reduced carbon dioxide is 112 kcal, therefore the efficiency at a particular wavelength equals 112 divided by the product of the number of kcal per quantum and the quantum or quanta, requirements (Figure 8).

Myers (1963) published the following formula for the calculation of the efficiency:

(2)

$$\text{Efficiency} = \frac{(\text{grams algae produced})(\text{kcal heat of combustion/gram})}{(\text{kcal absorbed light energy})}$$

Viewed thermodynamically, low energy quanta are more efficiently used than the high energy quanta. Hence, a higher thermodynamic efficiency is obtained in red light than in blue light. Most authors agree that 20 percent is a reasonable value for the maximum

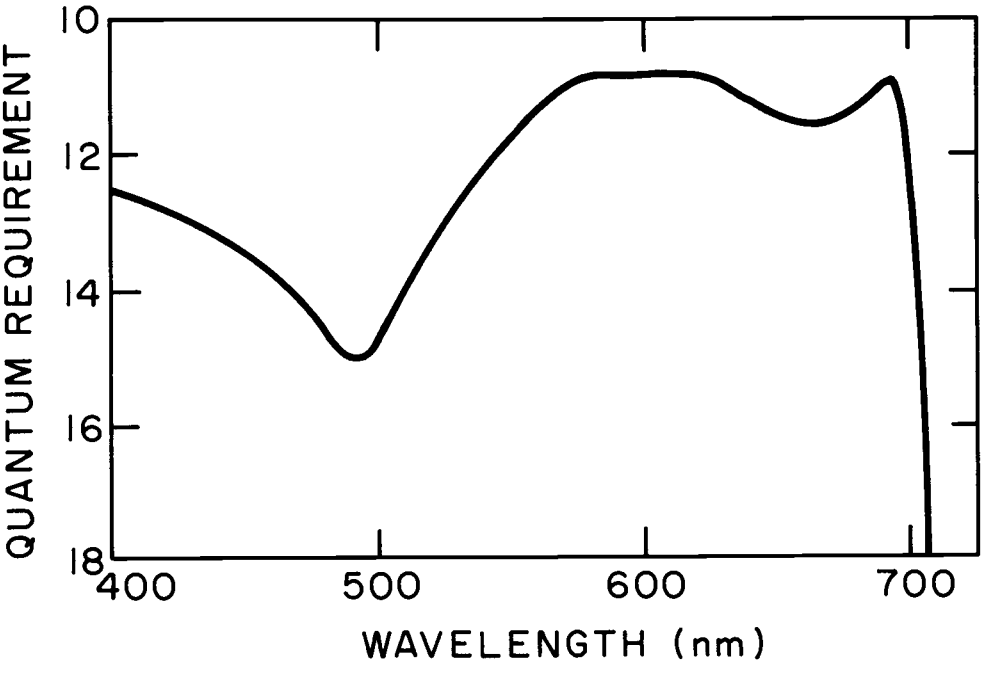

Figure 6. Quantum requirement of photosynthesis by Chlorella as a function of light wavelength (Emerson and Lewis, 1943).

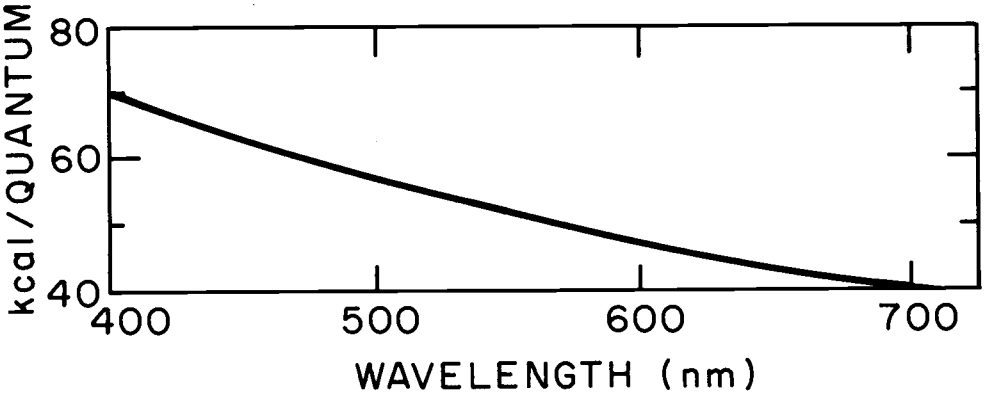

Figure 7. Energy per mole quantum of light as a function of wavelength (Tanada, 1951).

efficiency with which Chlorella use the energy of visible light. Myers and Graham (1958) reported a 19 ± 1 percent maximum efficiency value using a value of 5.5 kcal/g for the energy content of algae and 8 percent as the value for the maximum light utilization in outdoor basins. Forty percent of the total energy of sunlight is in the visible region so that a maximum overall efficiency of 8 percent (0.4 times 20 percent) can be expected. Kok and VanOorschot (1954) reported that 8 to 10 percent of the light energy absorbed was converted to cellular material when culturing algae at 30 C under an intensity of 0.6 cal/cm^2/sec. According to Graham, Phillips, and Myers (1951), Kok and VanOorschot reported a maximum efficiency of 20 ± 2 percent. Tamiya reported field-calibrated values of 3 to 7 percent. Differences are attributed to variations of light-saturation effects.

As defined by Myers (1963), maximum efficiency of light utilization implies that both growth rate and yield are limited only by irradiance. This condition exists only over the light-limited portion of the specific growth vs. illuminance curve, or that region where growth rate increases with light intensity (Figure 9). Sorokin and Krauss (1962) suggest that since algae have relatively high efficiencies of utilizing of solar energy at low light intensities, algal cultures may survive better in shaded or deep water. Kok and VanOorschot (1954) tried to alter saturation levels for Scenedesmus and Chlorella, but

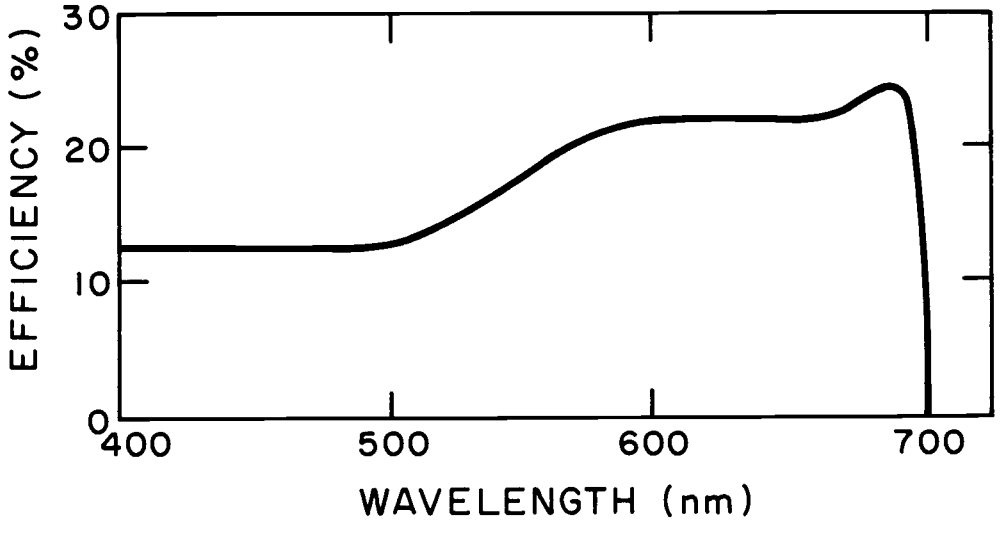

Figure 8. Efficiency of photosynthesis as a function of wavelength for Chlorella.

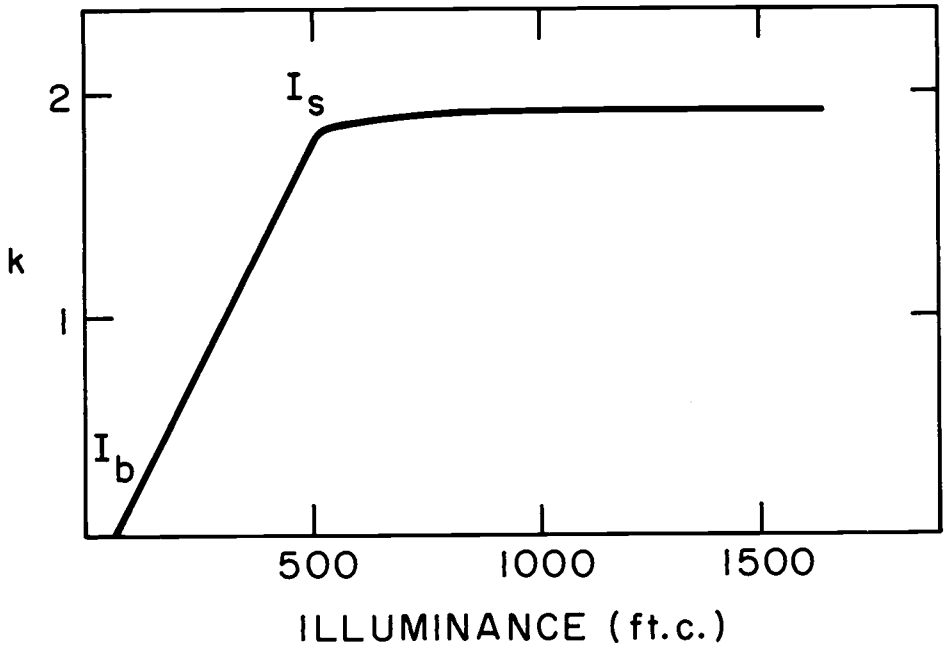

Figure 9. Specific growth rate as a function of illuminance. I_b is the illuminance to maintain basal metabolism of cells. I_s is the saturation intensity (Myers, 1963).

prolonged cultivation at specific light intensities did not result in significant changes.

Sorokin and Krauss (1962) defined three regions of the illuminance saturation curve shown in Figure 9. Region one would be the initial light-dependent portion where the growth rate increases with increasing intensity. Following saturation a plateau of light-independent results occurs. At high intensities the growth rate may actually decrease with increasing intensities, as do relative photosynthesis (Figure 10) and relative efficiency of photosynthesis (Figure 11).

Some researchers use the efficiency of light-energy conversion, calculated as follows:

$$\text{Efficiency} = \frac{\text{calories of harvested material}}{\text{calories of absorbed radiation}}$$

as a measure of growth activity. The caloric content of Chlorella ranges from 5.5 kcal/g of dry material to 5.8 kcal/g (Wassink, Kok, and vanOorschot, 1953). Tamiya (1957) points out that efficiency is more easily measured during the linear phase of growth where all the incident light is absorbed by the cell suspension. His results indicate that the efficiency of light-energy conversion increases with increasing temperature. Wesselius (1973) also describes energy conversion and claims that respiration is an

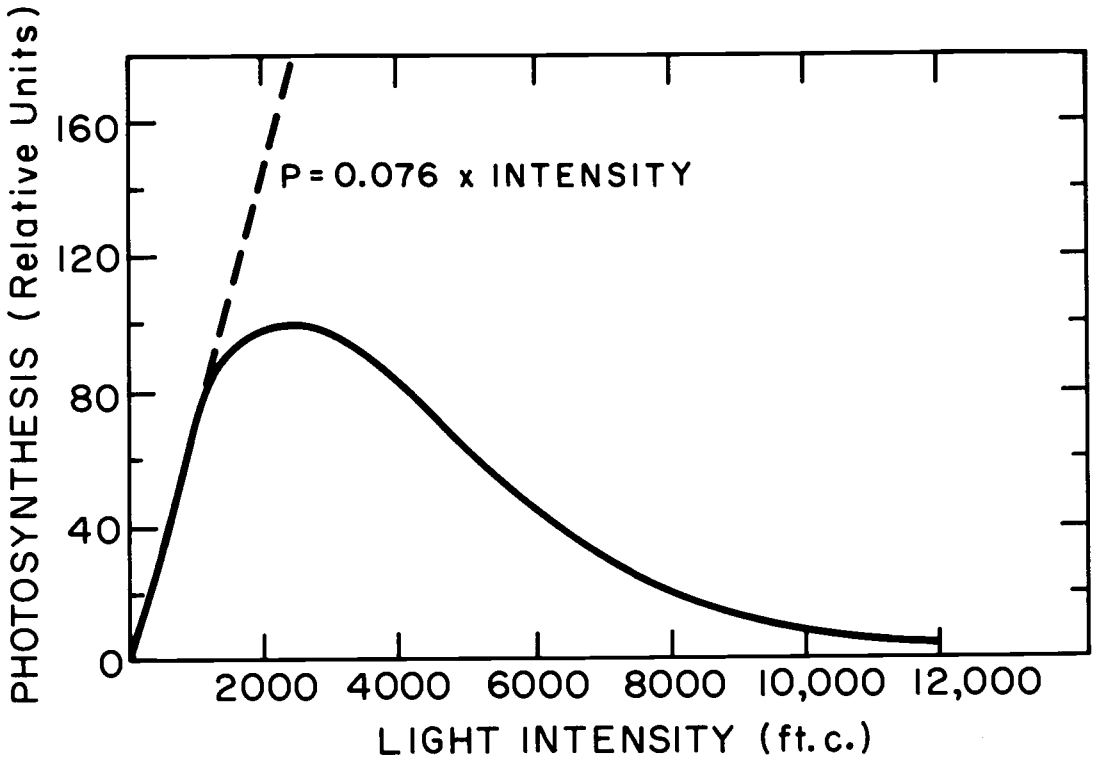

Figure 11. Efficiency of photosynthesis as a function of light intensity (from Figure 10).

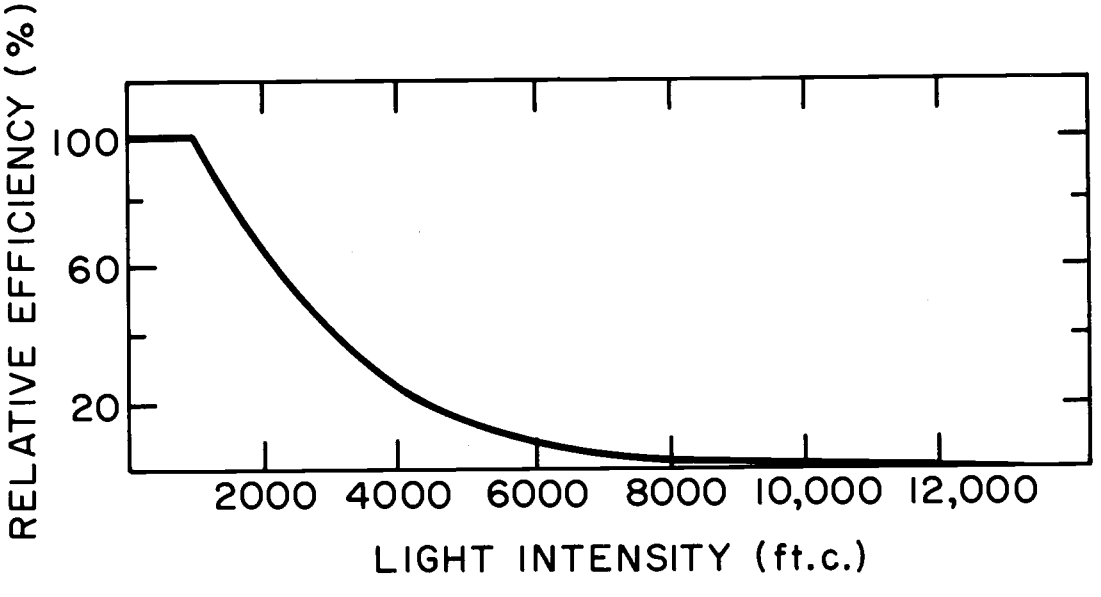

Figure 10. Photosynthesis of marine phytoplankton as a function of light intensity. Broken line is an extrapolation representing the hypothetical sustained maximum photosynthetic efficiency (Ryther, 1959).

important factor causing variability in rates of net photosynthesis under light-limited conditions.

Tamiya (1956) studied light-energy conversion efficiencies of a mesophile and thermophile. He concluded that using more than one strain throughout the year could provide for increased yields (Figure 12).

Luebbers and Parikh (1966) examined oxygen production by a mixed culture as a function of light intensity. Figures 13 and 14 illustrate their findings that net oxygen production increased to a maximum of about 13 mg/l/hr with increasing light intensity, then began to decrease slowly.

Soeder and Stengel (1974) state that relationships between light intensity and rates of photosynthesis or photoautotrophic growth can be represented by rectangular hyperbolic functions with an inhibition of growth occurring at supersaturating light intensities. Shapes of light vs. photosynthesis and light vs. growth curves as previously described are markedly affected by temperature (Sorokin and Krauss, 1962; Setlik, Berkova, and Kubin, 1969), salinity (McCombie, 1960), and nutrient level (Maddux and Jones, 1964). Setlik (1968), according to Soeder and Stengel (1974), demonstrated the relativity of the light vs. photosynthesis curves by demonstrating that the curve obtained by stepwise increases in light intensity was different from that obtained by gradually decreasing the light intensity from saturating levels. A

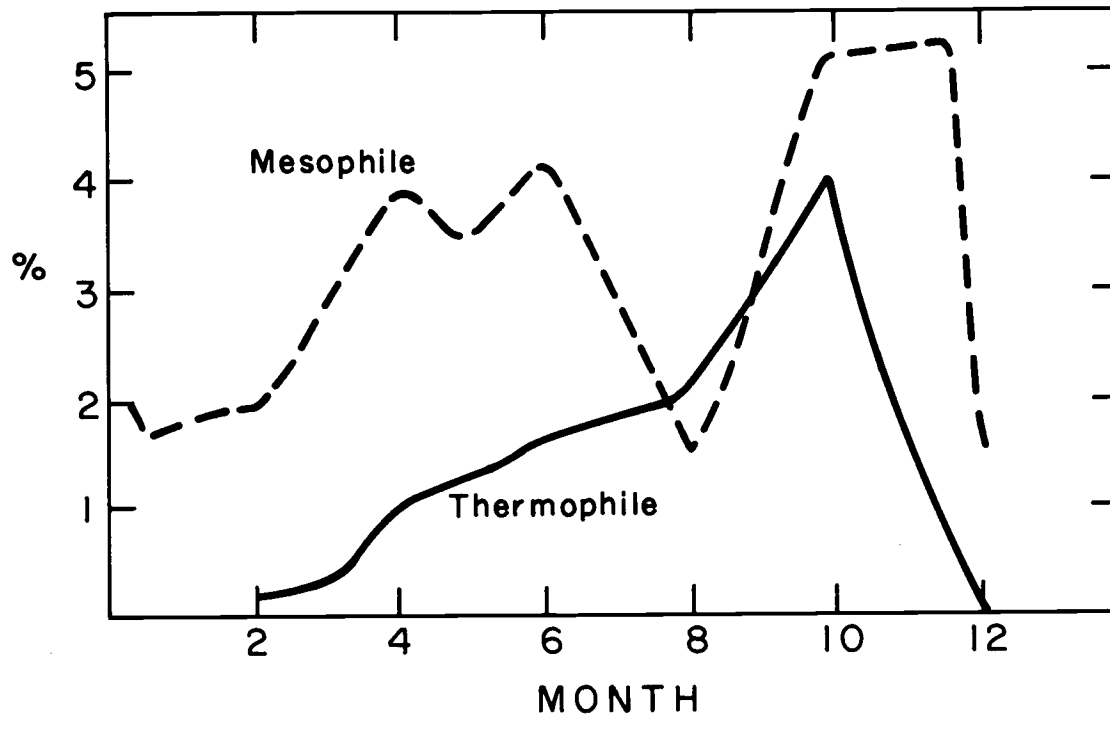

Figure 12. Monthly variation of the efficiency of solar-energy conversion (into cellular energy) effected by mesophilic and thermophilic Chlorella strains. Efficiency was calculated on a total radiation basis and the energy content of cells was considered to be 5.5 kcal/gram (Tamiya, 1956).

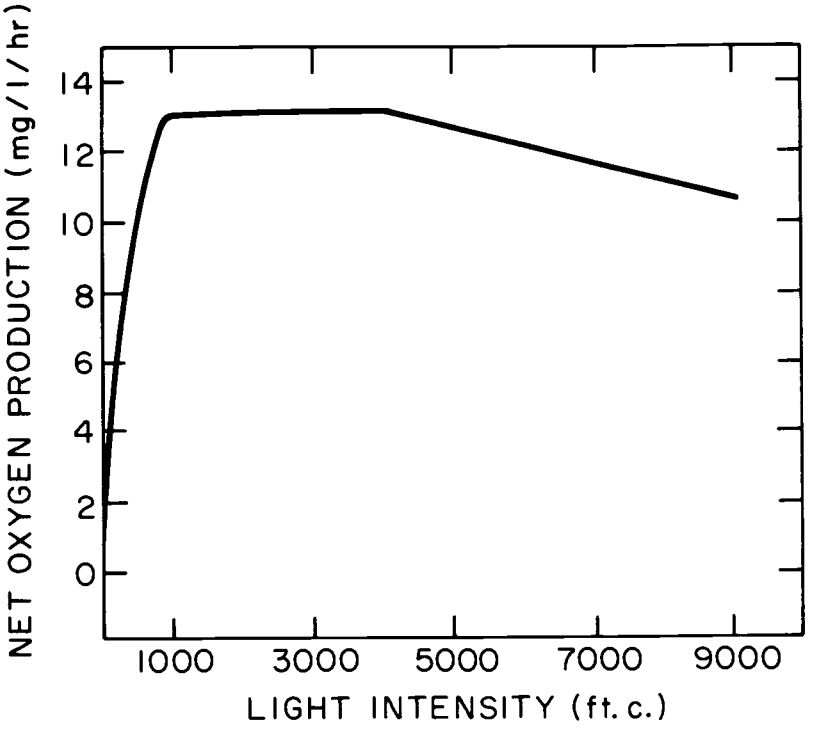

Figure 13. Effect of light intensity on oxygen production (Luebbers and Parikh, 1966).

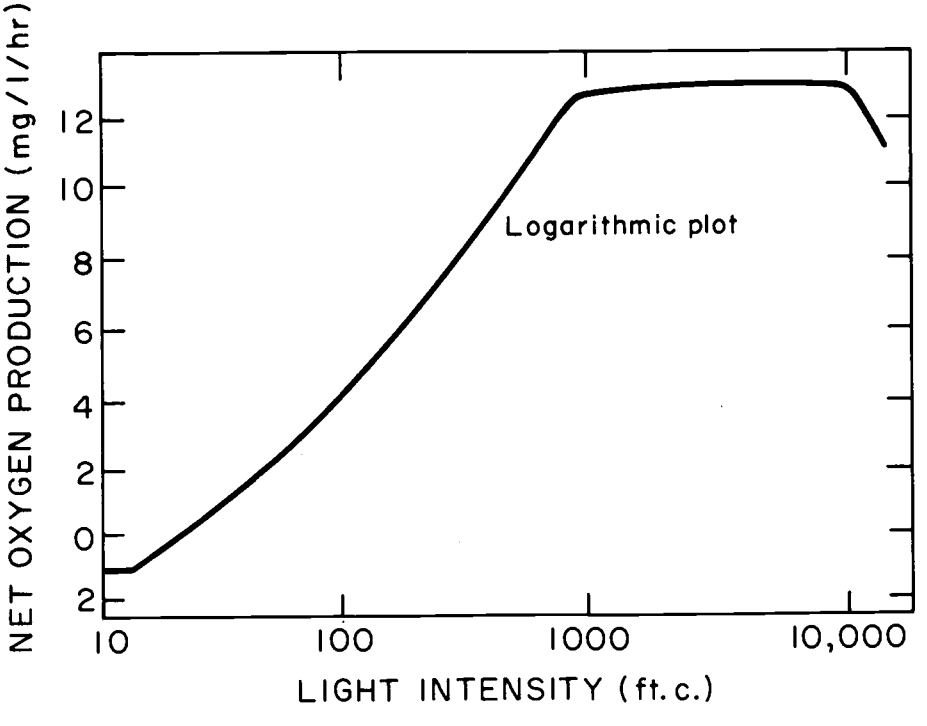

Figure 14. Effect of light intensity on oxygen production (Luebbers and Parikh, 1966).

mathematical model describing curves of light vs. photosynthesis rates and their integrals was assembled by Shelef, Oswald, and Golueke (1969).

Killam and Myers (1954) remind us that since Beyerinck's time, Chlorella have been grown in darkness with glucose provided as an organic substrate, although the rates of growth have been lower than those supported by photosynthesis. Chlorella can photosynthesize faster than it can grow, as Myers (1963) pointed out. Cells grown at a light intensity of 400 ft. c. or higher were more than twice as large as cells grown at 100 ft. c., but the chlorophyll content per cell was constant. He concluded that the maximum growth rate for Chlorella was not controlled by the rate of carbon or nitrogen assimilation. Myers added that the photosynthetic mechanism has been given an appearance of stability which may not be warranted.

Metabolic processes are also subject to light intensity affects. Ion transport (K^+, Na^+, and Cl^-) is light sensitive. Grant (1967) reported on the light dependency of the uptake of nitrite and nitrate. The activity of photosystem II in chloroplasts is linked to these two ions.

Tamiya (1957) proposes that generally the growth rate is directly proportional to the day-length at shorter day-lengths and calls this day-limited growth. This proportionality extends to longer day-lengths with lowering of the light intensity or raising of the day

temperature. With longer day-lengths and accompanying higher light intensities, growth becomes practically independent of day-length (day-saturation) or becomes smaller with increasing day-lengths (day-oversaturation).

Spoehr and Milner (1949) reported that a photo period of 12 hours produced 8 to 25 percent higher yields than continuous illumination. Many investigators (Ketchum and Redfield, 1938; Ketchum, Lillick, and Redfield, 1949; Tamiya, 1949; Tamiya et al., 1953; Myers and Phillips, 1955) have examined the potential for increasing culture yield with intermittent or flashing light. Myers and Phillips claimed that a dense culture growing under sunlight illumination will experience a significant increase in growth if turbulence is sufficient to move cells in and out of the front surface at such a rate to given flash times between 0.001 and 0.1 seconds. These findings, as well as those of Tamiya, were not predicted from the classic flashing-light theory. That theory, developed from experiments by Emerson and Arnold, predicts that for flashes of saturating intensity the yield per flash will increase with increasing dark times. This yield will approach a maximum yield which is independent of any characteristic of the flash itself.

Tamiya (1949) states that any growth-promoting effect of light-intermittency loses its significance as the temperature and light intensity drop to low levels. A study by Davis et al. (1953), showed

that linear growth rates of Chlorella could be increased as much as 70 percent by vigorous stirring.

Undoubtedly, temperature and light intensity interact to a great extent. High temperatures tend to favor higher light-saturation intensities. Cell bleaching results when excessive light intensities occur in combination with low temperatures. Sorokin (1971) warns that high-temperature tolerance does not automatically insure high performance by a particular Chlorella organism when cultured at optimal temperatures. Kok and VanOorschot (1954) reported that in outdoor experiments where cultures completely absorbed incident sunlight, temperatures of the cultures could climb as high as 20 C above the ambient reading, inducing a death phase in thermosensitive strains.

Sorokin (1971) claims that increases in light-saturation intensities at temperatures near 40 C are due to increased photosynthetic rates, or more specifically, to increased enzymatic carbon dioxide fixation. These enzymes draw and demand more energy from the light-absorbing pigment system, thus increasing light requirements. Low energy conversion efficiencies are due primarily to low light-saturation intensities. Tamiya (1957) related light intensity to temperature and light-energy conversion. He concluded that at high temperatures and high light intensities, higher linear growth rates occurred. However, the increased light intensity decreases

light-energy conversion while higher temperatures increase the conversion.

Most investigators are careful to point out limitations of artificial light. The spectrum of natural sunlight cannot be duplicated. Ketchum, Lillick, and Redfield (1949) tried using immersed fluorescent or neon tubes in bubbling cultures. More typical is the use of overhead incandescent bulbs and fluorescent tubes, making adjustments for heat changes and proper spectral emission.

Luebbers and Parikh (1966) determined the spectral distribution of several light sources (Figure 15). Sunlight is richer in blue radiation, where incandescent bulbs provide more red radiation. The amount of infra-red radiation from bulbs is several times higher than from sunlight. They claim that about 80 percent of the infra-red radiation is absorbed in approximately 5 centimeters of water. They further note that the intensity and spectral distribution of sunlight vary during the day. The atmosphere absorbs sunlight in varying degrees depending on the angle of the sun above the horizon and the composition of the atmosphere. The number of sunlight hours is a function of latitude and time of year. Figure 16 shows the wavelength distributions of the sun's photons incident at the upper limit of the atmosphere and at the surface of the earth. Planck's radiation distribution formula gives the idealized curve for the solar radiation spectrum reaching the upper limit of the atmosphere.

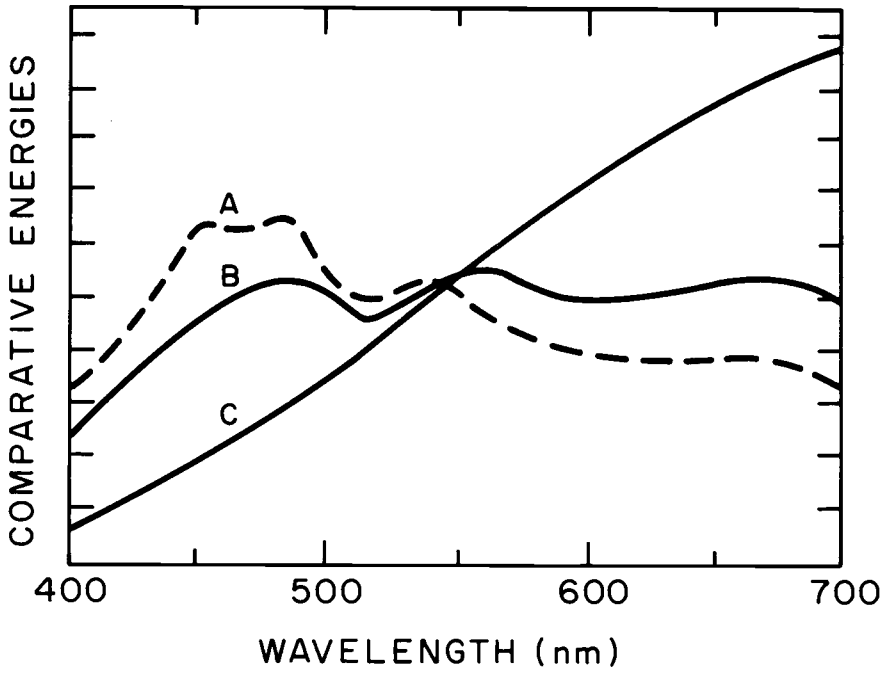

Figure 15. Spectral distribution of light. A: sun overhead; B: sun 30 degrees above horizon; C: light bulbs (IES Lighting Handbook, 1959).

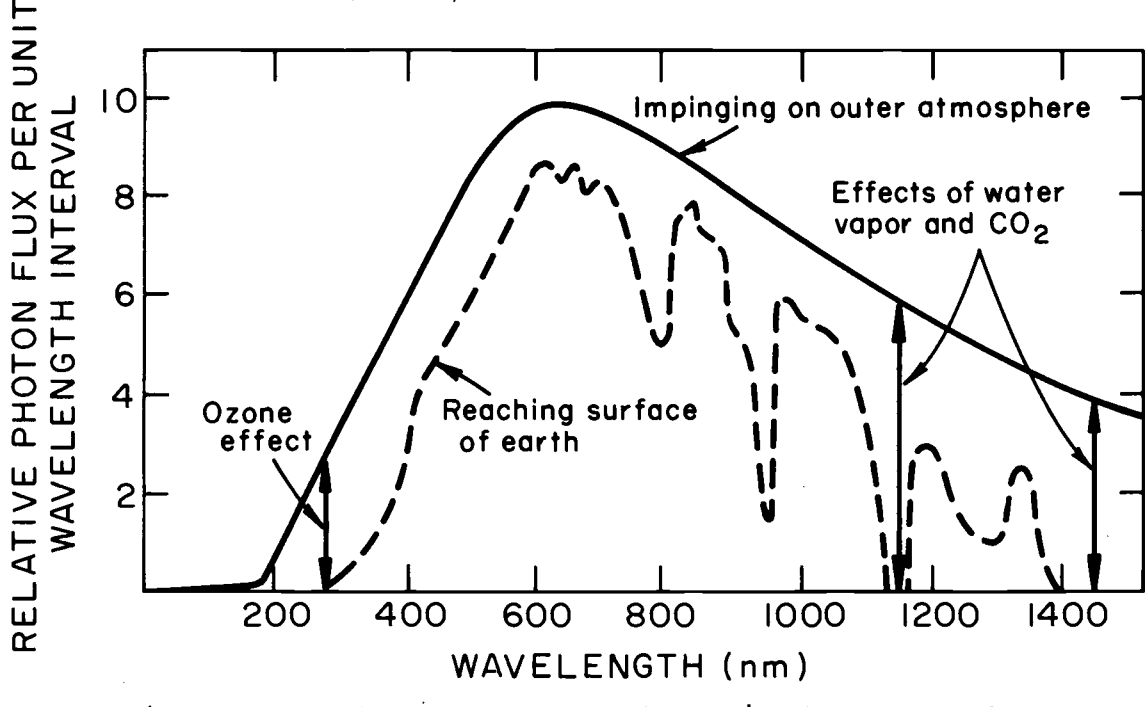

Figure 16. Wavelength distribution of the sun's photons incident on the earth's atmosphere and its surface. The pattern indicated by the lower curve is appropriate at sea level on a clear day with the sun nearly overhead (Nobel, 1974).

The photosynthetic rate of algae changes with age. Myers and Sorokin (1953) used Chlorella pyrenoidosa to show that during the initial three hours of growth, the photosynthetic rate increases much faster than dry weight, chlorophyll content, or respiration rate. They indicate that the slope of the light intensity vs. growth curve in the light-limited region changes with age.

Current knowledge about growth inhibition by light is being developed. According to Soeder and Stengel (1974), Gilet and Terrier (1969) showed that Chlorella possess ultraviolet damage-repair mechanisms and a capacity for photoreactivation. High light intensities may inhibit the respiration of actively photosynthesizing cells (Brown and Tregunna, 1967). Photo inhibition of respiration is evidently maximized in blue light. The extent of damage depends on the respiratory activity prior to illumination (Ried, 1969).

As mentioned previously, extreme light intensities can destroy chlorophyll. The process is enhanced by the presence of oxygen and evidently involves a stimulation of chlorophyllase activity (Ziegler and Schanderl, 1969). The sensitivity of Chlorella to high light intensities or to sudden increases in the light intensity varies with cell age (Pirson, Lorenzen, and Koepper, 1959; Sorokin, 1960). Older cells may have lost the ability to adjust to such changes.

Light intensity and wavelength affect the chemical composition of algae. Kowallik (1962) and Trukhin (1968) concluded that blue light

enhances protein synthesis whereas red light increases carbohydrate synthesis in Chlorella. Pickett (1971) decreased the protein content of Chlorella by culturing them in red flashing light. Blue flashing light kept the protein/carbohydrate ratio constant.

Kowallik (1962, 1963, 1965) performed detailed analyses of the properties of Chlorella cells grown in red and blue light. Cultures were adjusted to yield the identical amount of dry matter per unit volume. After transfer to monochromatic light, Kowallik found that cells grown in red light attained higher carbohydrate levels, lower protein levels, and lower RNA levels than cells grown in blue light. The cells grown in red light divided into a greater number of daughter cells (14) than did cells grown in blue light (10 to 12). The reduction in autospore number under blue-light was associated with partial inhibition of DNA synthesis. Kowallik added that these discrepancies between red- and blue-light cells disappeared after prolonged cultivation in monochromatic light.

Temperature

Thermophilic green algae have an optimum temperature for growth in the range of 38 to 39 C. They can be cultured at comparatively low temperatures, whereas cryophiles are more stenothermic. Sorokin (1971) claims that high and low temperature limits for algae depend particularly on the light intensity. Growth at higher

temperatures indicates an increased capacity to use higher levels of radiation energy.

Spoehr and Milner (1949) agree that light intensity is the primary parameter influencing the affects of temperature on the growth of Chlorella. Myers (1955) took a somewhat different view by suggesting that temperature optima for growth are governed, not by photosynthetic or respiratory proecesses per se, but by some more sluggish mechanisms such as the balance between rates of formation and denaturation of enzyme systems. Slobodskoi, et al. (1969) have formulated mathematical expressions for the interactions between temperature and light intensity for Chlorella, according to Soeder and Stengel (1974).

High-temperature algae are found in many environments ranging from hot springs where temperatures exceed 70 C, to polluted river waters (Sorokin, 1971). Pioneering work on the isolation of high-temperature strains for practical applications began in 1951 and was publicized in 1953 with the introduction of Chlorella 7/11/05, isolated from Waller Creek on the University of Texas campus. In 1967, Sorokin established Chlorella 1/9/30 as a worthy competitor. Both thermophiles are still being tested for their use in mass cultures.

Luebbers and Parikh (1966) claimed that photosynthesis may not be temperature dependent as suggested by Wassink (1956), but that the growth rate may be. If the growth-rate is temperature-dependent,

then algal concentration and rate of oxygen-production will also be temperature-dependent. Figure 17 illustrates their efforts to correlate temperature with oxygen production and respiration.

Soeder, Schulze, and Thiele (1967) established according to Soeder and Stengel (1974), that when temperature is growth-limiting, a more or less exponential increase with temperature occurs in the yield of Chlorella cells between 20 and 30 C. The rate of algal growth is affected more by the day temperature than by the night temperatures (Tamiya et al., 1955). The temperature dependence of the growth rate decreased and tends to disappear with decreasing light intensity and shortening of the day-length.

Since temperature affects yield at high light intensities, it is of interest to know that the light saturation intensities of thermophilic Chlorella may be increased by increasing the culture temperature. Table 11 shows that a shift from 25 to 39 C more than doubled the light saturation intensities of Chlorella pyrenoidosa 7/11/05.

At temperatures near 40 C, the rates of enzymatic fixation of carbon dioxide are increased to such an extent as to place increased demands on the pigment systems for chemical energy. The result is higher saturation intensities. This result corresponds with Tamiya's 1957 conclusion that the conversion efficiencies of light energy increase with increasing culture temperature.

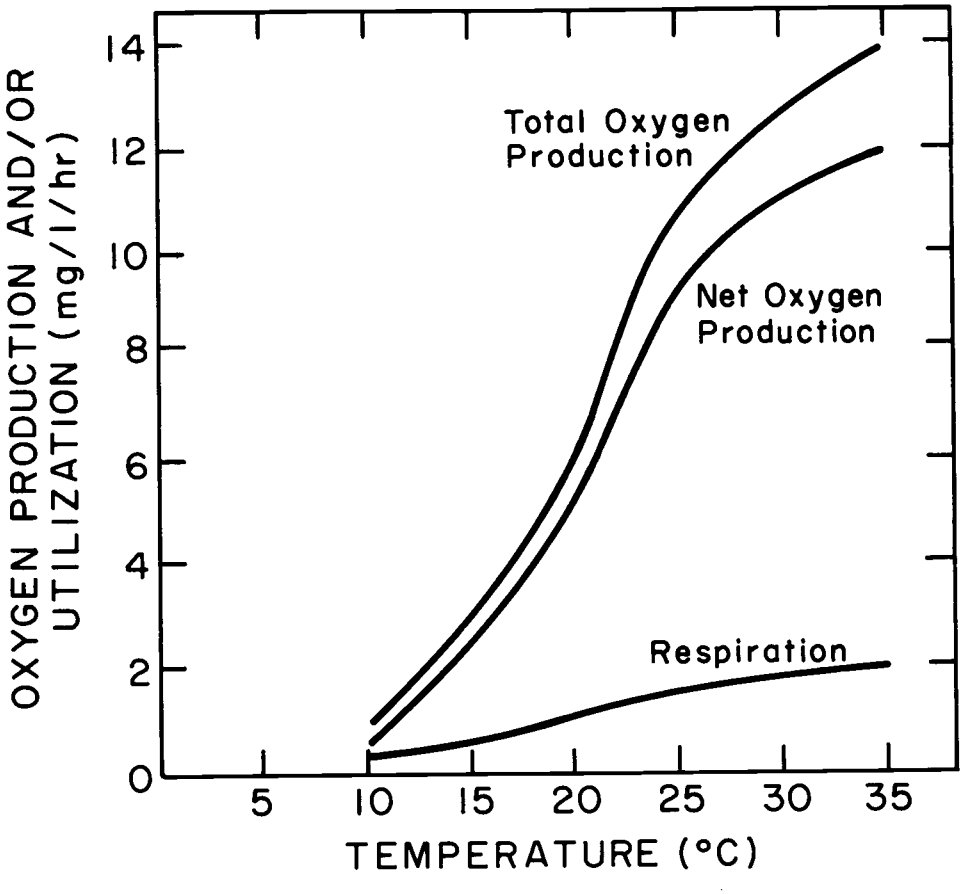

Figure 17. Effect of temperature on oxygen production and respiration (Luebbers and Parikh, 1966).

Table 11. Effect of light intensity and temperature on the growth of Chlorella pyrenoidosa 7/11/05 (Sorokin and Krauss, 1962).

	Growth Rate		Light Intensity	
Temperature	Half Saturation	Saturation	Saturation	Inhibition
°C	cell doublings per day		- - - - ft. c. - - - -	
25	2.3	3.0	500	3500
39	7.0	9.2	1400	8500

The effect of temperature on the growth and cell division of Chlorella cells has been studied by Sorokin and Krauss (1962), Lorenzen (1963) and Semenenko, Vladimirova, and Orleanskaya (1967). Sorokin (1971) discovered that tolerance to low temperatures markedly increased for synchronized cultures. He postulated that this resulted from the sensitivity of cell division to temperature and light intensity. Cell division is favored at high temperatures and accompanying low light intensities, or darkness. A combination of low temperature and high intensity actually delays or stops cell division. If the illumination is intermittent, cell division occurs in the dark while growth proceeds during the light periods. Processes involving DNA synthesis and subsequent cell division are more sensitive to sub-optimal temperatures than is the growth phase of the cellular life cycle. Semenenko, Vladimirova, and Orleanskaya (1967) transferred Chlorella with a temperature optimum of 33 C to 39 C in the light and found that photosynthesis and dry matter

production were stimulated for about 14 hours, although the capacities of the cells for division were irreversibly lost. The resulting giant cells had high protein contents.

Sorokin (1971) also investigated the effect of temperature on the metabolic activity by comparing a mesophilic Emerson strain with Chlorella 7/11/05, a thermophile (Figures 18 and 19). The rates of respiration and photosynthesis are higher for the thermophile even in the temperature range of 20 to 30 C.

Tamiya (1956) provides an interesting comparison between yields of a mesophilic culture of Chlorella ellipsoidea, and a thermophilic culture of Chlorella Ctm 37. Daily yields of both cultures varied over the year as functions of solar radiation and atmospheric temperature (Figure 20). Mixed cultures may prove necessary to maintain high productivity during the entire year.

Membrane potentials and therefore ion-transport phenomena are also sensitive to temperature. Fatty acid synthesis has been shown to be temperature dependent (Harris and James, 1969). Patterson's (1970) research indicated a rather complex temperature affect associated with fatty acid metabolism in a high-temperature Chlorella strain. The degree of unsaturation was maximized at 22 C, decreasing at either higher or lower temperatures which stimulated relative production of total fatty acids.

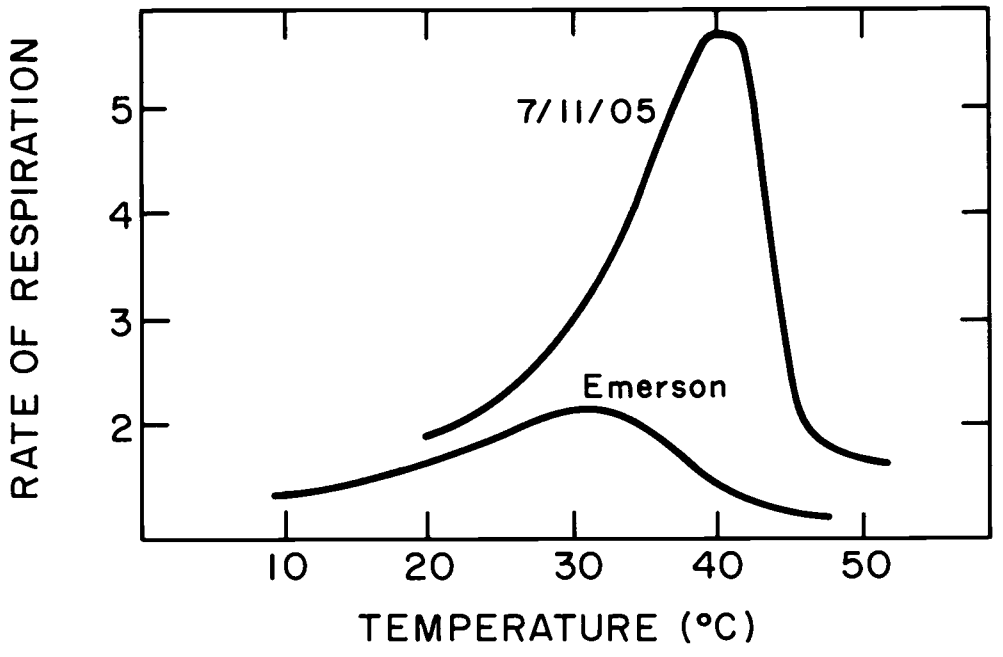

Figure 18. Rates of endogenous respiration for two strains of green algae measured at several temperatures. The respiration rate is presented as mm^3 oxygen/mm^3 packed cells/hour (Sorokin, 1971).

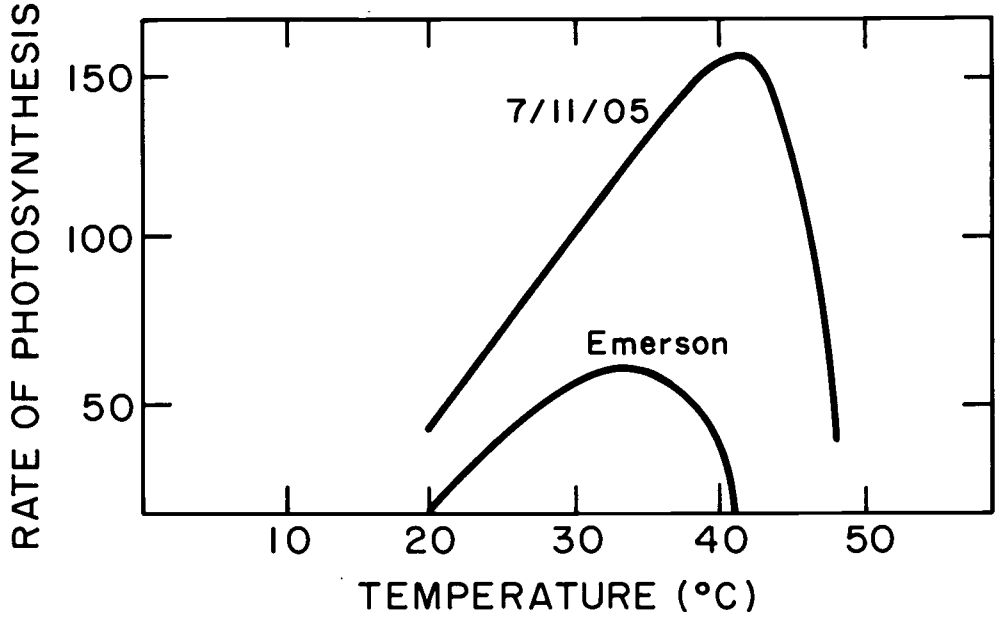

Figure 19. Rates of photosynthesis for two strains of green algae. The rate of photosynthesis is expressed as mm^3 oxygen/mm^3 packed cells/hour (Sorokin, 1971).

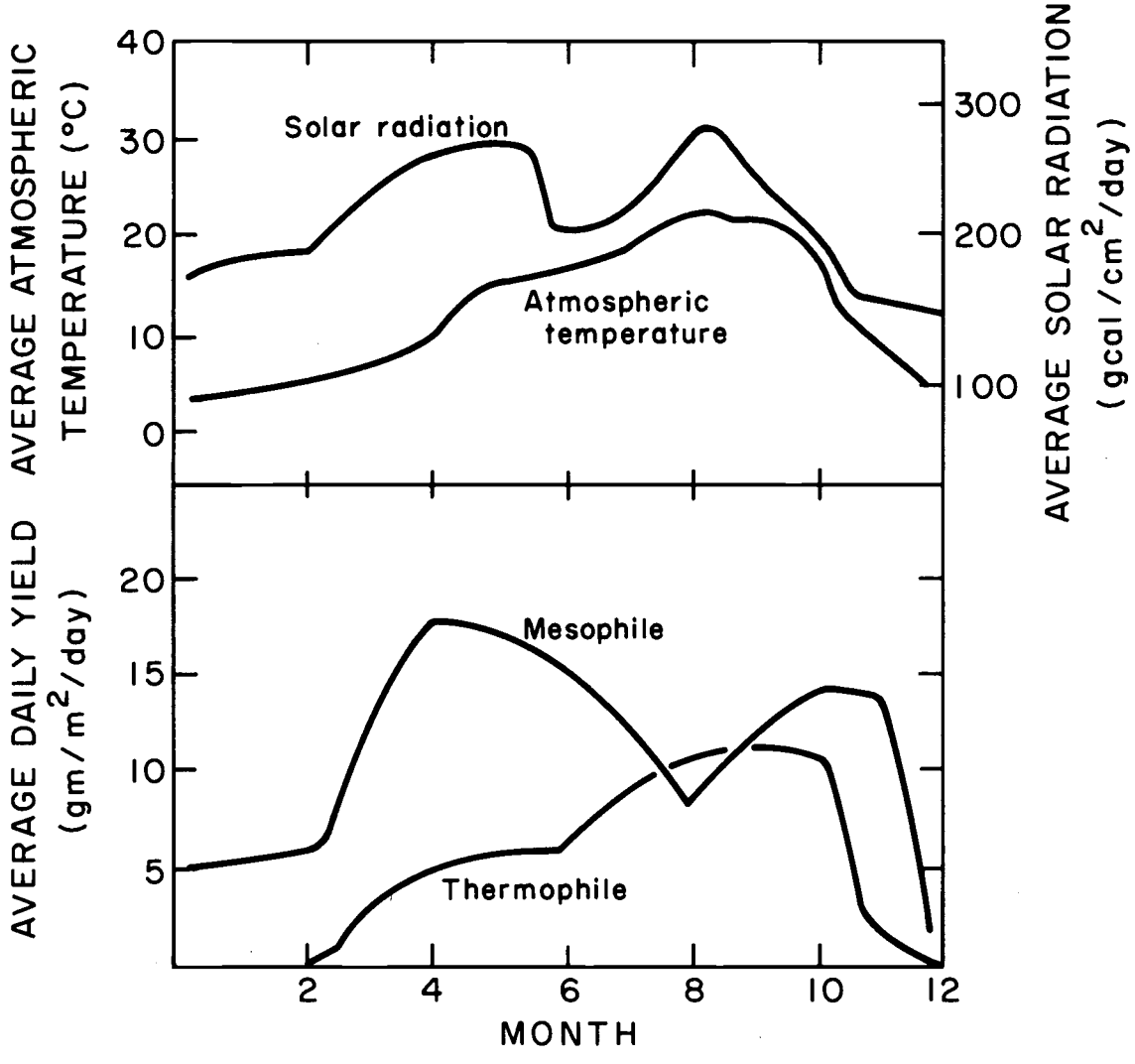

Figure 20. Monthly variation of average solar energy, atmospheric temperature, and the average daily yield obtained with mesophilic and thermophilic Chlorella strains (Tamiya, 1956).

Kok and VanOorschot (1954), reported drastic temperature changes in outdoor cultures in the Netherlands. By the end of the second week in August, the culture temperature had surpassed 50 C which led to decreased yields and cell bleaching. Temperature fluctuations may prove advantageous (Davis et al., 1953). Using Chlorella, it was noted that yields from cultures maintained continuously at 25 C were exceeded by using a day temperature of 25 C with a 10 C temperature decrease at night.

Organism Selection

Myers (1955) claims that the mass culture of algae is a problem of applied photosynthesis, where one seeks maximum utilization of sunlight toward the production of organic matter. The problem is also one of applied microbiology. Species used need to have advantageous responses to the environmental conditions, of which temperature, light intensity, and media composition are most influencial.

More specifically, the organism of selection should: (i) be nontoxic, (ii) have a biochemical composition suitable for use as a feed supplement, (iii) be capable of being harvested and processed with minimal expenditures of money and labor, (iv) be physiologically adapted for growth to the media used, (v) have a growth rate rapid enough to ensure economic feasibility of the culturing process, (vi) be substantially competitive to resist culture invasion, and

(vii) have a minimal fiber (undigestible polysaccharide) content.

Boughton and Krauss (1952) claim that Chlorella is a logical choice since it has a relatively high growth rate, as well as a capacity to alter its chemical composition to complement its environment. Other advantages of Chlorella are: (i) its tolerance to a rather wide range of salt concentrations, (ii) its high photosynthetic efficiency when compared to many crop plants, (iii) its high protein content, (iv) its potential for genetic manipulations, (v) the increasing numbers of isolated high-temperature strains, and (vi) its ability to use recycled nutrients.

Barlow et al. (1975) carried out a screening experiment using four algal species, namely: Chlorella vulgaris 211/8K (a high-temperature strain acquired through Dr. N.I. Bishop, OSU Botany and Plant Pathology Department), Selenastrum capriconia, and two species of Scenedesmus. They used Goran's and Bishop's media, as well as undiluted, diluted, and ferric chloride-clarified swine waste. Table 12 illustrated his findings and supports the conclusion that Chlorella vulgaris 211/8K is the most promising organism to grow on swine manure. Chlorella is of the order Chlorococcales and of the class, Chlorophyceae.

In attempting to evaluate total organic material produced by a Chlorella culture Spoehr and Milner (1949) introduced R-values. These describe the level of reduction which carbon of cellular

Table 12. Growth rates of four algal species at 30 C in 125 ml flat-bottomed flasks using three different culture mediums (Barlow et al., 1975).

Organism	Manhole Waste x 1			Manhole Waste x 4			Bishops Media		
	3 Days	7 Days	10 Days[2]	3 Days	7 Days	10 Days	3 Days	7 Days	10 Days
	mg DW per liter								
Chlorella vulgaris (211/8K)	956	945	1330	217	332	728	73	245	870
Scenedesmus obliq. D_3WT	520	700	1270	267	346	798	100	280	820
Scenedesmus quad.	-1	340	800	-1	232	747	-1	155	750
Selenastrum capriconia	-1	455	950	-1	177	648	-1	190	590

[1] Growth too low to measure at this time.

[2] Note that HCO_3^- was added to flasks at day 7.

material has attained. It also expresses the energy content as it is directly proportional to the heat of combustion per gram. The value for carbon dioxide is zero and methane, with carbon fully reduced, has a value of 100. Table 13 shows R-values ranging from 38 to 63 and the associated composition of Chlorella cells. Changes of the R-value during growth are continuous rather than stepwise, according to Spoehr and Milner (1949). These authors claim the R-value is a function of the environmental parameters present during growth, rather than being determined by selective growth of a different strain.

Table 13. R-values and corresponding composition of Chlorella (Spoehr and Milner, 1949).

R-Value	Calculated on Ash-Free Basis				
	% Ash	% C	% H	% N	% O
37.92	3.45	49.51	6.78	9.31	34.40
40.08	3.78	51.00	6.90	11.29	30.81
42.41	20.21	51.65	7.37	14.11	26.87
43.99	5.81	54.59	7.28	10.38	27.75
43.99	8.56	54.79	7.93	5.28	32.00
45.92	7.88	54.58	8.11	10.75	26.56
45.92	2.28	56.01	7.84	7.86	28.29
50.18	2.87	59.73	8.56	4.80	26.91
54.37	1.36	63.77	8.97	3.08	24.18
57.05	5.32	65.35	9.68	1.62	23.35
60.29	4.57	68.18	10.01	1.28	20.53
61.87	3.44	69.19	10.33	1.17	19.31
63.33	3.46	70.17	10.53	1.43	17.87

Using R-values and elemental analyses, Spoehr and Milner (1949) formulated methods to calculate lipid, protein, and carbohydrate

content of cultured algae. They used the following equations, keeping in mind that the R-values for protein, carbohydrate, and lipid are 42, 28, and 67.5 respectively.

$$R = (\%\ \text{protein})42 + (\%\ \text{CHO})28 + (\%\ \text{lipid})67.5$$

$$\%\ \text{protein} = (\%\ \text{nitrogen})6.25$$

Their calculations resulted in Table 14 which indicates that as the R-value changes, so do the proportions of these major components. The relationship between R-values and the percent lipid is nearly linear. As the R-value increases, the carbohydrate and protein percentages decrease almost linearly. Spoehr and Milner indicate that carbon and hydrogen have a linear relationship to the R-value. Nitrogen and oxygen decrease with increasing R-value, although not in a linear fashion.

Table 14. Constituents of Chlorella calculated from R-values (Spoehr and Milner, 1949).

R-Value	Protein	Carbohydrate	Lipid
	%	%	%
38	58.0	37.5	4.5
42	50.0	32.2	17.7
50	28.3	26.2	45.5
56	15.7	19.0	65.3
63	8.7	5.7	85.6

Myers (1963) reported that protein levels of Chlorella range from 40 to 60 percent unless the medium is nitrogen deficient. Table 15 shows that the essential amino acid content of Chlorella vulgaris 211/8K, grown on swine manure makes this species comparable to milk in nutritive value, except for the methionine deficiency.

Table 15. Essential amino acid content of Chlorella vulgaris 211/8K grown on untreated swine waste at 37 ± 1 C. The amino acid spectrum of Scenedesmus, Spirulina, milk and soybean are literature values (Barlow et al., 1975).

Amino Acids	Scenedesmus	Spriulina maxima	Chlorella vulgaris (211/8K)	Soybean	Milk
	- - - - - -grams per 100 grams protein- - - - - -				
Valine	7.2	6.2	6.2±0.1	5.2	7.0
Leucine	9.3	8.5	9.3±0.4	8.4	9.9
Isoleucine	4.4	6.0	4.8±0.2	5.3	6.4
Threonine	5.2	4.6	5.3±0.1	4.4	5.4
Methionine	1.4	1.4	1.8±0.2	1.7	2.5
Phenylalanine	4.6	5.0	6.6±0.4	5.8	4.8
Lysine	5.7	1.4	8.0±0.5	5.6	7.7
Tryptophan	1.4	4.6	N.A.	1.3	1.4

Myers (1963) notes that lipids normally constitute 15 to 20 percent of dried Chlorella. If nitrogen deficiencies exist, lipid levels may climb to 75 percent. About 20 percent of the dry weight of Chlorella vulgaris is starch, with about 5 percent carbohydrate in cell walls. Heitman and Hintz (1967) provide Table 16 which lists the composition of algae grown on sewage.

Table 16. Composition of sewage-grown algae (Hintz and Heitman, 1967).

Component	No. of Samples	Percentage of Dry Matter
Crude protein	25	50.93 ± 0.68
Crude fiber	25	6.20 ± 0.41
Ether extract	25	6.01 ± 0.41
Ash	25	6.24 ± 0.74
Calcium	10	1.93 ± 0.19
Phosphorus	10	2.22 ± 0.10

Elemental analyses are frequent in the literature. Their variations illustrate the fact that environmental parameters affect the elemental composition of algae. Myers (1963) claims that 1.8 grams of carbon are required per gram of algae produced. An overall analysis gave carbon, hydrogen, nitrogen and ash content in the following percentages: 50.44, 7.0, 9.58, and 8.48. This indicates an organic product with the formula $C_{6.14}H_{10.3}O_{2.24}N$. The ash content decreases as the content of organic matter increases, according to Spoehr and Milner (1949) who reported a range of ash contents from 1.5 to 20.0 percent.

The content of oxygen also varies widely. Spoehr and Milner (1949) reported a range of 18 to 31 percent for the oxygen content of Chlorella. Oswald (1962) claims that there is a relatively constant ratio of 1.6 pounds of dissolved oxygen per pound of oxygen used.

Chlorella vitamin content is shown in Table 17. Krauss (1962) claimed that less than 0.5 pound of algae would supply more than the

daily requirements of vitamins known to be essential for human nutrition. Kanazawa (1963) and Provasoli (1963) also provide information concerning algal vitamins, according to Provasoli and Carlucci (1974). More recently, Carlucci and Bowes (1972) showed that vitamin content of algae varies depending upon the vitamin level of the medium, with starved cultures containing fewer vitamins.

Table 17. Vitamin assay of dried Chlorella (adapted from Combs, 1952, by Krauss, 1962).

Vitamin	Lab Sample
	mg/lb
Carotene	218.0
Thiamin	4.5
Riboflavin	16.3
Niacin	109.0
Pyridoxine	10.4
Panthothenic acid	9.1
Choline	1370.0
	µg/lb
Biotin	67.0
Vitamin B_{12}	10.0

Micronutrient requirements have also been examined. Zinc and calcium are required by Chlorella. Manganese deficiencies affect nitrogen metabolism, in particular nitrate assimilation. Pirson and Wilhelmi (1950) showed that following that addition of manganese to deficient Chlorella cells, the photosynthetic rate increased three fold within two hours, according to Myers (1951). Myers reported that

experiments by Noack and Pirson (1939) and Alberts-Dietert (1941) indicated that nitrogen metabolism in Chlorella is sensitive to iron deficiencies. Mandels (1943) reported that sulfur deficiencies inhibit chlorophyll formation in Chlorella cells. According to Myers (1951), Pirson (1939) and Pirson and Wilhelmi (1950) showed potassium-deficient cells to have slower growth and lower photosynthetic rates, while maintaining higher respiratory rates than cells with adequate potassium. Copper has been shown to be toxic at low concentrations (10^{-7} M) for Chlorella due to inhibition of photosynthesis.

Myers (1963) reported that levels of chlorophylls a and b, normally present as 3 to 5 percent the total dry weight, are depressed by nitrogen deficiencies and by high average illuminance per cell. Spoehr and Milner (1949) claim that the chlorophyll and carotene content of Chlorella decreases as the R-value increases. In fact, the chlorophyll content may be reduced to about 0.012 percent. Although this minimal content is attained, the percentage may drop further due to further increases in cell mass. Hence, these authors speculate that at high R-values, any increase in cell yield may be independent of the chlorophyll concentration.

Lynch and Strehler (1955) report changes in the absorption spectrum of Chlorella which are induced by the light itself. Understanding the specific and complex relationship between light and algal production is far from complete, but progress is being made.

Hansen, Nielsen, and Jorgensen (1962) showed that Chlorella vulgaris adapts to new light intensities only when new cells are produced. With illumination higher than 3 klux, where cell size and chlorophyll content are reportedly maximized, cell size and chlorophyll content decrease while enzymes required for photosynthesis tend to increase in quantity. Decreasing the illumination from 3 klux results in decreased cell size as well as a reduction in the concentration of photosynthetic enzymes.

Tamiya (1957) describes the variation in cell type of Chlorella. He claims that there are two major cell forms, namely dark cells and light cells, each type capable of further division. Dark cells are smaller, richer in chlorophyll, maintain stronger photosynthetic activity and carry on a weaker form of respiration. Upon illumination these dark cells increase in mass while transforming into light cells. If aerobic conditions prevail, with or without light, these cells go through the autospore-production process to become the dark cells of the next generation.

Myers (1963) supports the common view that the Chlorella cell size and composition varies with environmental conditions. Sorokin (1971) claims that metabolic activity fluctuates during the growth cycle. Growth rates, photosynthesis, respiration, and generally, enzyme activity decline toward cell division. Furthermore, older and dividing cells cause the overall metabolic activity of an

asynchronous culture to be lower than that maintained by cells in synchrony where all cells are doing the same thing at the same time. Spoehr and Milner (1949) report that upon completion of cell division the cells' R-values increase and cell appearance changes. Not only will cells change to a lighter color, but their size may increase to be many times the size of the dark green cells associated with lower R-values. A similar effect may result upon depletion of the nitrogen supply. Spoehr and Milner (1949) and Kok and VanOorschot (1954) agree that cell count is an unreliable index of the production of organic matter.

Chlorella produces chlorellin, a water-soluble organic base which is converted to a salt in an acid medium. Chlorellin retards further growth once it reaches a threshold concentration (Pratt, 1942). The majority of Pratt's experiments were carried out at 18 to 22 C, 10,000 lux, 5 percent carbon dioxide and 95 percent air bubbled through the media which initially had a pH of 4.45.

Chlorellin can pass through colloidion membranes. Analyses suggest that the molecule is less than 15 Angstroms in diameter. As the culture ages the tendency for chlorellin to be excreted extracellularly decreases and the retarding molecules accumulate intracellularly. The concentration of the inhibitor within cells, as indicated by a reduction of the rate of photosynthesis, continued to increase despite the cessation of growth (Pratt, 1943). Fong and Pratt (1940)

reported growth being inhibited by a cell-free filtrate from a suspension in which Chlorella had been growing. This depression increased with the percent of filtrate added to the new media and with the physiological age of the filtrate.

Pratt (1944) claims that reduced growth rates in Chlorella cultures exposed to chlorellin are not a result of negative influences on the mechanisms of growth per se; but rather that they are "reflections of a general disturbance among the relative rates of different metabolic processes." More specifically, chlorellin decreases photosynthetic activity and respiration with effects increasing with culture age. The principle influence on the photosynthetic process is probably on the Blackman reaction. At low light intensities where the photochemical reaction rate limited the overall photosynthetic rate, there was little difference between the rates of oxygen production by cells from old and young cultures. However, at high light intensities where the rate of the dark, or Blackman, reaction limited the rate of photosynthesis, cells from older cultures showed less activity. Pratt reports this being consistent with Hille's (1938) observation that "it seems probable that the controlling factor of the photosynthesis of an 'aging' culture of Chlorella is in the Blackman reaction."

This inhibition has been reported for Chlorella vulgaris and Chlorella pyrenoidosa. Chlorellin also possesses antibiotic activity

in vitro against Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, E. coli, and Pseudomonas aeruginosa (Pratt et al., 1944). Pratt (1942) concluded that the inhibitor was more effective at pH values from 6 to 7, than at pH values less than 5.

The inhibition may be expected regardless of whether the available nitrogen is in the nitrate ion or ammonium ion form. Pratt (1942) adds that there is a possibility that chlorellin may actually stimulate the growth of Chlorella at very low concentrations.

Pratt (1948) examined the relationship of the surface tension of the medium to inhibition by chlorellin. Measuring the surface tension at 22 C by a deNouy precision tensiometer and by the capillary rise method, he found that surface tension increased with culture age. This finding was unexpected for two reasons. Cultures 10 days or more or age had shown foam formation when normal or carbon dioxide-enriched air was bubbled through them, with the amount of foam increasing with culture age. Pratt had correlated this with decreasing surface tension. The antibiotic action of chlorellin, which was more prominent in older cultures, was associated with unsaturated fatty acids being released into the medium. This would be expected to decrease the surface tension. Pratt rationalized this contradictory finding of surface tension increase by postulating that the foam may trap fatty acids and/or other surface active lipoidal

compounds. Removing them from the medium allowed the surface tension to increase.

Despite apparent universal use of Chlorella for mass-cultures other organisms may supplement or replace it for one reason or another. Blue-green algae with nitrogen-fixing capabilities, such as Anabaena and Tolypothrix, and filamentous algae like Ulothrix and Hormidium certainly are worth investigating. Sorokin warned that high light-saturation intensities achieved at higher temperature may be misleading since average irradiance in dense cultures may result in light intensities below the higher saturation intensities everyone strived to achieve. Other factors such as tendency to froth or precipitate must be examined.

Carbon Dioxide Fixation

Chlorella uses light energy by converting it to chemical energy in ATP and $NADPH_2$, most of which finds use in reducing carbon dioxide. Articles dealing specifically with algal carbon dioxide fixation are those by Bassham (1964), Levedahl (1968), Raven (1970), and Gibbs et al. (1970).

Chlorella readily absorbs carbon dioxide which diffuses across the cell membrane. The bicarbonate ion can also be used. It is accumulated by active transport. The bicarbonate ion is reportedly converted to carbon dioxide prior to use in photosynthesis. Raven

(1974) states that it is possible that the rate at which inorganic carbon enters the algal cell can limit the rate of photosynthesis under natural conditions. Schindler (1971) supports this claim. Inorganic carbon is made available to algae through decarboxylation reactions, as well. These occur within cells in the light and in the dark.

Enzyme studies and labeled carbon dioxide techniques indicate that the photosynthetic carbon reduction cycle, or Calvin cycle, is indeed the mechanism of autotrophic carbon dioxide fixation. Figure 21 illustrates this cycle. With Chlorella the light intensity determines at what rate components of the cycle are produced (Ogasawara and Miyachi, 1970).

The rate of photosynthetic-carbon dioxide fixation by algae depends on several external and internal factors. Internal factors examined are algal species (McAllister, Shah, and Strickland, 1964; Brown and Tregunna, 1967), the stage of growth of rapidly dividing cells (Tamiya, 1966; Pirson and Lorenzen, 1966), and the circadian rhythms of slowly dividing cells (Hellebust, Terborgh, and McLeod, 1967; Cumming and Wagner, 1968). External factors are inorganic carbon supply and pH (Brown and Tregunna, 1967; Raven, 1970), intensity and wavelength of available light (Paasche, 1966; Raven, 1969), the oxygen level (Turner and Brittain, 1962), and organic and inorganic nutrient supply (Russell and Gibbs, 1966; Kanazawa, Kirk, and Bassham, 1970; Kanazawa et al., 1970). These factors influence

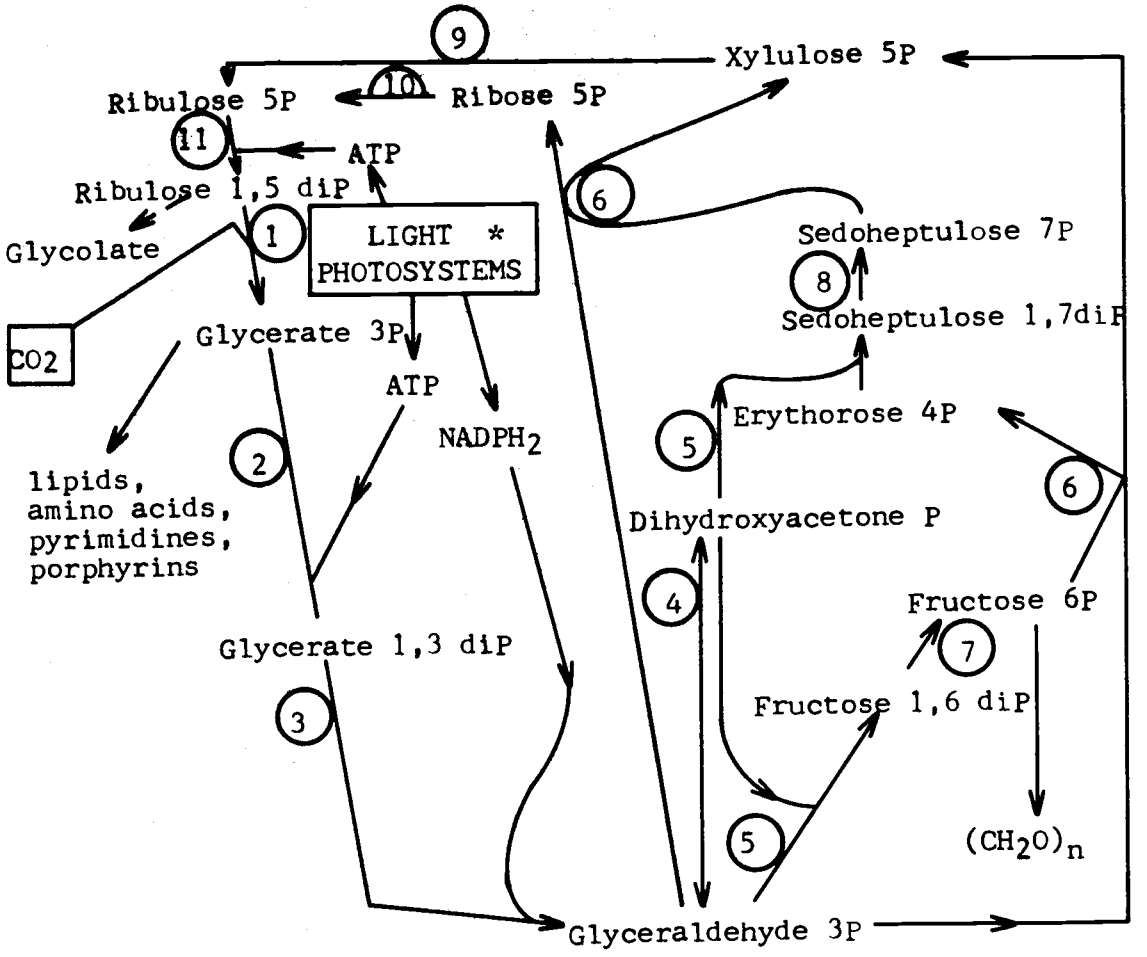

Figure 21. The photosynthetic carbon reduction cycle. The enzymes of the cycle, referred to by numbers, are: (1) ribulose diphosphate carboxylase, (2) 3-phosphoglycerate kinase, (3) 3-phosphoglyceraldehyde dehydrogenase, (4) phosphotriose isomerase, (5) fructose diphosphate aldolase, (6) transketolase, (7) fructose-1,6-diphosphate-1-phosphatase, (8) sedoheptulose-1,7-diphosphate-1-phosphatase, (9) phosphopentose epimerase, (10) phosphoribose isomerase, (11) phosphoribulokinase. * = catalytic role of carbon dioxide (Nobel, 1974).

fixation by controlling enzyme activity (Preiss and Kosuge, 1970), enzyme quantity (Russell and Gibbs, 1966; Smille, 1968), and substrate (carbon dioxide, ATP, $NADPH_2$) supply (Raven, 1970).

Light intensity and spectral distribution affect carbon dioxide assimilation in many ways. Latzko and Gibbs (1969) grew cells heterotrophically and in the dark to demonstrate that some enzymes of the carbon reduction cycle and of photoproduction of ATP and $NADPH_2$ require light for their production. Light activation of enzymes is associated with irreversible steps (Preiss and Kosuge, 1970; Raven, 1970). Raven (1974) summarizes the process by stating that is is possible to account for the regulation of the rate of the photosynthetic carbon reduction cycle by cofactor supply at various light intensities.

Dark carbon dioxide fixation occurs in both heterotrophic and autotrophic algal growth. A typical example of a carbon dioxide requiring reaction is beta-carboxylation, an anaplerotic pathway associated with the TCA cycle which supplies carbon skeletons for biosynthesis (Wood and Utter, 1965; Kornberg, 1966). Beta-carboxylation also aides in the regulation of intracellular pH. Synthesis of carbomoyl phosphates, precursors in arginine and pyrimidine synthesis, also requires carbon dioxide.

Carbon dioxide also performs as a catalyst. Any carbon dioxide requirement by Chlorella for carbon dioxide-catalyzed fatty acid

synthesis must be fulfilled at low levels of the gas since Paschinger (1969) found glucose being converted to lipids even when cells were illuminated in the presence of a carbon dioxide trap. Urea assimilation by Chlorella proceeds via a biotin-dependent carboxylation of urea (Thompson and Muenster, 1971). The gas also catalyzes photosystem II of photosynthesis.

Osmotic Effects and pH

Osmoregulation and osmoadaption are based on the activity and specificity of certain ionic pumps. Chlorella responds to osmotic stress by accumulating oligosaccharides, according to Dedio (1966, 1968) as reported by Soeder and Stengel (1974). High salinity stress on Chlorella results in cells discharging TCA-soluble, organic, phosphates into the medium (Antonyan and Pinevich, 1967). If synchronized Chlorella are transferred to a medium of higher salt concentration, the formation of daughter cells is more strongly inhibited than is biomass synthesis. This results in an increase of dry matter per cell for one or two days (Soeder, Schulze, and Thiele, 1967).

For Chlorella, the sensitivity of photosynthesis to any increase in osmotic pressure varies depending upon its stage of development. Schmidbauer and Ried (1967) report that cells just beginning to release autospores are most sensitive. Effects of osmotic stress on cellular respiration depend on the age of the cell. Respiration of mature

autospore-mother cells is inhibited by transfer to a more concentrated medium (Schmidbauer and Ried, 1967). Inhibition reportedly stems from direct inhibition of respiratory enzymes. Respiration of new autospores is stimulated by such a transfer to a more concentrated medium. This may come from indirect stimulation of ATP turnover as with salt respiration.

Kanazawa and Kanazawa (1969) claim that changes in culture pH will alter pigment composition, rates of synthesis of polyphosphates and some organic phosphates, and states and mobilities of heavy metals. These authors reported an increase of copper toxicity for Chlorella with decreasing pH. The pH dependency shown by dissociation rates and ionic states of polar inorganic and organic compounds affects availabilities of many nutrients like carbon dioxide, iron (Stengel, 1970), and organic acids (Cook, 1965). Also affected are cell wall surface charge (Hegewald, 1972), ion transport systems at the plasmalemma, and the associated membrane potentials (Bentrup, 1971).

Nitrogen Assimilation

Ammonium- and nitrate-nitrogen are the two most common sources of nitrogen used by algae for growth. Nitrite may be used although it becomes toxic at higher concentrations. Syrett (1962) claims that ammonium is preferred by the majority of algae.

However, he points out that growth rates are similar for algal growth on the two common nitrogen sources. The concensus is that nitrate nitrogen is converted to ammonium prior to incorporation into organic forms. According to Morris (1974), Czygan (1965) suggests that oximes are formed from nitrates prior to reduction which gives amino acids.

Algal research has confirmed that two enzymes catalyze the reduction of nitrate nitrogen to ammonium (Hattori and Myers, 1966, 1967; Zumft, Paneque, Aparichio, and Losada, 1969; Aparichio et al., 1971). Nitrate reductase, containing a flavomolybdo-protein, converts nitrate ions to nitrite nitrogen. Nitrite reductase completes the conversion to ammonium. In Chlorella the latter enzyme does not require molybdenum (Cardenas et al., 1971), but iron does seem to be a necessity (Aparichio et al., 1970). If nitrate is consumed as nitric acid, the pH of the culture may increase slightly according to Myers (1951).

The preferrential use of ammonium nitrogen over nitrate sources by algae is related to the control of nitrate assimilation. Ordinarily, ammonium assimilation results in feedback inhibition and repression of enzymes responsible for nitrate reduction. Several Chlorella species have shown this behavior (Syrett and Morris, 1963; Knutsen, 1965; Losada et al., 1970; Smith and Thompson, 1971). It was concluded that some product of ammonium assimilation actually

is the inhibitor because ammonium did not inhibit nitrate reductase in cell-free extracts (Syrett and Morris, 1963; Smith and Thompson, 1971).

Myers (1951) explained how algae show a careful conservation of nitrogen. Chlorella ordinarily reduce nitrate only at the rate determined by the requirements of cellular synthesis (Cramer and Myers, 1948). As the nitrogen source is being depleted, the nitrogen content of the Chlorella cells decreases and the rate of photosynthesis decreases (Hille, 1938). These same cells can assimilate nitrogen at relatively high rates (Ketchum, 1939).

Morris and Ahmed (1969) postulate that the presence of carbon dioxide does not affect nitrate and nitrite assimilation by Chlorella. Generally it is believed that light not only stimulates nitrate assimilation by producing electron donors for assimilation, but also aides photosynthetic carbon dioxide assimilation enabling carbon skeletons to form for organic nitrogen-compounds.

Sims, Folkes, and Bussey (1968) list three reactions whereby ammonium nitrogen is organically bound. Reductive amination of certain keto acids yields various amino acids. Further amino acid amination leads to amide compounds. A reaction between carbon dioxide, ATP, and ammonium yields carbamoyl phosphate, a precursor of citrulline.

Algae have been shown to use certain organic nitrogen compounds as sole sources of nitrogen. Eight species of Chlorophyceae were able to use urea and five used uric acid and xanthine (Birdsey and Lynch, 1962). Bollard (1966), after culturing Chlorella vulgaris on a variety of organic-nitrogen substances, concluded that of 20 protein-amino acids, all except cysteine, histidine, hydroxyproline, lysine, methionine, phenylalanine, and tryptophan supported growth, that D-glutamine, D-serine, and D-threonine allowed slight growth, that several dipeptides (e.g., glycyl-glycine, glycyl-serine) and amides supported growth, and that amines (except putrecine) and amino alcohols did not support growth. Den Dooren De Jong (1967, 1969) claimed 23 amino acids were utilized by three Chlorella vulgaris strains, although each strain showed unique behavior.

EXPERIMENTAL DESIGNS AND PROCEDURES

Introduction

Experiments were performed under laboratory conditions of controlled light intensity, temperature, culture depth, retention time, and aeration. Algae was cultured in rectangular miniponds at depths of 5, 10, and 15 cm. By using different combinations of the above parameters, optimal growth conditions could be found. Growth was measured in terms of culture dry-weight density determined by collecting a given aliquot on a millipore filter. Following are specific discussions of the procedures used to culture Chlorella vulgaris 211/8K and to measure its growth.

Minipond Culture

For culture depths of 5 and 10 cm, rectangular fiberglass basins were used. These basins had a surface area of 0.1 m^2. Because these basins were less than 15 cm deep, plastic containers with a surface area of 0.059 m^2 were used for studying production from ponds with a 15 cm depth. Figure 22 shows a typical fiberglass minipond in which algae were cultured.

The miniponds were inoculated with algae cultured in swine waste under the conditions to be tested. This allowed prior adjustment of the algae to the experimental conditions. Adjustment periods are

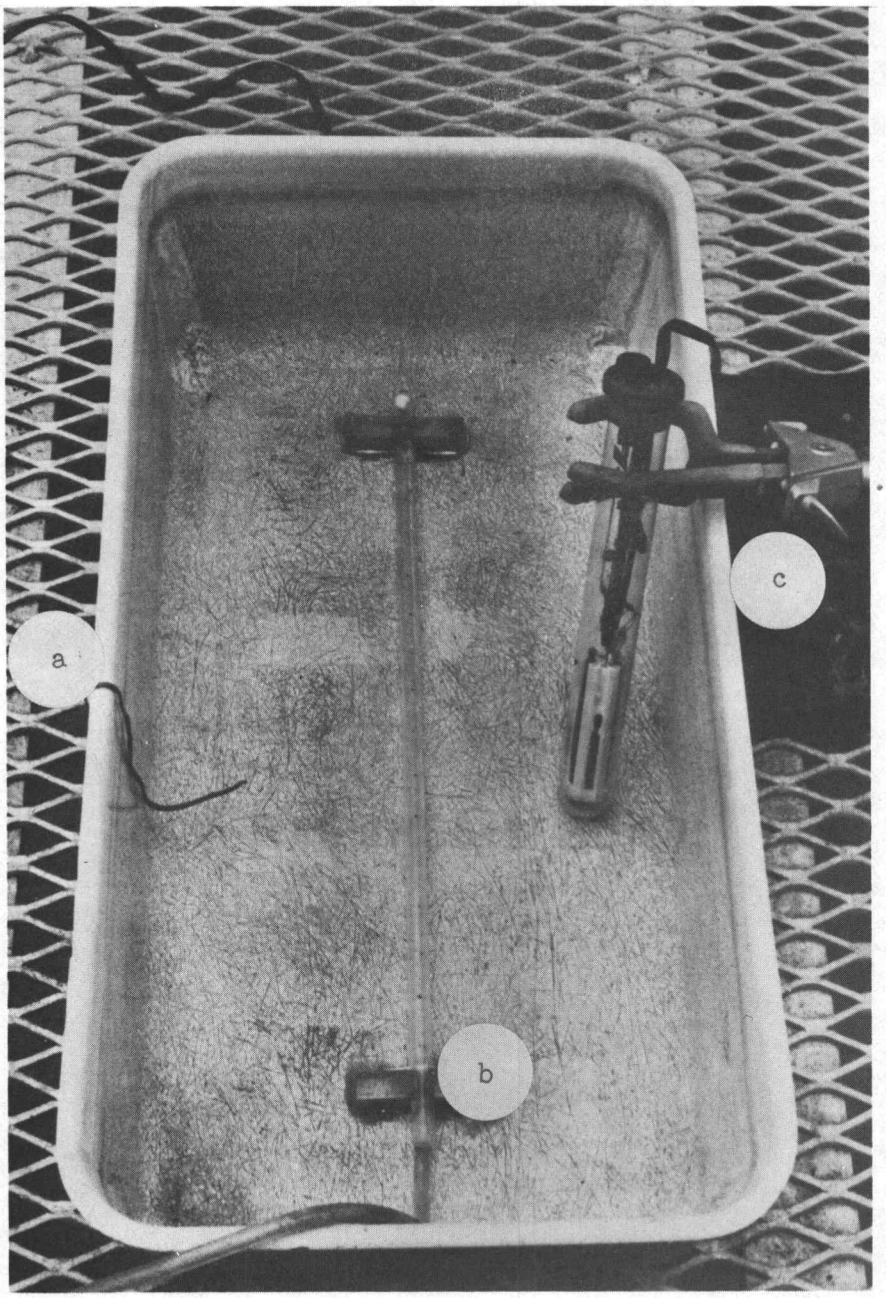

Figure 22. Minipond culture apparatus. a = temperature recorder, b = bubbler, and c = heater.

mandatory when altering environmental conditions. Soeder and Stengel (1974), besides mentioning effects of enzyme producation and developmental rhythms on adaptation, state the importance of pre-cultivation temperatures when transferring cells to new conditions. Kok and VanOorschot (1954) had earlier made a similar claim, linking atypical growth to pretransfer temperatures. As early as 1938 it was reported that the age of an algal culture used to start a new culture may exert an effect on the daughter culture for quite some time. Pratt (1943) reported an extension of the effect into granddaughter cultures. Figures 23 and 24 were taken from Pratt's publication discussing effects of culture age of inoculum on photosynthetic and respiratory rates. A relative rate was defined as the rate of growth in the new culture inoculated with a 27-day old culture divided by the rate of growth in the culture inoculated with algal cells from a 3-day old culture. The appreciable effect of the age of the parent culture can be recognized even after six days. Pratt suggested the data demonstrated the potency of chlorellin, a growth-limiting compound produced by the Chlorella cells.

With regard to changes in light intensity and their effect on algal growth, Nielsen, Hansen, and Jorgensen (1962) showed increasing the intensity from 3 to 30 klux decreased the rate of photosynthesis slightly, if any, and increased the number of enzymes while chlorophyll content dropped. Beale and Appleman (1971) support the common

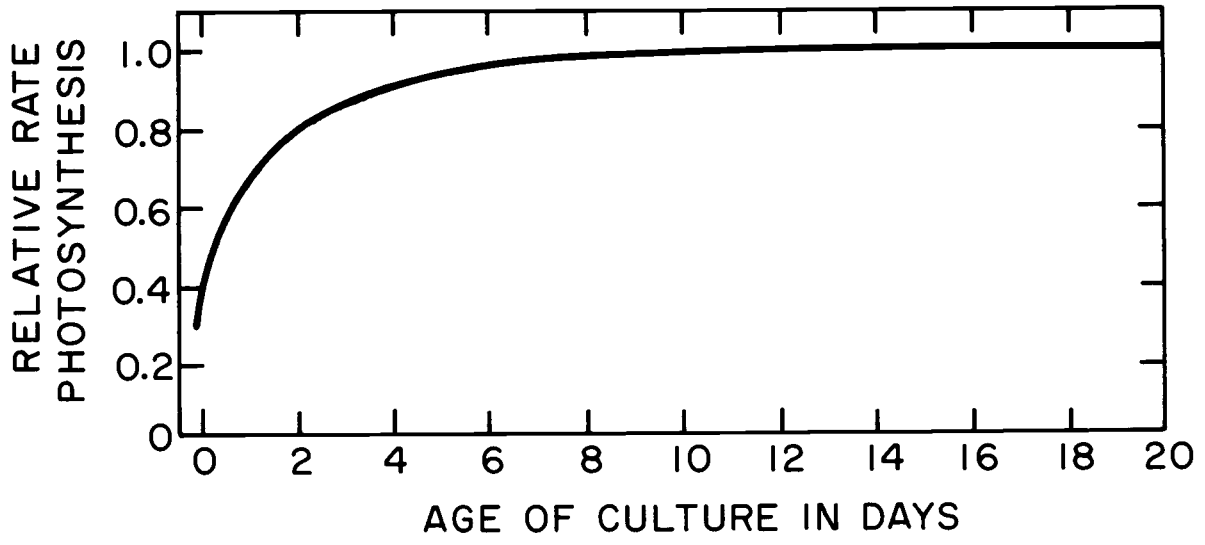

Figure 23. Relative rates of photosynthesis in cultures of Chlorella vulgaris of the same age but seeded with inocula from cultures of different ages (Pratt, 1943).

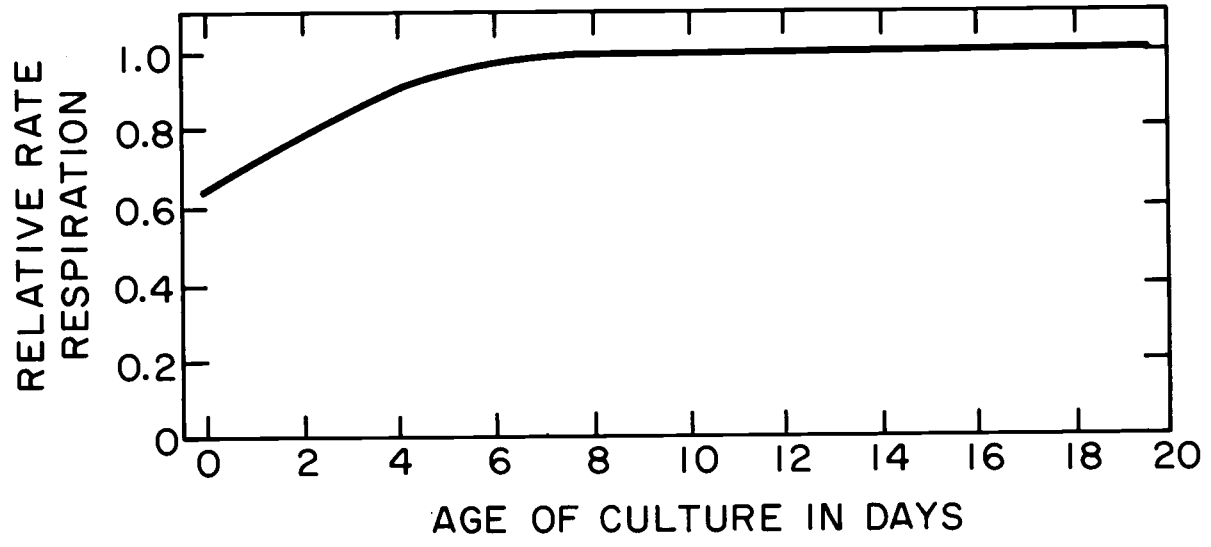

Figure 24. Relative rates of respiration in cultures of Chlorella vulgaris of the same age but seeded with inocula from cultures of different ages (Pratt, 1943).

Chlorella-type of light adaptation where adjustments to changes in light intensity are made through changes in pigment concentrations. Steeman-Nielsen et al. (1962) claim that Chlorella vulgaris adapts to new light intensities in less than 30 hours. The above points make clear the reasons for allowing at least 48 hours of adjustment to new environmental conditions by the algae prior to making measurements of culture density.

Water losses due to evaporation were replenished with distilled water. The losses averaged less than 0.5 l/day for most cultures, and were substantial only at 40 C with accompanying high light intensities (1200 and 1800 ft. c.).

The miniponds, aerators, and heaters were thoroughly cleaned between trials to prevent accumulation of bacteria and nonmetabolized waste ingredients. This helped to standardize measurements since sludge and slime layers often coated the sides and bottoms of the miniponds. Thermophilic organisms also accumulated over the surfaces of the heaters, interferring with efficienty of heat transfer.

Pond Aeration and Agitation

Four possible means of agitation are (i) bubbling air, (ii) stirring with a motor-driven stirrer, (iii) shaking or rolling a culture, and (iv) using a pump or paddle-wheel for agitation (Tamiya, 1957). For experiments discussed herein, compressed air at room

temperature was passed through a filter into single teflon tube bubblers. A Matheson flowmeter was used to measure the air flow rate directed to each pond. Approximately 0.5 liter of gas per minute passed through the cultures. The bubbling device was a 3/8 inch teflon tube with air-outlet holes drilled 2 cm apart with a #80 drill. The tube was placed along the center of the dish about 1 cm from the bottom. Teflon tubing was selected over plexiglass tubing after experiments showed teflon to be more resistant to coating and plugging by sludge and bacterial growth. Teflon bubblers required cleaning only 1/3 as often as did plexiglass bubblers.

Aerating in the described manner also provided agitation of the culture through the action of air curtains where bubblers move fluid by viscous drag. The expected result is slowly moving cells. Mixing the culture increases the change for equal exposure of all cells to available light and increases the efficiency of heat transfer. Mixing also decreases the thickness of the cell boundary layer, thus diminishing concentration gradients.

In response to claims that algal cultures need periods of rapid agitation in order to maintain high productivity, Barlow et al. (1975) examined the effects of agitation and found that periodic mechanical stirring actually decreased dry-weight densities in miniponds similar to those employed here. He also mentioned accompanying decreases in light penetration, citing sludge resuspension as a cause. The only

stirring done in this project was that just prior to measuring the culture density. Appendix A describes an experiment performed to determine the effect of stirring on the measured culture density.

To determine the number of bubblers to use and the duration of bubbling time, various combinations (Table 18) were tested for their influence on culture density. These experiments suggested optimal densities are attained with continuous single bubblers. This combination was used for trials discussed herein.

Table 18. Design of aeration experiment.

Type of Aeration	Duration	Lighttime Flow Rate[1] (l/min)	Nighttime Flow Rate (l/min)
Single bubbler	continuous	0.54	0.72
Single bubbler	lighttime	0.54	0.0
Two bubblers	continuous	1.08	1.44
No aeration	continuous	0.0	0.0

[1]Flow rates determined roughly with soap film apparatus.

Carbon Dioxide and pH

The pH was monitored five times a week through several months of experimentation and was determined using samples obtained in the morning after stirring the culture. The optimum pH range for Chlorella vulgaris 211/8K is 6.5 to 7.0. The pH of the swine waste added daily was in this range. The predicted pH increase accompanying growth alters nutrient availability through processes such as

precipitation of phosphates and volatilization of ammonia. It should be kept in mind that the pH decreases when darktime respiration exceeds photosynthesis.

It reportedly takes 1.8 grams of carbon dioxide to allow production of one gram of algae. Because no carbon dioxide enrichment was made for the experiments discussed herein, carbon dioxide should be regarded as a limiting nutrient. The carbon dioxide content of the air bubbled through the culture is approximately 0.03 percent. Even if all this gas was solubilized for algal use, the level would still be less than the saturation level of 0.05 ± 0.10 percent of CO_2 reported for Chlorella. Because carbon dioxide uptake is high in dense cultures and gas readily escapes into the atmosphere, researchers enrich Chlorella cultures with air containing 5 percent carbon dioxide. Barlow et al. (1975) reported such enriched air was insufficient to maintain optimal pH conditions, therefore affording no advantage in that respect.

Sampling

Total productivity of the cultures in the minipond was of primary concern, hence separation of harvested material into biomass components was not performed routinely. Several methods were considered for obtaining representative measurements of dry matter concentration. Centrifugation proved too time consuming and posed the

problem of variable speeds giving different results. When compared to millipore densities, centrifugation densities were consistently lower. Maximum densities could be calculated through flocculation with aluminum sulfate. The problem here is removing the flocculant from the product. Using millipore filters appeared to be the logical method to meet the needs of this project.

The dry weight of the harvested aliquot was measured daily by drawing 5 ml of sample through a tared, preleached, 1.2 μ millipore filter with a diameter of 47 mm. The filter was then dried at 30 C for 30 minutes and allowed to cool in a dessicator overnight prior to weighing. This method of obtaining dry-weight densities of the cultures proved to be consistent and reliable as the statistical analysis shows.

The factors studied and their values are listed in Table 19. All possible combinations of factors were used in culturing the algae. Dry-weight densities of the cultures were calculated by the procedure outlined above. Depths of 5, 10, and 15 cm were selected to show the response of the algal cultures under different levels of light penetration. Light intensities ranged from below the saturation intensity of 500 ft. c. for Chlorella to well above this intensity. All temperatures tested were known to support the growth of Chlorella vulgaris 211/8K in the diluted swine manure.

Table 19. Factors under study and their values. All combinations were tested for their effect on the growth of Chlorella vulgaris 211/8K in the diluted swine manure.

Depth	Light Intensity	Temperature	Retention Time
cm	ft. c.	°C	days
5	300	25	1.8
10	800	30	2.2
15	1200	35	2.8
	1800	40	3.7
			5.5
			11.0

Swine Manure

Untreated swine waste was obtained from a manhole draining pens at swine barns of Oregon State University. The drain from the manhole was plugged, the swine barn floors were shoveled relatively clear of solid and liquid materials, and the suspension in the manhole pool was stirred prior to obtaining the manure. The manure was filtered through two layers of cheesecloth into plastic trash drums for transport to and storage at the laboratory. Materials continued to settle for at least 24 hours. No stirring of the waste followed except to remove overlaying scum.

After settling, the waste was analyzed for ammonium nitrogen and nitrate nitrogen by Kjeldahl methods. The ammonium nitrogen concentration was adjusted to 300 ± 20 mg/l by adding distilled water

to decrease, or urea to increase, the concentration of the waste. This nitrogen level should have assured excess nitrogen for all experiments. Spoehr and Milner (1949) reported that it required nitrogen concentrations of less than 30 mg/l before growth and protein production of Chlorella was limited. Barlow et al. (1975) suggested 250 mg/l as a lower limit of ammonium nitrogen when using Chlorella vulgaris 211/8K in miniponds similar to those employed during this research.

The swine waste used here has been reported to be a splendid medium for Chlorella. Barlow et al. (1975) concluded that Chlorella vulgaris 211/8K grew better in the untreated manhole waste than in waste clarified by adding ferric-chloride. They established that the untreated manhole waste diluted to 25 percent its original concentration was as good as any of the other medium tested, including the inorganic media.

Temperature

The temperatures selected for testing in this research were 25, 30, 35, and 40 C. Lower and higher temperatures were also considered. Because Chlorella vulgaris 211/8K is a thermophile, 35 and 40 C were assumed to be optimal for maximum algal densities. Temperatures were maintained with automatic aquarium heaters, located opposite in position to the thermocouple suspended midway through the

culture (Figure 22). Thermocouples outside the ponds provided measurement of the ambient temperature, which remained within two degrees of pond temperatures.

Light Intensity

Four intensities were used during this research as Table 20 shows. Measurements were made at the culture level.

Table 20. Light intensities used for experimentation.

Photometric Sensor	Foot Candles	Lux	Photon Sensor	Photons/cm^2/sec ($\times 10^{-15}$)
.058	1800	19,268	.037	22.29
.039	1200	12,912	.022	13.25
.026	800	8,608	.016	9.64
.009	300	3,228	.006	3.61

Measurements were made with a Lambda Photometer (Model LI180), employing photometric and photon sensors. The photometer responds to visible region photons and readings are in foot candles or lux. The candle is a unit of luminous intensity with the true international unit referred to as a candela. A candela equals 1/60 of the total light intensity emitted by a cm^2 of a black body radiator at 2042°K, the melting temperature of pure platinum. The total light emission from a one candela source equals 4π lumens. Lux are the metric equivalents to foot candles as the following conversions indicate:

(1) 1 foot candle = 1 lumen/ft^2

(2) 1 lux = 1 lumen/m^2

(3) 1 lux = 1 ft. c. x 10.764 ft^2/m^2

It is important when using lux to designate the light source and its wavelength distribution. For example, at the same lux illumination, a fluorescent lamp produces about three times as much blue light as a tungsten lamp. For this research both fluorescent and incandescent lights positioned at the growth chamber's ceiling were utilized.

Algae utilize a limited portion of the light spectrum for photosynthesis in the 400 to 700 nm waveband. An integral relationship exists between the number of molecules changed photochemically and the number of photons absorbed (Rabinowitch and Govindjee, 1969), regardless of the energy of the photon, provided it falls within the requisite waveband. Excess energy is primarily released as heat. Accurate estimations of photosynthetically active radiation must then rely on sensors which measure only photons in the 400 to 700 nm waveband. The photon sensor used here meets this requirement as Figure 25 shows.

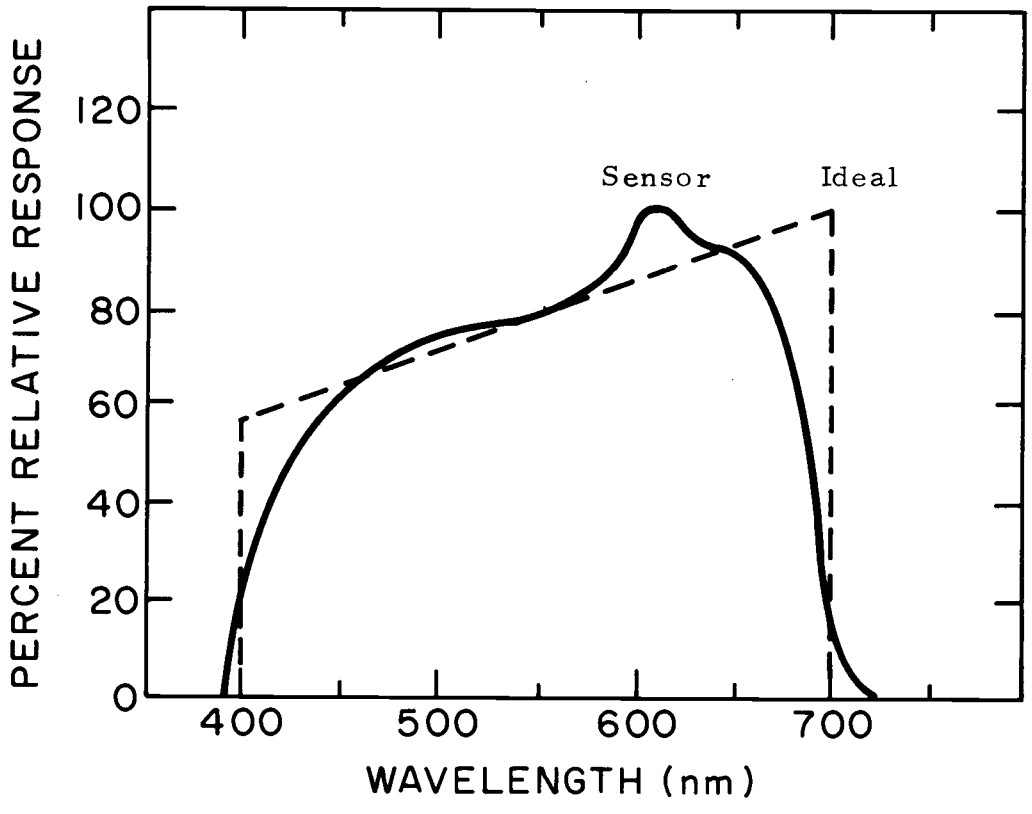

Figure 25. Spectral response curve for photosynthesis response light sensor and ideal photon response sensor.

EXPERIMENTAL RESULTS

Statistical Procedures

Assumptions

The statistical procedures to be used are based on the assumptions that the biomass in the minipond cultures were growing at a constant rate at the time of sampling and that algae in the cultures were included in the density measurement. The biomass of the cultures consisted primarily of Chlorella, with bacteria and zooplankton also contributing. The relative numbers of these organisms are a function of culture depth and temperature, the waste retention time, and the light intensity.

Experimental results support these assumptions. A constant rate of growth in the cultures at the time of measurement was assured by allowing the cultures to reach equilibrium conditions during a three-day period for each set of experimental conditions. Preliminary experiments were performed to establish that three days was adequate time for development of steady-state conditions. Density determinations began on the day of inoculation of the experimental cultures and were made daily thereafter. The densities were found to be constant after three days. Table 21 shows results typical of these preliminary experiments. Day-one densities vary due to estimated inoculums

based on culture volumes remaining after the different harvests have been made. For example, the inoculum for the culture having a 5-day retention time would be eight liters of Chlorella culture. This inoculum would be added to two liters of waste to give the final volume of ten liters.

Table 21. Culture density in grams per liter as a function of time for 10 cm deep cultures at 30 C and 1800 ft. c.

Retention Time	Days Since Inoculation							
	1	2	3	4	5	6	7	8
Days	- - - - - - - - - - - - - g/l - - - - - - - - - - - - -							
10.0	1.52	1.55	1.67	1.67	1.65	1.65	1.68	1.66
5.0	1.38	1.45	1.61	1.61	1.61	1.61	1.63	1.61
3.3	1.36	1.40	1.63	1.63	1.63	1.61	1.61	1.62
2.5	1.35	1.36	1.51	1.43	1.46	1.40	1.41	1.42
2.0	0.73	0.61	0.57	0.55	0.50	0.57	0.61	0.56
1.7	0.90	0.51	0.45	0.35	0.38	0.38	0.40	0.38

Observations supported the second assumption that the algae in the cultures was included in the density measurement. Following filtration of minipond culture samples through millipore filters used for density measurements, filtrates were subjected to microscopic observation. The conclusion was that the filter effectively retained the Chlorella cells.

Microscopic observation of the culture showed that, in addition to Chlorella, a variety of bacteria were growing in the cultures. The exact proportion of algae to bacteria was not determined. The

importance of defining the culture population is realized, and subsequent studies will include size differential particle counting using a Coulter counter. Used in conjunction with microscopic counting of algal cells, fluorometric readings, and dry-weight determinations, the particle counts should provide an estimate of relative proportions of algae and bacteria in the total biomass. A third assumption must be that observed differences in dry-weight densities are a result of different levels of algal growth.

Standard Deviation of Density Measurements

The precision of the density measurements made during the four day experimental periods was determined by calculating sample variances for all trials. The mean sample variances for 5, 10, and 15 cm deep ponds were 0.0159, 0.0044, and 0.0095 g^2/l^2, respectively. The pooled variance was 0.01 g^2/l^2, suggesting that the pooled standard deviation of the density measurement was 0.1 g/l.

Statistical Analysis

The design of the experiment to determine the effects of culture depth, light intensity, temperature, and retention time on the growth of Chlorella vulgaris 211/8K in diluted swine manure was a 3 x 4 x 4 x 6 factorial. This implies that the factors of depth, temperature, light intensity, and retention time had 3, 4, 4, and 6 levels,

respectively. Although the mathematical model expressing this four-way classification is lengthy, once understood it simplifies data analysis. Symbols and their definitions are given in Table 22.

Table 22. Equation symbols and their definition used in the statistical analysis.

Symbol	Definition
i	light level; i = 1, 2, 3, 4
j	temperature level; j = 1, 2, 3, 4
k	depth level; k = 1, 2, 3
ℓ	retention time level; ℓ = 1, 2, 3, 4, 5, 6
m	sample number; M max = 1152
Y	an observation described by i, j, k, ℓ, m
μ	general mean of all observations
λ	light effect
τ	temperature effect
δ	depth effect
γ	retention time effect
E	experimental error
e	sampling error

Any observation of culture density hypothetically depends on the general mean of all observations, the main effects, the interactions, and the experimental and sampling errors as follows:

$$
\begin{aligned}
Y_{ijk\ell m} = \mu + \lambda_i + \tau_j + \delta_k + \gamma_\ell + (\lambda\tau)_{ij} + (\lambda\delta)_{ik} \\
+ \ldots (\lambda\gamma)_{i\ell} + (\tau\delta)_{jk} + (\tau\gamma)_{j\ell} + (\delta\gamma)_{k\ell} + (\lambda\tau\delta)_{ijk} \\
+ \ldots (\lambda\tau\gamma)_{ij\ell} + (\lambda\delta\gamma)_{ik\ell} + (\tau\delta\gamma)_{jk\ell} \\
+ \ldots E_{ijk\ell} + e_{ijk\ell m} .
\end{aligned}
$$

Since the experiment was not repeated in its entirety, it was not possible to distinguish between $(\lambda\tau\delta\gamma)_{ijk\ell}$ and $E_{ijk\ell}$. Hence, as is commonly done, the experimental error term was used alone with the assumption that $(\lambda\tau\delta\gamma)_{ijk\ell} = 0$. This assumption implies that the four way interactions are negligible.

Analysis of variance was used for the interpretation of the data. The basic assumption to this technique is that the errors, $E_{ijk\ell}$, are normally distributed with a mean of zero and an unknown variance, σ^2. The mean square of $E_{ijk\ell}$, given as 0.187 in Table 23, is the best estimate of this variance. Analysis of variance is a systematic way of partitioning the total sum of squares of the variable of interest, main effects or interactions in our case, into component parts, each of which is associated with a recognized source of variation. The resulting components are then arranged in tabular form to systematize the computations and to facilitate hypothesis testing. Table 23 is the analysis of variance table for this experiment. To establish whether or not a component part of the variable of interest is significant, the F value is determined. This test statistic is given by:

$$F = \frac{\text{mean square of the component part}}{\text{mean square of } E_{ijk\ell}},$$

with degrees of freedom corresponding to the component part and to the experimental error. Throughout this discussion, "significant"

implies that the F test showed significance at the $\alpha = .001$ level. This level corresponds to a confidence coefficient of 0.999, with a value of 1.0 being the maximum confidence coefficient.

Table 23. Analysis of variance table, where D is depth, T is temperature, L is light, R is retention time, E is experimental error, and e is sampling error.

Source	df	Sum of Squares	Mean Square	F Value	Significant ($\alpha = .001$)
Depth	2	23.124	11.562	61.829	+
linear	1	17.328	17.328	92.663	+
nonlinear	1	5.796	5.796	30.995	+
Temperature	3	14.256	4.752	25.412	+
linear	1	6.117	6.117	32.711	+
quadratic/lin.	1	3.147	3.147	16.829	+
remainder	1	5.002	5.002	26.749	+
Light	3	10.877	3.626	19.390	+
quadratic	1	9.823	9.823	52.529	+
lin. /quadratic	1	1.016	1.016	5.433	-
remainder	1	0.38	0.038	0.203	-
Retention	5	54.080	10.816	57.840	+
log	1	51.528	51.528	275.551	+
$\sqrt{}$ /log	1	2.544	2.554	13.658	+
remainder	3	0.008	0.008	0.016	-
D x T	6	9.678	1.613	8.626	+
D x L	6	14.022	2.337	12.497	+
L x T	9	23.976	2.664	14.246	+
T x R	15	8.430	0.562	3.005	+
D x R	10	3.110	0.311	1.663	-
L x R	15	2.985	0.199	1.064	-
L x T x D	18	50.148	2.786	14.898	+
L x D x R	30	2.580	0.086	0.460	-
L x T x R	45	9.045	0.201	1.075	-
D x T x R	30	5.130	0.171	0.914	-
E	90	16.830	0.187		
e	864		0.013		

To test if any of the effects of the four factors could best be expressed by an exponential term, further analyses were made. Through regression analyses, equations containing the factors and their squares were obtained. In order to determine the actual effect of each term in the equation, the regression coefficients and their standard errors were used to compute the T values as follows:

$$T = \frac{\text{regression coefficient}}{\text{standard error of the regression coefficient}}.$$

For this experiment a T value greater than 3.3 suggests that the associated equation term is significant ($\alpha = .001$) in terms of describing the data.

Effects of Individual Factors

Culture Depth

Culture depth significantly affected the culture density as shown in Table 24 where the average culture densities associated with each depth are shown. Figure 26 illustrates the effect of depth on culture density. The relationship between culture density (Y) and depth (D) can be described by the linear equation:

$$Y = 1.4464 - .03D.$$

Because the culture density decreased at 15 cm, it was thought that a

quadratic equation might improve the mathematical description of the data. The ANOVA table (Table 23) indicates that both a linear and a nonlinear term significantly describe the effect of depth on culture density. Table 25 shows that T values support the claim that the following quadratic equation surpasses the above linear equation in describing the data:

$$Y = 0.9448 + 0.0903D - 0.006D^2 .$$

Table 24. Effect of depth on average culture density.

Depth	Average Culture Density
cm	g/l
15	0.9456
10	1.2463
5	1.2460

Table 25. A test for significance of the quadratic equation describing the effect of depth on culture density.

Variable	Regression Coefficient	Standard Error	T Value	Significant $\alpha = .001$
D	0.0903	$1.909\ (10^{-2})$	4.729	+
D^2	0.006	$9.451\ (10^{-4})$	6.368	+

Temperature

No obvious correlation between temperature and density can be derived from a comparison of average densities and the associated temperatures. Table 26 shows that the variation in density over the range of 25 to 35 C is within the standard deviation of the measuring procedure. Figure 27 illustrates the change in density with increasing temperature. The temptation is to consider the mean density at 35 C on outlier, since without this point the figure would indicate a direct relationship between culture density and temperature. Repeating the experiment is necessary to establish whether or not such a direct relationship exists.

Table 26. Effect of temperature on average culture density.

Temperature	Average Culture Density
°C	g/l
25	1.0710
30	1.1494
35	1.0380
40	1.3254

Linear and quadratic equations were computed in an attempt to describe the data relating temperature and culture density. The ANOVA table (Table 23) and Table 27 show that both linear and quadratic expressions significantly describe the data. However, the

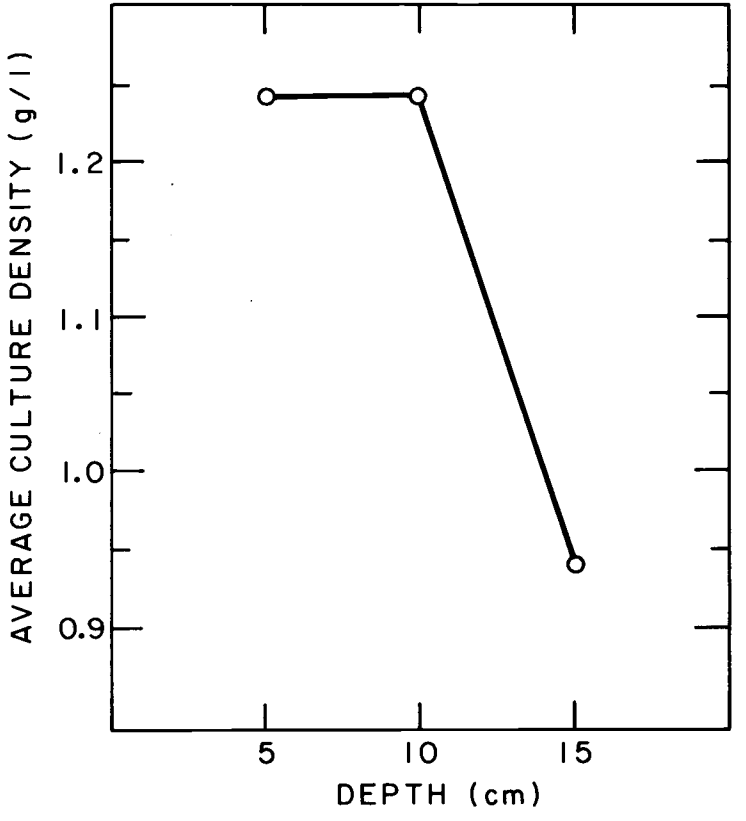

Figure 26. Average culture density as a function of culture depth.

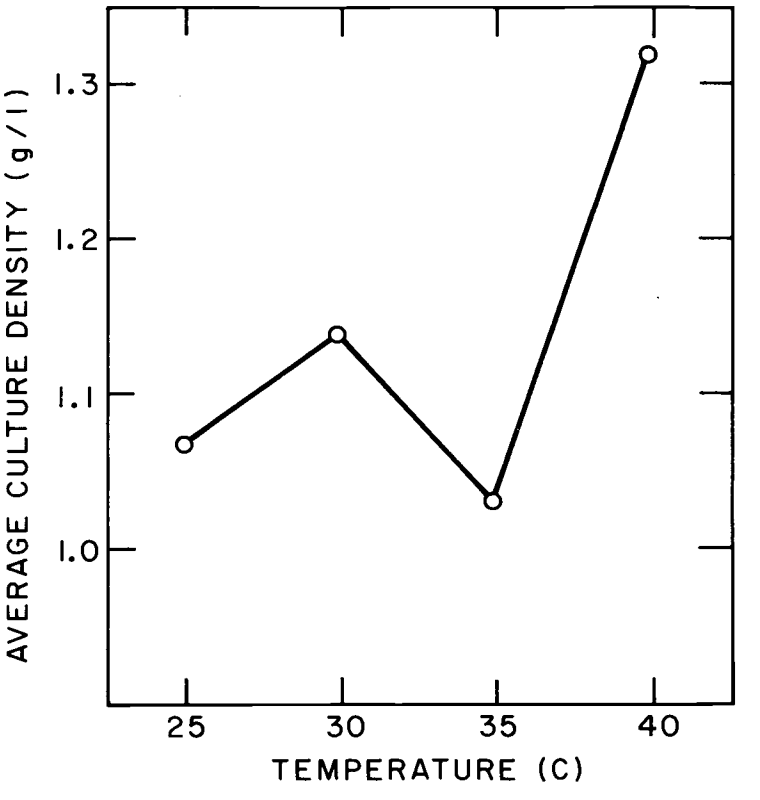

Figure 27. Average culture density as a function of culture temperature.

ANOVA table shows clearly that a cubic equation might also fit the data since the remainder component of the sum of squares for temperature had a significant F value. Fitting an equation to the data becomes less meaningful as general trends fail to appear.

Table 27. A test for significance of the quadratic equation describing the effect of temperature on culture density.

Variable	Regression Coefficient	Standard Error	T Value	Significant $\alpha = .001$
T	0.1228	$2.902\ (10^{-2})$	4.232	+
T^2	0.0021	$4.455\ (10^{-4})$	4.692	+

Light Intensity

Before fitting an equation to the data relating culture density to light intensity, it is helpful to observe Table 28 and Figure 28 which show the effect of light intensity on the average culture density. The variation in culture density is just greater than the standard deviation of the measurement when either extreme is compared to the density reached at 800 or 1200 ft. c.

Figure 28 suggests that a quadratic equation best fits the data. A statistical test on a regression model led to the conclusion that a quadratic expression significantly describes the data. The results of this test are shown in Table 29. The regression model is best estimated by the following equation:

$$Y = 0.8013 + 8.0445(10^{-4})L - 3.5481(10^{-7})L^2 ,$$

where Y is the culture density and L is the light intensity in foot candles.

Table 28. Effect of light intensity on average culture density.

Light Intensity	Average Culture Density
ft. c.	g/l
300	1.0083
800	1.2259
1200	1.2482
1800	1.1015

Table 29. A test for significance of the quadratic equation describing the effect of light intensity on culture density.

Variable	Regression Coefficient	Standard Error	T Value	Significant $\alpha = .001$
L	0.0008	$9.275\ (10^{-5})$	8.672	+
L^2	$3.5481\ (10^{-7})$	$4.279\ (10^{-8})$	8.290	+

Along with regression analysis, analysis of variance supports the conclusion that the effect of light intensity on culture density can best be described by a quadratic equation. The ANOVA table (Table 23) shows that the quadratic component of the light factor has an F value of 52.529 and is the only significant component.

Retention Time

Retention time significantly affects the culture density. Table 30 and Figure 29 shows that density increases with retention time. This increase can be described by the following equation:

$$Y = 0.8698 + 0.0613R,$$

where Y is the culture density and R is the retention time in days. The F value associated with this equation is 227.540 which implies the linear equation significantly describes the data.

Table 30. Effect of retention time on average culture density.

Retention Time	Average Culture Density
days	g/l
1.8	0.8389
2.2	0.9534
2.8	1.0765
3.7	1.1947
5.5	1.3441
11.0	1.4682

A statistical test, the results of which are given in Table 31, indicated a quadratic equation could also significantly describe the data. However, quadratic expressions often fit data which may be better explained in terms of a logarithmic function. Analysis of variance showed this to be the case here. Computer analysis described

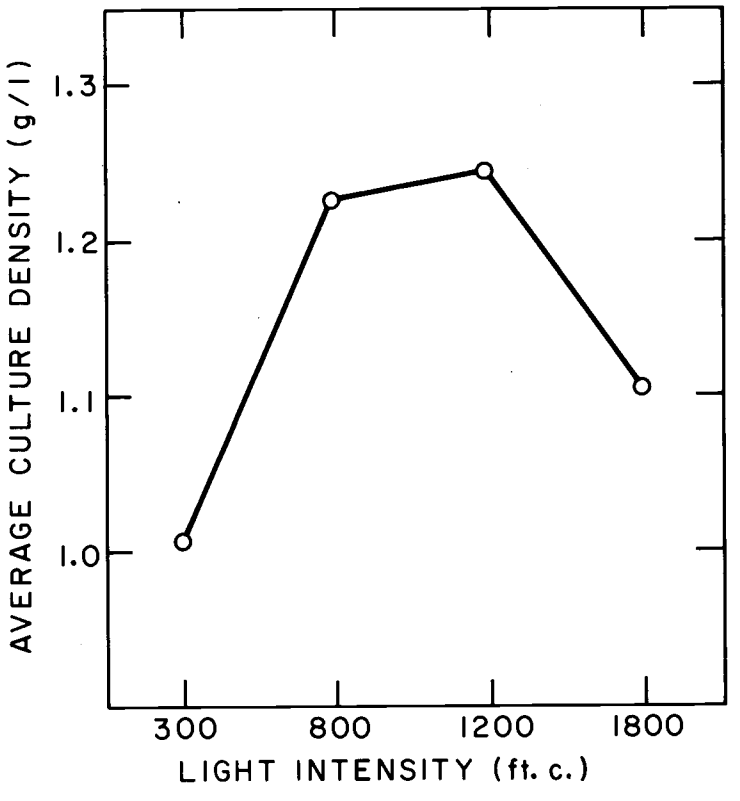

Figure 28. Average culture density as a function of light intensity.

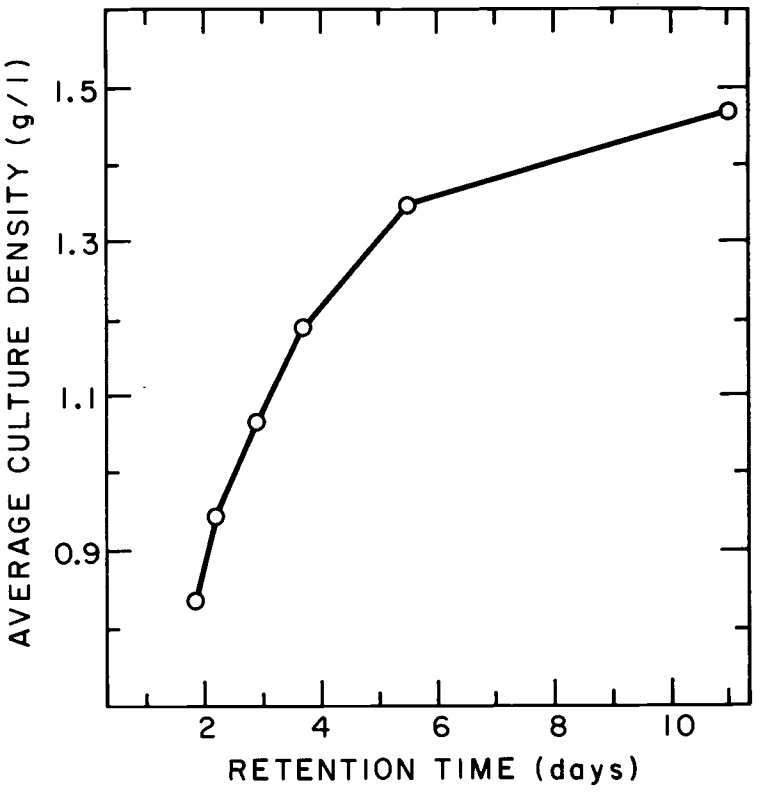

Figure 29. Average culture density as a function of retnetion time.

the effect of retention time (R) on culture density (Y) as follows:

$$Y = 0.6921 + 0.3483 \ln R.$$

A highly significant F value of 284.289 is associated with this equation.

Table 31. A test of significance of the quadratic equation describing the effect of retention time on culture density.

Variable	Regression Coefficient	Standard Error	T Value	Significant $\alpha = .001$
R	0.2279	$1.981\ (10^{-2})$	11.504	+
R^2	0.0128	$1.498\ (10^{-3})$	8.544	+

To determine which, if any, additional terms add significantly to the description of the data, a comparison of R, $\sqrt{R}$, and R^2 terms was made. Computer analysis showed that the description of the data was significantly enhanced only by a $\sqrt{R}$ term. Therefore, the best equation for describing the data on the change in density as a function of retention time is:

$$Y = 1.0095 - 0.5321\sqrt{R} + 0.9273 \ln R.$$

Interactions

Whereas in the analysis of an individual factor all other factors were considered to cancel one another, computer analysis can provide an equation predicting the culture density as a function of all four factors. The culture density can be obtained as follows:

$$Y = 0.6911 - 0.03D + 0.013T + 5.4057(10^{-5})L + 0.0613R.$$

This equation had an F value of 100.369, which implies that it significantly describes the data.

Light Intensity and Temperature

Mean densities for each temperature-light intensity combination tested are shown in Table 32 and Figure 30.

Most of the significance of this two factor interaction stems from the observation that at 40 C, the culture density is relatively independent of the light intensity. There is also a crossing interaction due to the fact that at 35 C the maximum density occurs with a light intensity of 1200 ft.c. rather than 800 ft.c. as it does with lower temperatures of 25 and 30 C.

A crossing interaction simply refers to the physical crossing of the lines plotted in Figure 30. This is in contrast to a spreading interaction shown by lines that diverge from a common area. Both

types of interactions suggest two variables act concommitantly to influence one parameter, like culture density being affected by the combination of light intensity and temperature.

Table 32. Mean densities for all temperature-light intensity combinations tested.

Temperature	Light Intensity	Main Density
°C	ft. c.	g/l
25	1800	0.8829
	1200	1.1093
	800	1.2883
	300	1.0037
30	1800	1.1409
	1200	1.1791
	800	1.3184
	300	0.9593
35	1800	1.0818
	1200	1.4398
	800	0.9794
	300	0.6509
40	1800	1.3005
	1200	1.2645
	800	1.3175
	300	1.4193

Light Intensity and Depth

Table 33 lists the mean densities for all depth-light intensity combinations. The crossing interaction shown in Figure 31 indicates that depth and light intensity interact significantly to influence the culture density.

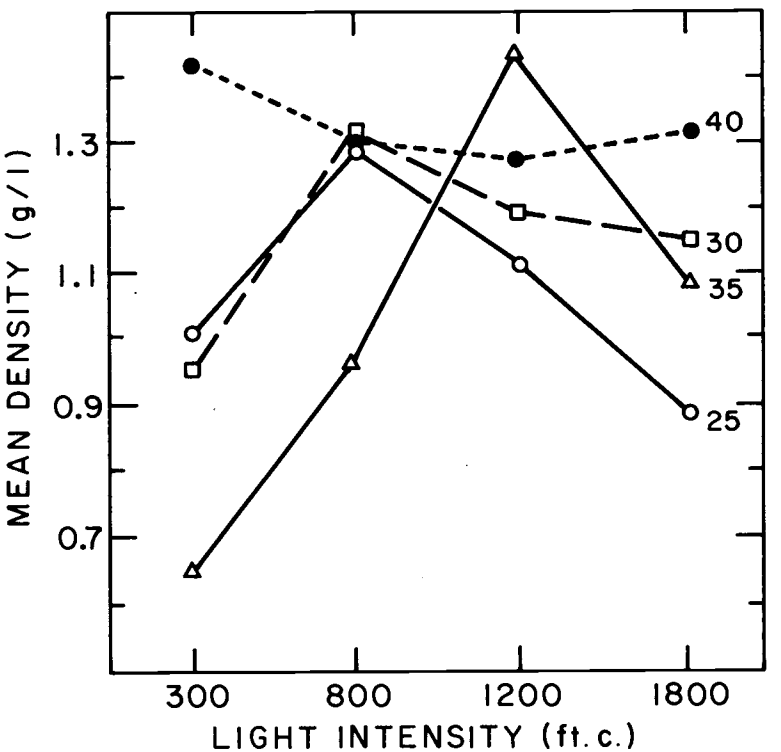

Figure 30. Mean densities associated with all temperature-light intensity combinations tested. Average culture density as a function of light intensity at culture temperatures of 25 C (-o-o-), 30 C (-□--□-), 35 C (-△-△-), and 40 C (-●--●-).

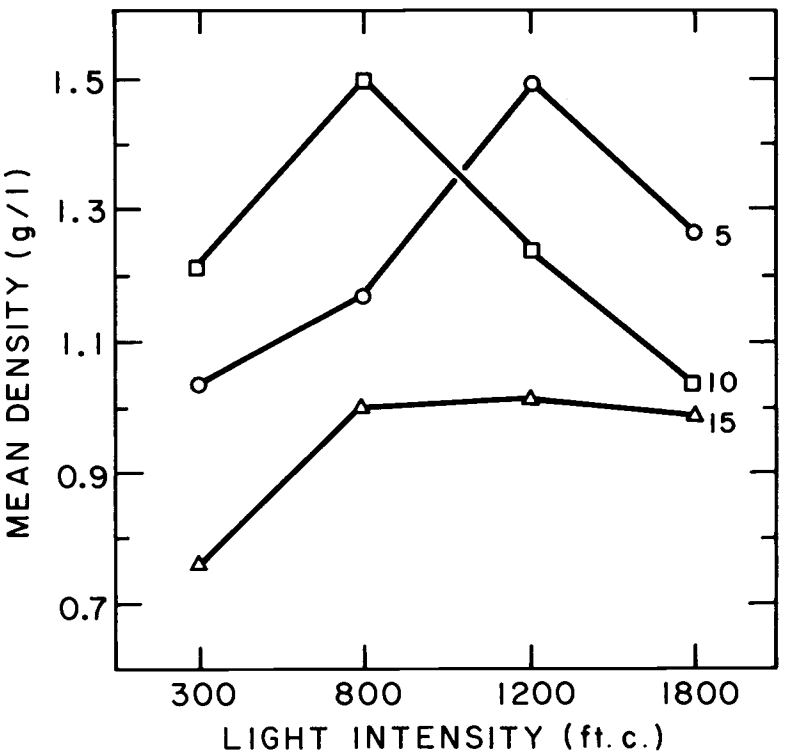

Figure 31. Mean densities associated with all depth-light intensity combinations tested. Average culture density as a function of light intensity at culture depths of 5 cm (-o-o-), 10 cm (-□-□-), and 15 cm (-△-△-).

Temperature and Depth

The interaction of depth and temperature was significant in terms of affecting culture density. The culture densities associated with the various combinations are shown in Table 34 and Figure 32. Higher temperatures affect the density of cultures that are 5 cm deep more than cultures 10 to 15 cm deep. The interaction is weak and has less influence on the culture density than did those combinations discussed previously.

Temperature and Retention Time

The final two factor combination showing significant interaction is that of temperature and retention time. Mean densities for all temperature-retention time combinations are listed in Table 35. As Figure 33 shows, both crossing and spreading interactions occurred, suggesting temperature and retention time act together to affect the culture density. Their combined effect is similar to that effect of retention time alone. The combination of temperature and retention time exerts a weaker influence on the culture density than other two-factor combinations.

Table 33. Mean densities for all depth-light intensity combinations tested.

Depth	Light Intensity	Mean Density
cm	ft. c.	g/l
5	1800	1.2714
	1200	1.4962
	800	1.1738
	300	1.0427
10	1800	1.0415
	1200	1.2301
	800	1.4981
	300	1.2155
15	1800	0.9916
	1200	1.0183
	800	1.0058
	300	0.7667

Table 34. Mean densities for all depth-temperature combinations tested.

Depth	Temperature	Mean Density
cm	°C	g/l
5	25	1.1360
	30	1.2547
	35	0.9761
	40	1.6172
10	25	1.1954
	30	1.2725
	35	1.2259
	40	1.2914
15	25	0.8817
	30	0.9211
	35	0.9119
	40	1.0677

Table 35. Mean densities for all temperature-retention time combinations tested.

Temperature	Retention Time	Mean Density
°C	days	g/l
25	11.0	1.3616
	5.5	1.2137
	3.7	1.1168
	2.8	1.0087
	2.2	0.9097
	1.8	0.8156
30	11.0	1.6993
	5.5	1.4431
	3.7	1.2177
	2.8	1.0458
	2.2	0.8343
	1.8	0.6564
35	11.0	1.3272
	5.5	1.1620
	3.7	1.0983
	2.8	1.0162
	2.2	0.8675
	1.8	0.7566
40	11.0	1.4845
	5.5	1.5577
	3.7	1.3460
	2.8	1.2354
	2.2	1.2022
	1.8	1.1268

Summary of Analysis

Analysis of variance provides a basis for simplifying the hypothetical equation which predicted the value of the culture density, $Y_{ijk\ell m}$, as previously described. The experimentally determined equation is:

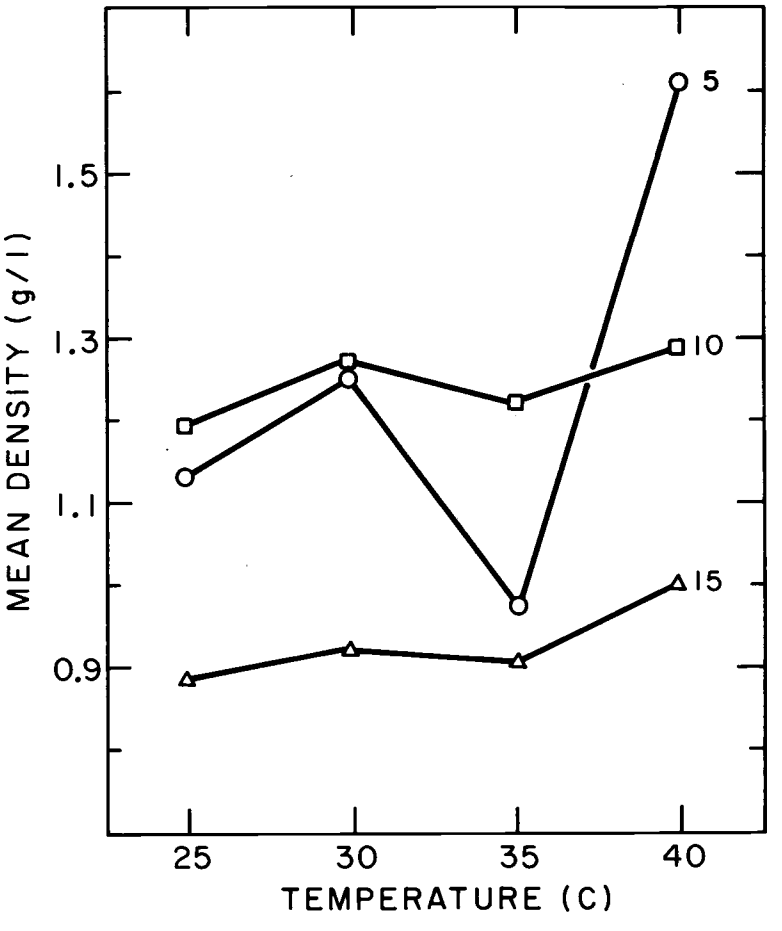

Figure 32. Mean densities associated with all depth-temperature combinations tested. Average culture density as a function of temperature at culture depths of 5 cm (-o-o-), 10 cm (-□-□-), and 15 cm (-△-△-).

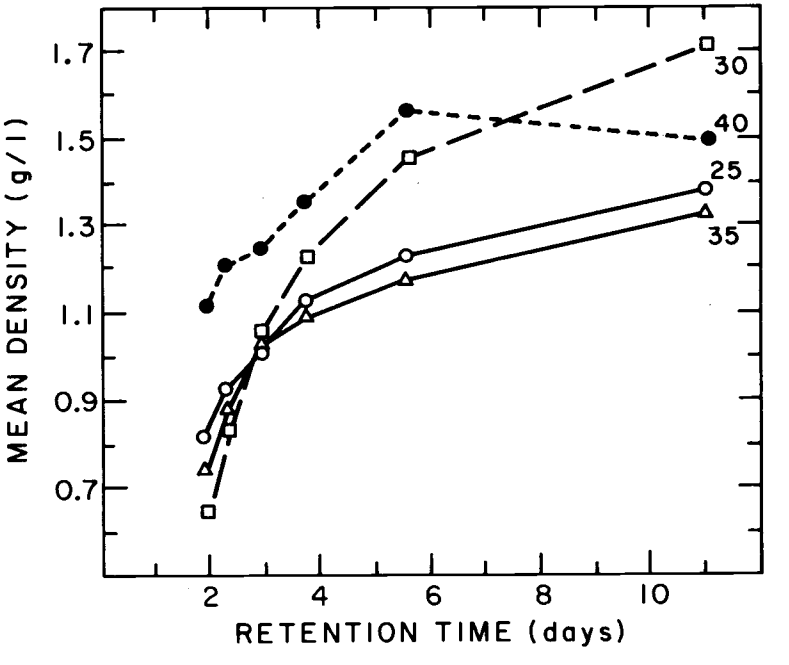

Figure 33. Mean densities associated with all temperature-retention time combinations tested. Average culture density as a function of retention time at culture temperatures of 25 C (-o-o-), 30 C (-□--□-), 35 C (-△-△-), and 40 C (-●--●-).

$$Y_{ijk\ell m} = \mu + \lambda_i + \tau_j + \delta_k + \gamma_\ell + (\lambda\tau)_{ij} + (\lambda\delta)_{ik}$$
$$+ \ldots (\tau\delta)_{jk} + (\tau\gamma)_{j\ell} + (\lambda\tau\delta)_{ijk} + E_{ijk\ell} + e_{ijk\ell m} .$$

The experiments indicate that five combinations of factors ($\lambda\gamma$, $\delta\gamma$, $\lambda\tau\gamma$, $\lambda\delta\gamma$, and $\tau\delta\gamma$) are insignificant in terms of influencing culture density. Instead, the culture density depends on culture depth and temperature, the light intensity, the retention time, and particular combinations ($\lambda\tau$, $\lambda\delta$, $\tau\delta$, $\tau\gamma$, and $\lambda\tau\delta$).

Generally the culture density increases as temperature, light intensity, and retention time increase. Increasing the depth decreases the culture density.

Worth noting is the fact that the small sampling error, $e_{ijk\ell m}$, confirms that any error associated with the repetitive sampling for density determination under a particular set of conditions was insignificant.

Comparison of Cell Numbers with Millipore Densities

Following inoculation of 10 cm deep cultures with Chlorella vulgaris 211/8K cell counts and dry weights were determined daily (Tables 36 and 37). Culture temperatures studied were 30, 35, 40, and 45 C. The light intensity was 800 ft. c.

Figures 34 and 35 illustrate that the increase in dry weight at 30, 35, and 40 C can be attributed to an increase in cell numbers.

After 48 hours the dry weight remained relatively constant despite continued increases in cell number. Microscopic observation showed that after 72 hours the cell size was reduced, thus accounting for the discontinuity. Because fresh media were not supplied to the cultures, the reduction in cell size may be a result of nutrient limitations.

The temperature of 45 C reduced both cell numbers and dry weight. This temperature caused cell death and cell lysis.

Table 36. Cell counts following inoculation of minipond cultures.

Temperature	Days Following Inoculation					
	1	2	3	4	5	6
°C	- - - - - - cells/ml x 10^{-6} - - - - - - -					
30	1.3	3.0	7.3	10.6	14.4	17.9
35	1.1	1.0	3.1	5.2	7.8	19.5
40	1.4	1.1	7.6	13.6	14.8	15.8
45	1.3	1.0	0.8	0.4	0.5	0.3

Table 37. Dry weights following inoculation of minipond cultures.

Temperature	Days Following Inoculation					
	1	2	3	4	5	6
°C	- - - - - - - - - g/l - - - - - - - - -					
30	0.37	0.66	0.83	0.72	0.70	0.76
35	0.20	0.42	0.70	0.71	0.69	0.63
40	0.20	0.72	0.72	0.68	0.67	0.66
45	0.22	0.18	0.12	0.13	0.09	0.07

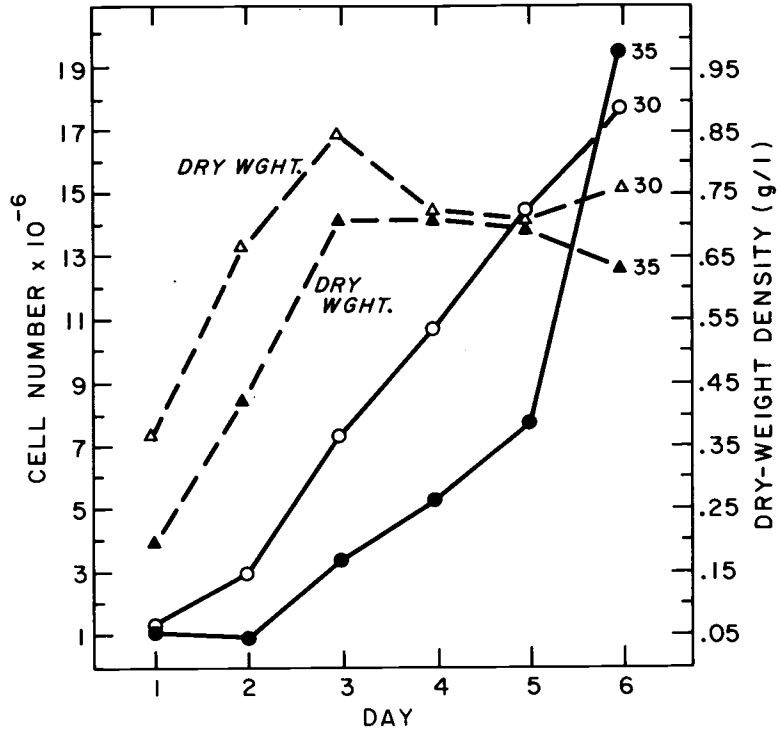

Figure 34. Cell number and dry weight as a function of time for 10 cm deep cultures at 30 and 35 C. Cell numbers are shown by solid lines. Broken lines show the dry weight densities.

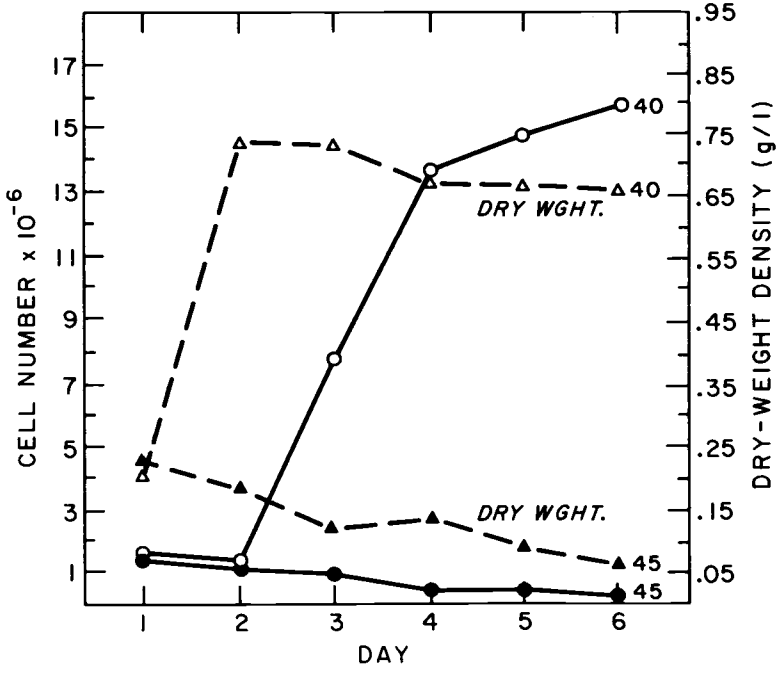

Figure 35. Cell number and dry weight at a function of time for 10 cm deep cultures at 40 and 45 C. Cell numbers are shown by solid lines. Broken lines show the dry weight densities.

DISCUSSION

The data analysis indicated that the growth of Chlorella vulgaris 211/8K in diluted swine manure is affected by culture depth, light intensity, retention time, and to a lesser extent by temperature and interactions of these factors. A discussion of these factors follows with regard to possible physiological and physical limitations imposed by them on the growth of Chlorella vulgaris 211/8K.

Culture Depth

The optimum depth for exploiting photosynthetic activity in diluted swine manure toward the goal of maximizing algae production is 10 cm. The upper limit of 10 cm agrees with the earlier prediction of Hemens and Stander (1969). They claimed that beyond this depth the light intensity dropped below 100 ft. c., the minimum light intensity allowing effective nutrient assimilation in a lagoon.

Decreasing the depth to 5 cm can increase the maximum culture density slightly (Figure 36). However, the limitations imposed on production by the accompanying decrease in harvest capacity makes this impractical (Figure 37). Under higher sunlight intensities photoinhibition of algal growth occurs more quickly in ponds less than 10 cm in depth.

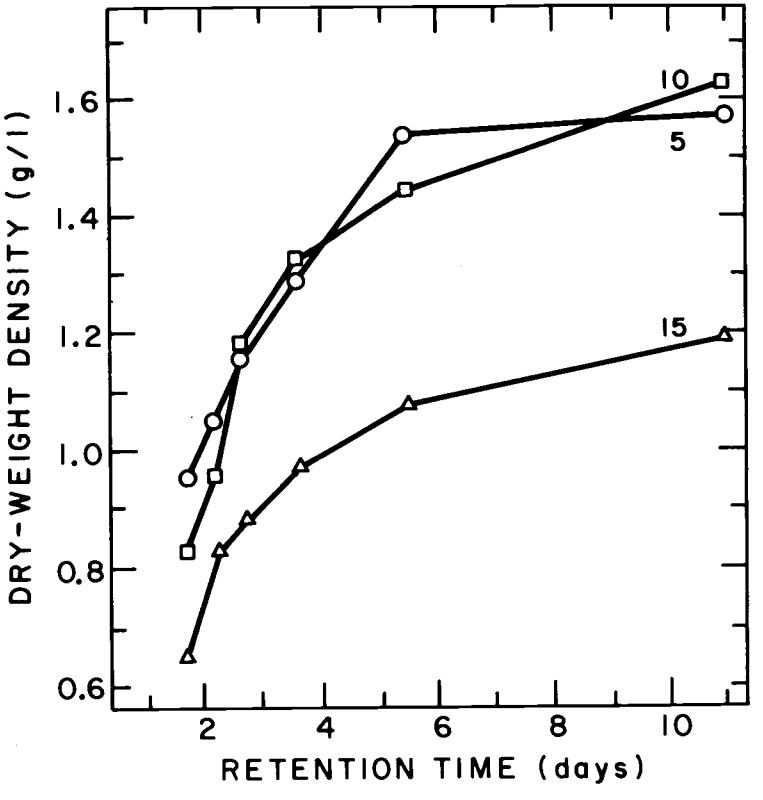

Figure 36. Average culture density as a function of retention time at culture depths of 5 cm (-o-o-), 10 cm (-□-□-), and 15 cm (-△-△-).

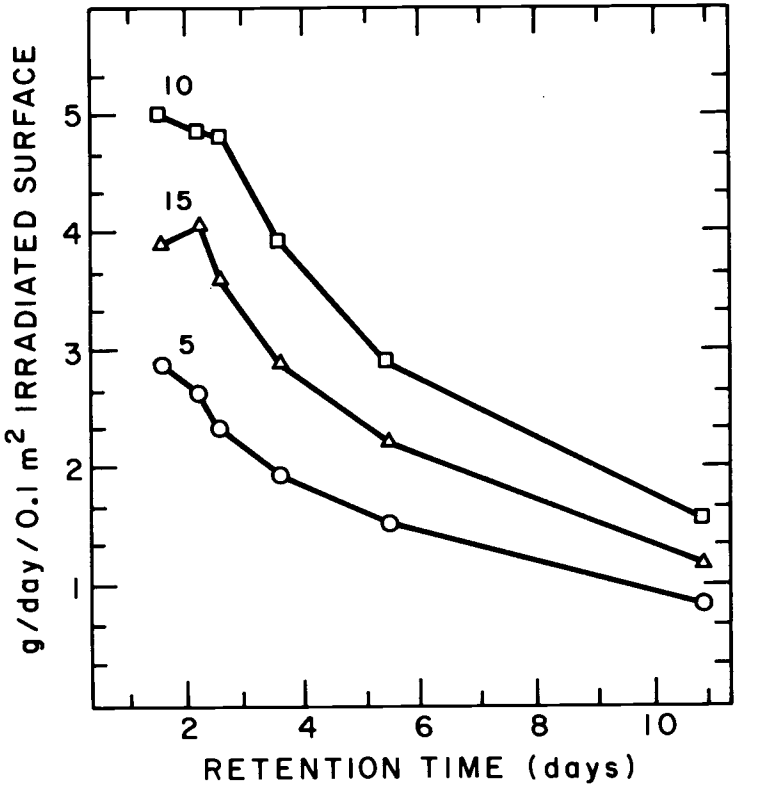

Figure 37. Yield in terms of g/day/0.1 m^2 irradiated surface as a function of retention time at culture depths of 5 cm (-o-o-), 10 cm (-□-□-), and 15 cm (-△-△-).

The lowest densities at all light intensities and temperatures occurred at 15 cm. Increasing the culture depth will decrease the culture density once the depth exceeds the depth to which light can penetrate. Other possible causes of the decreased culture density at the 15 cm depth are changes in the light spectrum with increasing depth, inefficient culture agitation by the bubbling apparatus, and a critically low surface to volume ratio. Water absorbs red and blue light readily, allows further penetration of green light. Since the former wavelengths are essential for photosynthesis, the photosynthetic efficiency decreases as the depth increases. The importance of maintaining an optimal surface to volume ratio was pointed out by Tamiya (1957) who stated that at higher densities, any increase in the number of cells becomes proportional to the area of the illuminated surface. The surface to volume ratios were 0.020, 0.010, and 0.005 m^2/l for the 5, 10, and 15 cm ponds respectively. The data suggests 0.01 m^2/l is the minimum surface to volume ratio allowing maximum growth of Chlorella vulgaris 211/8K in the diluted swine manure.

Light Intensity

At light intensities below the saturation intensity of 500 ft.c. the photo-chemical reaction rate limits the rate of photosynthesis and therefore the growth of Chlorella vulgaris 211/8K. The light intensity

of 300 ft. c. resulted in lower densities for several other reasons. This intensity slowed the light-dependent production of enzymes needed in the carbon reduction cycle. This subsaturating intensity decreased the growth rate by decreasing enzyme activity at those steps in the carbon reduction cycle where ATP or $NADPH_2$ are consumed irreversibly. Limiting light intensities will decrease the growth rate further by limiting the light-sensitive production of cofactors like flavoproteins, ferrodoxines, and pyridine nucleotides. At 300 ft. c. light-dependent cyclic and noncyclic photophosphorylation are limited, which in effect limits the availability of ATP and $NADPH_2$ substrates.

Maximum culture densities were attained at light intensities near 500 ft. c., the reported saturation intensity for Chlorella. Evidently the turbidity of the waste brought the light intensities of 800 and 1200 ft. c. closer to the saturating level. Under these conditions growth and dry weight production become functions of factors like retention time, nutrient availability, and temperature.

At 1800 ft. c. the culture density declined. This may have been the result of the extracellular release of photosynthetic carbon products, which is maximized at high or low intensities. Carbon is shunted away from cellular carbon to excretory products such as glycolic acid. The major product (reportedly over 50 percent of the total ^{14}C) of photosynthesis by algae under conditions of high oxygen concentrations, low carbon dioxide levels, and pH 8.5 or higher, is

glycolate. These conditions were typical of 5 and 10 cm cultures growing under 1800 ft. c. of light.

By aerating the cultures continually, respiratory loss was enhanced through the stimulation of photorespiratory oxygen uptake. It should be noted that higher light intensities may counter any stimulation of respiration by increasing photophosphorylation which reduces the rate of respiration via an ADP-drain from mitochondria.

Because chlorophyll a is the primary pigment involved in Chlorella photosynthesis, the "Chlorella-type" light adaptation should be understood. This adaptation is characterized by an inverse relationship between the chlorophyll a content of the cell and the light intensity to which the cell is exposed. At 300 ft. c. there is an overproduction of chlorophyll a in an attempt to compensate for the deficient energy input. On the other hand, high light intensities lead to chlorophyll destruction. The high oxygen levels attained in the algal cultures stimulated chlorophyllase activity, which normally increases with rising light intensities. In either case, growth is reduced below maximum levels due to energy expenditures for chlorophyll synthesis and degradation.

Temperature

Temperature is not a primary factor influencing culture density. Chlorella vulgaris 211/8K, like many thermophiles, can grow over a

wide range of temperatures. It grew in diluted swine manure over the range of 15 to 40 C. Under the test conditions, temperature would rank behind culture depth, light intensity, and retention time in affecting the culture density. The temperature range of 25 to 40 C may have been too narrow to predict accurately the effect of temperatures outside this range on culture density.

Retention Time

Culture density increases with retention time. Several facts account for the higher densities associated with longer retention times. There is less competition from heterotrophic bacteria since the smaller harvest volumes mean that less organic substrate is introduced into the cultures upon nutrient replenishment. The high oxygen levels and pH associated with longer retention times may also inhibit bacterial growth. Chlorella species are known to produce chlorellin and chlorellin-like substances which are bacteriostatic.

An adjustment period to waste which replenishes the harvested culture volume is required. The longer the retention time, the less the harvest volume, and the less affected the Chlorella culture would be by the added waste. Natural precipitation of cells occurs in spite of the aeration, and occurs to a large extent as the culture density increases. Such precipitation could enhance the photosynthetic efficiency of the culture by allowing greater light penetration.

The problem with long retention times is that the harvest volumes are too small for economical gains. A compromise must be reached, taking into consideration the culture density at the various retention times. Beyond a 4-day retention time the gain in culture density is not worth the decrease in harvesting capacity. Figures 36 and 37 show this relationship for cultures at different depths.

Interactions

Analysis of the data indicates that temperature and light intensity act together to determine the culture density. As the literature review indicated, light intensity is the primary factor influencing the effects of temperature on the growth of Chlorella. The temperature dependency of growth rate decreases and tends to disappear as the light intensity drops. In other words, temperature has little effect on photosynthesis and growth in light-limited systems.

With the achievement of light saturation, temperature does influence growth. As the temperature increases from 25 to 35 C, the light intensity resulting in maximum culture density increased from 800 to 1200 ft. c. This points out that once the light intensity reaches saturating levels, increasing the temperature can increase the light-saturation level of Chlorella vulgaris 211/8K. This effect ceases as the temperature rises above the optimum growth temperature. This claim is supported by the observation that at 40 C, culture

density is relatively independent of the light intensity.

The combination of low temperature and high light intensity prevents rapid growth of Chlorella. Cell division is inhibited and cells are bleached rapidly.

SUMMARY AND CONCLUSIONS

Experiments were designed to determine the effects of depth, light intensity, temperature, and waste retention time on the dry weight density of a culture of Chlorella vulgaris 211/8K. The indigenous bacterial populations were not examined. Minipond basins with a surface area of 0.1 m^2 were used to culture the algae. Agitation and carbon dioxide was supplied by a single air bubbler running along the basin floor. Lighting was provided by fluorescent and incandescent bulbs at the ceiling of the growth chamber. Temperatures were maintained by submersible aquarium heaters and were monitored continuously.

The depths of 5, 10, and 15 cm were compared. Of these, 10 cm was determined to be the optimum culture depth giving maximum yield of algae on a grams/day basis. Growth at culture depths of 15 cm was light-limited. This depth resulted in the lowest densities at all temperatures and light intensities.

Light intensities of 300, 800, 1200, and 1800 ft. c. were tested. This range includes intensities lower than and greater than the reported saturation intensity of 500 ft. c. for Chlorella. At 300 ft. c. the growth of Chlorella vulgaris 211/8K is limited by the photochemical reaction rate. The maximum culture densities were attained with intensities of 800 and 1200 ft. c. The turbidity of the swine waste

probably reduced these levels to near-saturating levels. The culture density decreased when the light intensity was increased further to 1800 ft. c., suggesting photo-inhibition of growth and/or photorespiration occurred at this higher light intensity.

Over the temperature range of 25, 30, 35, and 40 C, culture density was not influenced in a major way by temperature. Like many thermophiles, Chlorella vulgaris 211/8K grew equally well at all of these temperatures because temperature was not the limiting factor. Of the four factors studied, temperature was the least important in determining the culture density.

Culture density increased as the retention time increased from 1.8 to 11.0 days. As the retention time increases the harvest volume decreases. A compromise must be reached between culture density and harvest volume when optimum yield is desired. A 2.0 to 4.0 day retention time results in optimum yield on a grams/day basis. The fact that culture density increased with retention time implies that with time the diluted swine waste becomes a medium more suitable for algal growth. One reason for this may be less competition with bacteria due to a decreased supply of organic nutrients, high pH and oxygen tension, and possible bacteriostatic products from the Chlorella cells. The diluted swine waste was shown to be an excellent medium for supporting prolonged growth of Chlorella.

The four factors also interacted to affect the culture density. Statistical analysis provided the following equation to describe main effects and interactions determining culture density. See Table 22 for definitions of the symbols.

$$Y_{ijk\ell m} = \mu + \lambda_i + \tau_j + \delta_k + \gamma_\ell + (\lambda\tau)_{ij} + (\lambda\delta)_{ik}$$
$$+ \ldots (\tau\delta)_{jk} + (\tau\gamma)_{j\ell} + (\lambda\tau\delta)_{ijk} + E_{ijk\ell} + e_{ijk\ell m}$$

A more practical equation containing only the four factors shows the general effects of each variable on the culture density:

$$Y = 0.6911 - 0.03D + 0.013T + 5.4057(10^{-5})L + 0.0613R.$$

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APPENDICES

APPENDIX A

Effects of Stirring on Measured Culture Densities

When cultures are not stirred prior to sampling for density determinations, the densities will decrease with time. This effect first became apparent when eight cultures of varying temperatures (10, 15, 20, 25, 30, 35, 40, and 45 C) were measured for culture density under four light intensities (300, 800, 1200, and 1800 ft. c.). The cultures were 10 cm deep. Densities of these cultures declined after initial increases. Tamiya (1957) reported a tendency for precipitation within a suspension as the density increased. This effect was evident in this series of experiments.

To confirm these suspicions experiments were performed. Samples were taken before and after stirring from 10 cm deep cultures for density measurements. Temperatures of 30 and 35 C were used in conjunction with light intensities of 300, 800, and 1200 ft. c. Table 38 shows the variations in densities as a function of retention time which varied from 1.8 to 11.0 days.

The average increase in density due to stirring prior to sampling was computed to be 0.56 g/l. The increases ranged from 0.2 to 1.1 g/l, with the larger increases associated with longer retention times. This is expected since higher culture densities were associated with longer retention times.

Both 30 and 35 C temperatures allowed substantial growth of Chlorella to occur at all three intensities. Effects of stirring would be different as one tested temperatures further away from these since temperature-light intensity relationships are more critical at either end of the temperature scale.

Table 38. Variations in millipore densities due to stirring of the culture prior to sampling for density measurements.

Light Intensity	Temperature	Stirring (+ = yes)	Densities of Cultures with Given Retention Times (days)					
			11	5.5	3.7	2.8	2.2	1.8
ft.c	°C		- - - - - - - - - - g/l - - - - - - - - - -					
1200	35	-	1.50	1.00	0.90	0.60	0.26	0.20
		+	2.21	1.96	1.92	1.71	0.70	0.46
	30	-	0.52	0.40	0.36	0.30	0.28	0.23
		+	1.42	0.97	0.83	0.70	0.65	0.50
800	35	-	1.17	1.15	1.01	0.67	0.80	0.56
		+	1.74	1.60	1.42	1.37	1.00	0.96
	30	-	1.43	1.18	1.02	0.97	0.66	0.59
		+	1.98	1.53	1.49	1.29	1.18	0.97
300	35	-	0.96	0.83	0.73	0.67	0.70	0.69
		+	1.96	1.68	1.51	1.23	1.15	0.97
	30	-	1.23	1.25	1.16	1.01	0.77	0.62
		+	2.18	1.80	1.64	1.63	1.18	1.03

APPENDIX B

Changes in Culture Density and pH Associated with an Infinite Retention Time

Low-density minipond cultures at eight temperatures under four light intensities were sampled daily for density determinations. Routine harvesting and stirring prior to sampling were not performed for these experiments. Therefore, the results showed combined effects of natural precipitation and nutrient exhaustion. Table 39 shows the initial rates of increase of the culture densities for the 32 different combinations of temperature and light intensity. Figure 38 illustrates the results.

Table 39. Initial rates of increase in culture density (g/l/day) in ten centimeter cultures having infinite retention times.

Light Intensity (ft. c.)	Culture Temperature (°C)							
	10	15	20	25	30	35	40	45
300	--	0.02	0.03	0.04	0.12	0.14	0.15	0.19
800	--	0.025	0.03	0.32	0.22	0.167	0.175	0.14
1200	0.07	0.07	0.10	0.145	0.25	0.167	0.14	0.06
1800	0.125	0.167	0.17	0.20	0.08	0.09	0.13	0.03

These results suggest that when minipond cultures of Chlorella vulgaris 211/8K are subjected to a range of light intensities of 300 ft.c. to 1800 ft. c. and temperatures ranging from 10 to 45 C there appears to be a gradual shift downward of the temperature at which maximum

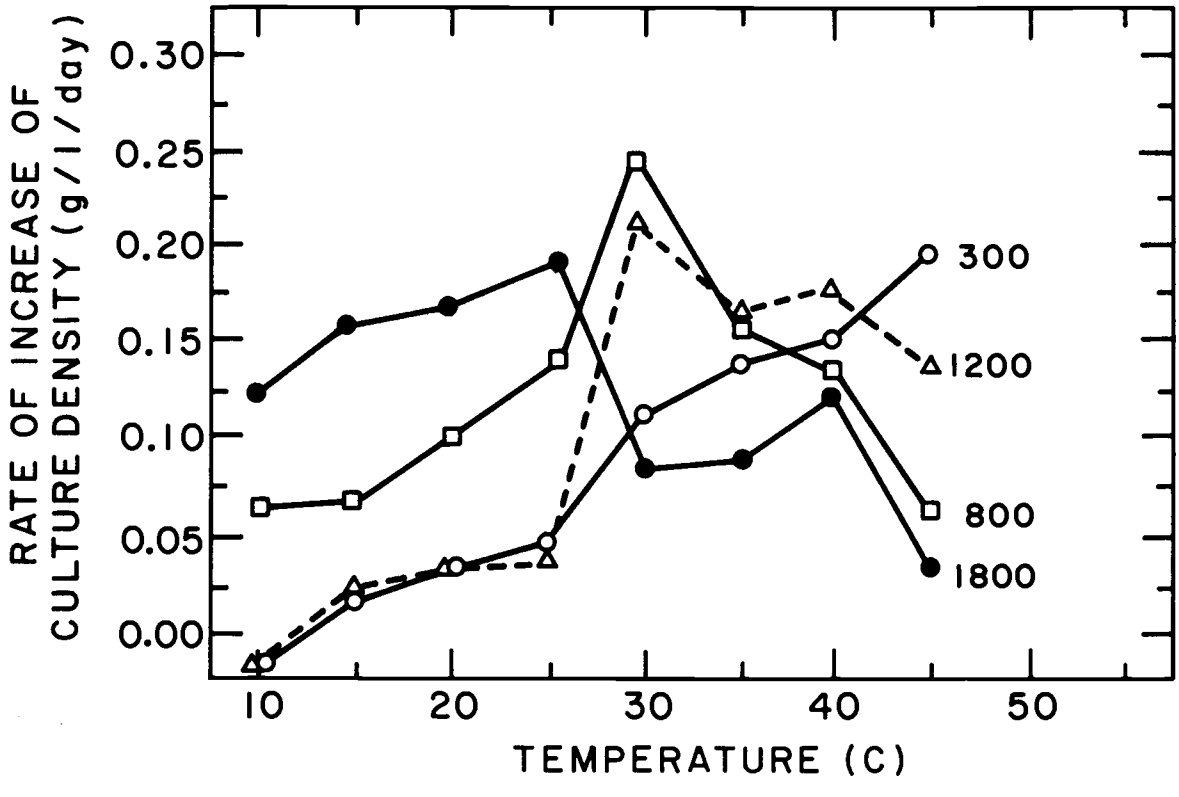

Figure 38. Rate of increase of culture density as a function of temperature at different light intensities. Light intensities are 300 ft. c. (-O-O-), 800 ft. c. (-□-□-), 1200 ft. c. (-Δ--Δ-), and 1800 ft. c. (-●-●-).

growth occurs as the light intensity increases. The higher growth rate occurs at higher light intensity up through 1200 ft. c. While the progression of the curves to lower temperatures as the light intensity increases is a temperature effect, the lower peak at 1800 ft. c. is probably a matter of photoinactivation of the chlorophyll. The addition of two to five percent carbon dioxide might alleviate this effect by reducing the oxygen concentration.

At an intensity (300 ft. c.) below the reported light-saturation intensity (500 ft. c.) for Chlorella, temperature apparently affected growth rates. The density of 10, 15, and 20 C cultures was still increasing after nine days, whereas higher temperatures allowed attainment of maximum densities in one-third the time. With intensities of 800 and 1200 ft. c. high temperatures (35, 40 C) caused maximal densities to be achieved in less time than in cultures of lower temperatures, but precipitation and nutrient exhaustion also increased more rapidly.

In outdoor cultures with higher light intensities (10,000 ft. c. in full sunlight) it may be advantageous and/or necessary to find algae more resistant to photoinactivation than is Chlorella vulgaris 211/8K. As the cell density increases, shading within the pond increases. Such mutual cell shading or artificial shading during peak daylight hours may prove worthwhile to mitigate inhibitory effects of high light intensity.

Without routine harvesting the pH tended to increase with the density. Over the temperature range of 10 to 30 C the pH increased with temperature. As the temperature increased to 45 C, the pH decreased (Table 40).

Table 40. Changes in pH of minipond cultures grown at eight temperatures under four light intensities with an infinite retention time.

Temperature (°C)	Original pH	Maximum pH	Order of Light Intensities (ft. c.) Giving Maximum pH to Minimum pH			
10	7.6	9.5	1200	1800	300	800
15	7.5	9.7	1200	1800	800	300
20	7.5	9.8	1200	1800	800	300
25	7.5	10.0	1200	1800	800	300
30	7.5	10.4	1800	1200	800	300
35	7.6	10.0	1800	800	300	1200
40	7.6	9.5	1800	800	300	1200
45	7.6	8.8	300	800	1800	1200

For maximum growth the pH should be maintained at optimal values for the particular algae. Chlorella vulgaris 211/8K has a reported optimum range of 6.5 to 7.0. To prevent drastic pH increases routine harvesting, with fresh waste (pH 7.5) replacing the harvested volume, should be performed. Alternatively, mixed algal cultures might prove useful in that a second algae, such as a swine-waste adapted Spirulina, could grow when the pH reached higher values optimal for its growth.

APPENDIX C

Effect of Retention Time on Ammonium-Nitrogen Levels in Minipond Cultures

Single bubblers provided continuous agitation to cultures 10 cm deep. After steady state conditions were achieved at a given set of conditions, sample aliquots were centrifuged to obtain supernatants for analyses. Table 41 shows the results of the analyses.

The ammonium-nitrogen content of the culture supernatant decreased with increasing retention time at 30 and 35 C as is shown by Figures 39 and 40. The rates of ammonium-nitrogen recoverability at both light intensities for either temperature decreased equally from short to long retention times.

The influent waste ammonium-nitrogen is soluble to the extent that ordinarily over 96 percent remains in solution following centrifugation at near 38,000 m/sec^2 for 15 minutes. The 35 C temperature was more effective than 30 C in reducing recoverable ammonium-nitrogen values over the entire retention time range. This is expected considering higher temperatures favor evaporation and thermophilic enzyme systems.

Table 41. Effect of retention time on ammonium-nitrogen content of minipond cultures.

Parameter	Light Intensity			
	800 ft. c.		300 ft. c.	
	35°C	30°C	35°C	30°C
	- - - - - - - - mg/l - - - - - - - -			
Influent waste	370.14	352.60	340.72	352.60
Supernatant of centrifuged waste	340.88	341.62	338.61	346.72
Supernatant of centrifuged culture with retention time				
1.8 days	249.4	295.5	201.4	297.4
2.2	245.9	264.1	188.8	274.6
2.8	196.9	273.7	179.6	265.7
3.7	175.9	243.0	136.9	235.0
5.5	154.9	217.3	134.1	204.5
11.0	127.0	180.6	96.2	175.3

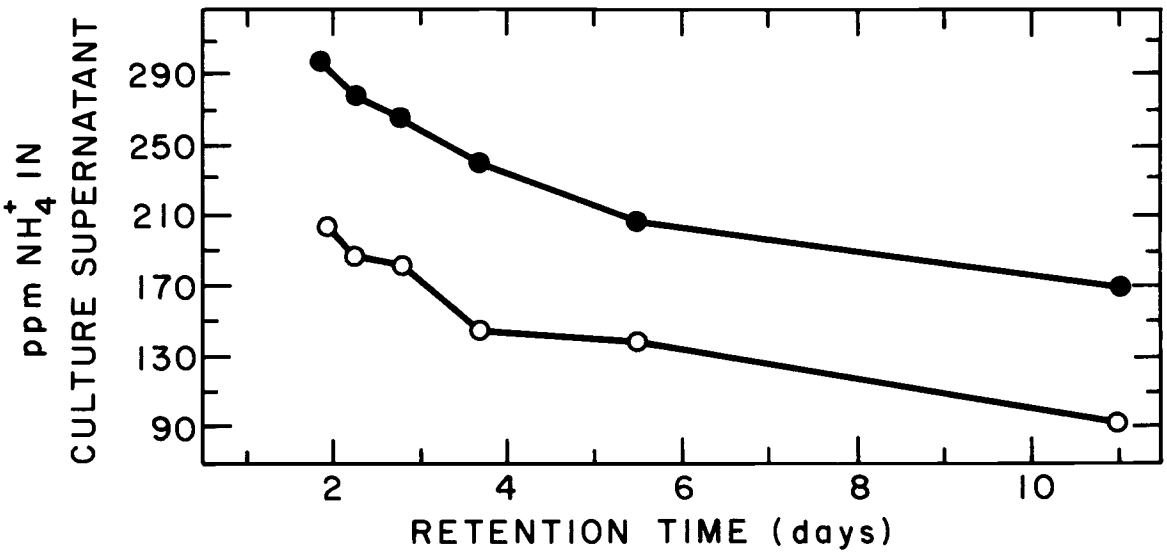

Figure 39. Effect of retention time on NH_4^+ nitrogen remaining in culture solution at 30 C (●—●) and 35 C (○—○). Light intensity was 300 ft. c.

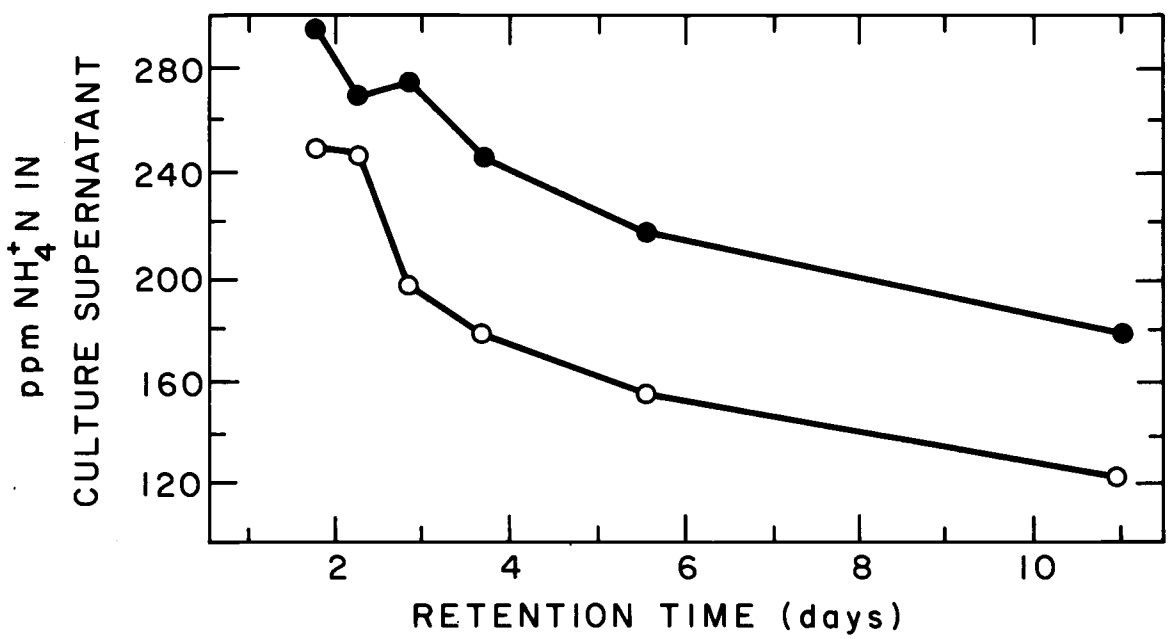

Figure 40. Effect of retention time on NH_4^+ nitrogen remaining in culture solution at 30 C (●—●) and 35 C (○—○). Light intensity was 800 ft. c.

APPENDIX D

Biomass Analysis

Samples from various cultures were centrifuged in a steam-powered Sharple's centrifuge at near 38,000 m/sec^2 to obtain centrifugates for biomass analyses. To determine the crude protein content of the biomass samples, nitrogen percentages were found. The standard conversion factor of 6.25 was used to estimate the crude protein percentage from the nitrogen content. Table 42 shows that the crude protein content remains relatively constant despite changing temperatures and light intensities. The range of 50 to 60 percent crude protein agrees with that range found by Barlow et al. (1975).

Table 42. Nitrogen and crude protein content of culture biomass.

Temperature	Light Intensity	Nitrogen Content	Crude Protein
°C	ft. c.	%	%
25	1200	9.60	60.00
30	1200	8.50	53.13
35	1200	8.04	50.25
40	1200	9.14	57.13
35	300	7.90	49.38
35	800	8.63	53.94
35	1200	7.77	58.46
35	1800	8.75	54.69