THE EFFECTS OF LINEAR ALKYLATE SULFONATE AND LINEAR ALKYL ETHOXYLATE ON THE GROWTH OF GLOEOCAPSA SP. LB 795

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An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Andrew Neal Barrass

August 1976

ABSTRACT

Gloeocapsa sp. LB 795 was used to assay the effects of Linear Alkylate Sulfonate (LAS) and Linear Alkyl Ethoxylate (LAE) surfactants on production. A very limited amount of information is present on the toxicity of surfactants on aquatic microorganisms. Therefore, the toxicity of LAS and LAE was determined using the Environmental Protection Agency (EPA) Algal Assay Procedure bottle test.

Gloeocapsa sp. was exposed to both surfactants for 20 to 40 days at concentrations of 0.1 ppm, 1.0 ppm, 5.0 ppm, 10.0 ppm and 20.0 ppm. Experiments were carried out with bacteria-free and bacteria-associated cultures as well as cultures aerated with 5% CO_2, v/v, air/CO_2.

Five growth parameters were used to monitor production including: final cell count, optical density at day 20 and the maximum optical density, growth rate and dry weight biomass. Data indicate inhibition in growth at all levels of surfactant and a toxic effect of the surfactants prior to or during log phase of growth when 10.0 ppm LAS and 5.0 ppm LAE were added to the media.

In addition to the toxicity bioassay, a carbon assimilation bioassay was conducted to test for heterotrophy. The herbicide 3,4 dichlorophenyl -1, 1 dimethyl urea (DCMU) was used to inhibit CO_2 intake. Bacteria-free cultures were grown with added surfactant and with the addition of 0.5% M glucose. Results implied that neither surfactant, glucose, nor combinations of surfactant and glucose could

be photoassimilated by Gloeocapsa sp. in the presence of DCMU. Both surfactants also inhibited growth when added to the media.

THE EFFECTS OF LINEAR ALKYLATE SULFONATE AND LINEAR ALKYL ETHOXYLATE ON THE GROWTH OF GLOEOCAPSA SP. LB 795

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LIST OF FIGURES

To the Graduate Council:

I am submitting herewith a thesis written by Andrew Neal Barrass entitled "The Effects of Linear Alkylate Sulfonate and Linear Alkyl Ethoxylate on the Growth of Gloeocapsa sp. LB 795." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Diane T. Findley
Major Professor

We have read this thesis and recommend its acceptance:

Diane T. Findley
Minor Professor
or
Second Committee Member

David W. Bath, Jr.
Third Committee Member

Accepted for the Council:

Wayne E. Stamps
Dean of the Graduate School

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INTRODUCTION

Americans use over a million tons of detergents in various forms and concentrations annually. The detergent industries are a major force in the chemical manufacturing enterprise. Most commerical detergents are composed of seven basic ingredients. These ingredients vary with manufacturers, cleaning functions and regional distribution. The detergents include: a fabric whitener or optical brightener, the rinse clean chemical; a perfume; a protection agent, the chemical used to protect machinery parts; an antiredeposition agent, this prevents the redepositing of dirt; a manufacturing additive, to improve powder or liquid qualities; a builder, the water softener and sequestering agent; and a surfactant, the surface-active agent or dirt lifter (Davidsohn and Milwidsky, 1972 and Cahn, 1974).

Excluding the surfactant the other six ingredients are called additives. These additives, specifically the builders, have recently been the focal point of harsh criticism related to their effects on the aquatic environment (Huchinson, 1969).

The most widely used builder is sodium tripolyphosphate (STP). This complex ion is added to the detergent, as are other builders, to reduce the water hardness by coupling with metal ions present in solution. By reducing the metal ions in solution the builders increase several fold the effectiveness of the surfactant that adhere to the dirt particles. Dirt is arbitrarily classified as any foreign substance that is present about the surface of the object being cleaned.

Therefore, builders reduce by chelations metal ions that would interfere with the surfactant.

The environment concern arises due to the effect of builders on two areas related to nutrient availability in the aquatic environment. One area of interest is the increase in phosphate in lakes and streams primarily due to the use of STP as a builder. The second concern is related to the effect that STP has on nutrient chelation. The argument about nutrient chelation as yet is still unresolved. Substitutes for STP such as nitrilotriacetic acid (NTA), sodium carbonate, citric acid and others may cause hazardous conditions that are worse than the problems associated with STP usage. In 1970 the Surgeon General of the United States warned that in tests with mice NTA caused birth defects when added to the drinking water (Davidsohn and Milwidsky, 1972). This and other problems such as nutrient chelation in aquatic environments (Strum and Payne, 1973) has caused the closing of several potential NTA manufacturing facilities (Davidsohn and Milwidsky, 1972).

Sodium carbonate could be used as a replacement for NTA. However, its presence in association with surfactants in solution increases the alkalinity to such extremes that warnings were placed on the packages of commerical detergents which used them (Cahn, 1974). Citric acid is also used as a builder. Payne (1973) found that growth inhibition occurred in Microcystis sp. and Selenastrum sp. due to the chelation of trace metals by citrate. He also concluded that citrate would not effect nutrient concentrations in natural waters or effect normal algal growth. Lange (1974) found that when ferric citrate, ethylenediaminetetraacetic acid (EDTA) and citric acid were

added to media, species of blue-green algae grew better when filtrates of "chelator-forming species" were added to the media. Hall (1974) suggests that metal chelators such as zinc and iron could be used to assay the effects of natural chelators in relation to added chelators by means of the Algal Assay Procedure (AAP), (USEPA, 1971).

The question of chelators either natural or man-made is yet undecided. However, the potential hazard of increased phosphate has been thoroughly explored. In 1969 the proceedings of the International Symposium on Eutrophication were published (National Academy of Science, 1969). This work and the events following led to the suggestion by the United States Government and the Canadian Government that phosphate particularly as STP was one of the major causes of eutrophic processes. Legge and Dingeldein (1970) offer an alternative argument to the proponents that wish to see an end to the phosphate increase. Studies by Francisco and Weiss (1973) also Porcella et al. (1973) demostrated that phosphate increases yielded little significant change in algal production. This data further emphasizes the work of Lange (1967), Kuentzel (1969) and Kerr (unpublished data) referred to by Legge and Dingeldein (1970). The thesis basically conceived by these researchers discusses the intricate relationship between the bacteria, heterotrophic blue-green algae and the photoautotrophic blue-green algae. They also imply that in order for large populations of algae to occur, concurrent amounts of carbon dioxide must also occur and be available for algal metabolism. Conflicting views of the naturally occurring amounts of carbon in lakes exist (King, 1970). Bacteria as well as the heterotrophic blue-green algae, by means of

carbohydrate degradation, increase dissolved carbon dioxide which is readily used by the photoautotrophic species. Oxygen from algal metabolism is then used by the bacteria completing the mutualistic relationship. The major focus on both sides of the rebuttal seems to be the economics of controlling any substance that may cause a eutrophic condition to occur in lakes or streams (Middlebrook et al., 1974; Porcella and Bishop, 1975 and Kirov, 1975). Vallentyne (1970) suggests that among all the growth controlling nutrients phosphorus is the only one that could feasibly be controlled at all levels of human usage. Porcella and Bishop (1975) used computer programming to theoretically model phosphorus removal and evaluate the cost of this removal.

However, the importance of carbon as a limiting factor cannot be ruled out as an potential source of algal blooms and lake eutrophication. King (1970) gives a detailed review of the theories of carbon limitation that emphasize the relationship between algal blooms and dissolved carbon. McDonald and Clesceri (1973) demostrated that organic material and domestic sewage enhanced algal growth.

Lange (1970 and 1971) has demostrated that added carbon, in the forms of organic substrates, will increase algal production in bacteria-algal systems. Although Schindler (1971) offers a rebuttal to the work of Lange (1970) and King (1970), his own data showed increased production in bottle tests of algae with added carbon sources.

Droop (1974) discusses the range and chemical peculiarities of heterotrophy in many species of algae. The utilization of various forms of carbon and organic substrates offers a unique problem not researched prior to this date. Could carbon present in the form of a

biodegradable surfactant increase algal production either in bacteria-algal systems or by a mode of heterotrophy?

The surfactant is commonly called the detergent block. The surfactant consists of a head and tail chemical chain. This chemical chain may be anionic or cationic depending upon the specific chemical nature. Surfactants with either of these ionic chains are called ionic detergents and their specific mode of action is to electrostatically couple with dirt particles.

Ionic detergents consists of a long chain alkyl group either branched or unbranched. The branched variety seem to be less biodegradable or "hard". This long chains alkyl group is very insoluable; this is called hydrophobic. In ionic surfactants, specifically anionic surfactants, the head consists of a benzene ring with a sodium sulphate group attached in the para position (Figure 1). The benzene ring and sodium sulphate act as the water solvent region or the hydrophilic group and allows the detergent to dissolve in water. In the nonionic surfactants the benzene ring is replaced by a long chain of ethylene oxide groups. The oxygen tends to enhance water soluability and the alkylate group ($-CH_2-CH_2-$) tends to be somewhat insoluable. The net effect is towards soluability of the entire molecule; however, many more ethoxylate groups are needed to overcome the hydrophobic alkylate tail.

Another type of surfactant that is relatively new to the consumer market is a nonionic chemical chain. This chemical chain is still composed of a head and tail region as well as a hydrophobic (water repelling) and hydrophilic (water attracting) region as in the ionic detergents (Davidsohn and Milwidsky, 1972).

Several workers have explored the biodegradability of Linear Alkylate Sulfonate (LAS) and Alkyl Benzene Sulfonate (ABS) type surfactants. Renn et al. (1964) and Swisher (1963) used a Pseudomonas sp. and a shake-flask technique similar to the more recent AAP (USEPA, 1971) to test the biodegradability of straight chain LAS and branched-chain ABS detergent.

Brenner et al. (1965) outlined procedures for determining the biodegradability of surfactants using both a shake-flask and continous culture bioassay. Malaney and Gerhold (1969) found that chemical structures effect the relative rates of biodegradation and these chemical determinates were related to various enzymes present.

Due to less foaming quality and the greater biodegradability of LAS, ABS-type detergents are no longer manufactured in the United States (Davidsohn and Milwidsky, 1972). Surfactants are now being used extensively for other uses besides that of detergent blocks such as the cleaning up of oil spills (Smith, 1970). Emphasis is now being placed on studies of the role of detergent blocks in biodegradation and nutrient procurement (Bunch, personal communication).

The utilization of various carbon forms by bacteria, and heterotrophic and autotrophic blue-green algae creates a unique niche within the process of biodegradation of carbon compounds each of which may only be divisible by means of specific nutritional categories (Stanier, 1973).

The incorporation of organic substrates by blue-green algae and some thiobacilli has been demostrated to be a light dependent process (Hoare and Moore, 1965; Carr and Pearce, 1966; Smith et al., 1967; Stanier et al., 1971; Pelroy et al., 1972 and Rippka, 1972). Stanier

et al. (1971) isolated and taxonomically grouped many coccoid blue-green strains according to the presence or absence of various deoxyribose nucleic acid (DNA) bases and the DNA base composition. Rippka (1972) using Aphanocapsa sp. strains isolated by Stanier et al. (1971) screened 38 species of the Chroococcales for their ability to grow in the presence of glucose and 3-(3,4 dichlorophenyl)-, 1, 1 dimethyl urea (DCMU) which inhibits electron flow preventing carbon dioxide uptake. Pelroy et al. (1972) outlined the biochemical pathway of photoassimilated glucose in Aphanocapsa sp. 6714 which Rippka (1972) had categorized as a chemotrophic strain due to its ability to grow in the presence or absence of light. He found that Aphanocapsa sp. 6714 could utilize glucose as a source of energy. Stanier (1973) discusses the biochemical pathway traced by Pelroy et al. (1972) and infers that other species as yet not tested may have similar pathways of carbon utilization.

Extracellular products have been found to occur in many algal species. These extracellular products resulted from the utilization of reduced carbon or in response to varied carbon forms in the media (Hellebust, 1974). The production of gluculate products has been reported in response to carbon compounds by Wang and Tischer (1973). In addition Watt and Fogg (1966) found that as a results of CO_2 limitation that Chlorella pyrenoidosa excreted glycolic acid as an extracellular product.

Surfactants such as LAS or Linear Alkyl Ethoxylate (LAE) could possibly be used to replace CO_2 by photoheterotrophic or chemotrophic strains of blue-green algae. Although, as Stanier (1973) points out, the known carbon sources of the species studied is fairly limited.

However, if the surfactant were primarily degraded by a bacteria then the intermediate fragment could possibly be utilized chemotrophically or photoheterotrophically. Hall (personal communication) points out that the research on the utilization of the intermediates and surfactants by bacteria alone is still in an infant stage of development. He also indicates that studies on the toxicity of surfactants on aquatic organisms have only recently begun to be investigated.

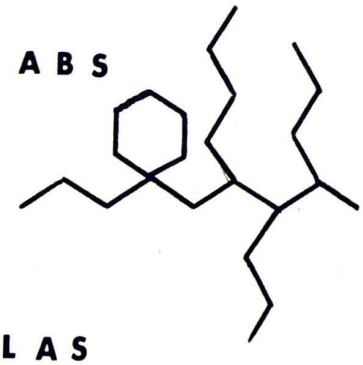
MOLECULAR STRUCTURES

GENERAL FORMULAE

Head Region

Tail Region

A B S

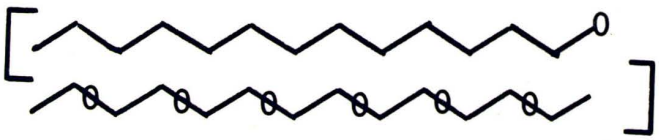


$CH_3-CH_2-CH(C_6H_5NaSO_3)-CH_2-CH(CH_2-CH_2-CH_2-CH_3)-CH-(CH_2-CH_2-CH_2-CH_3)-CH-(CH_2-CH_3)-CH_3$

L A S

$CH_3-(CH_2)_2(C_6H_5NaSO_3)-(CH_2)_6-CH_3$

L A E



$CH_3-(CH_2)_{13}-O(CH_2CH_2O)_7-H$

Figure 1. General Formulae and Molecular Structures of ABS, LAS and LAE Surfactants

METHODS AND MATERIALS

Liquid cultures of Gloeocapsa sp. LB 795 were obtained from the Indiana University Culture Collection (Starr, 1964). Gloeocapsa sp., a known obligate phototroph (Rippka, 1972), as well as a nitrogen fixing organism (Gallon et al., 1975), was used as a bioassay organism for testing the toxicity of both surfactants. Bacteria-free cultures of LB 795 were prepared using the methods of Stanier et al. (1971). Gloeocapsa sp. LB 795 has been described by Rippka et al. (1971) as Gloeocapsa sp. 6501 and later work indicates these are independent isolates of the same species (Stewart, 1973). Gloeocapsa sp. LB 795 was also used to assay the surfactants LAS and LAE as a possible source of carbon in photoassimilation.

Culture Conditions

Stock Cultures. The algae were maintained on agar slants, 5% agar and EPA media, (USEPA, 1971), (Tables I and II) in a Percival model I137 day/night incubator. Stock cultures were grown in 500 ml of liquid EPA media in 1,000 ml pyrex Erlenmeyer flasks at 25 C, and illuminated from the side with cool white fluroscent light (General Electric) at 4304-4842 lux measured by a General Electric light meter, type 213 (Allen, 1973 and Holm-Hansen, 1968). The stock cultures were bubbled with water saturated air at 400 ml/min. through Pasteur pipets inserted through the foam plugs. This induced mixing and prevented self-shading (Stanier et al., 1971; Starr, 1973 and USEPA, 1971). Stock cultures

TABLE I

EPA MEDIA MACRONUTRIENTS

Compound	Concentration*	Element	Concentration*
$NaNO_3$	25.500	N	4.200
K_2HPO_4	1.044	K	0.469
$MgCl_2$	5.700	P	0.186
$MgSO_4 \cdot 7H_2O$	14.700	Mg	2.904
$CaCl_2 \cdot 2H_2O$	4.410	S	1.911
$NaHCO_3$	15.000	C	2.143
		Ca	1.202
		Na	11.001

* Concentration expressed as milligrams per liter of media (USEPA, 1971).

TABLE II

EPA MEDIA MICRONUTRIENTS

Compound	Concentration*	Element	Concentration*
H_3BO_3	185.520	B	32.460
$MnCl_2$	264.264	Mn	115.374
$ZnCl_2$	32.709	Zn	15.691
$CoCl_2$	0.780	Co	0.354
$CuCl_2$	0.009	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3$	96.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000		

* Concentration expressed as micrograms per liter of media (USEPA, 1971).

were used to inoculate experimental batch cultures with viable cells acclimated to growth in liquid media at 3.5×10^6 cells/ml.

Glassware for all culture conditions was washed in mild detergent by hand (Simpson, 1966), rinsed in tap water, then soaked in a 20% HCL/H_2O solution. After soaking in HCL the glassware was rinsed in tap water 6 times and rinsed in glass distilled water 6 times. The glassware used for all experiments was then sealed with aluminum foil, steam autoclaved (15 psi and 121 C) and dried at 102 C for 24 hours (USEPA, 1971 and Hamilton, 1973).

Toxicity Bioassay Cultures. Bacteria-associated cultures were grown in 60 ml of sterile liquid, autoclaved, EPA media plus 0.1 ppm, 1.0 ppm, 5.0 ppm, 10.0 ppm and 20.0 ppm of bacteria static technical grade LAS and LAE surfactants (Hall, personal communication) which was added after sterilization of the media.

Three replicate flasks of each concentration of surfactant and controls were grown 20-40 days. During the growth period flasks were placed on a Eberbach oscillating shaker, set as approximately 100 oscillations/min., and incubated at 25 C with overhead illumination by cool-white fluroscent light at 3766-4304 lux.

Groups were aerated by using Pasteur pipets inserted through the foam plug sealed flasks; however, the tip of the pipet was suspended above the media to prevent bubbling (Stanier et al., 1971). Aeration was with 5% CO_2, air/CO_2, which was saturated with water. Other flasks were aggitated by adding 30 to 40, 4 mm glass beads which also increased mixing and assisted in breaking up of cell clumps. Liquid to volume ratios were 60 ml/250 ml Erlenmeyer flasks and

100 ml/500 ml Fernbach flasks (USEPA, 1971).

Bacteria-free cultures were grown in 500 ml Fernbach flasks with 100 ml of Millipore (0.45 micron filter) filtered EPA media plus surfactants. Cultures of the control, and 1.0 ppm and 5.0 ppm of both surfactants, were grown with and without CO_2 aeration.

Cultures were not considered bacteria-free unless nutrient broth inoculated with culture solution at the end of the growth period were negative after 48 hours of warm incubation in the light and dark. Other tests for bacteria were performed according to Hoshaw and Rosowski (1973).

Carbon Assimilation Bioassay. The carbon bioassay was conducted to test for carbon utilization in the form of a biodegradable surfactant on algal production. Bacteria-free cultures were grown as in the toxicity bioassay.

Surfactant concentrations, however, were equated to 2.0 mg/l of additional carbon (King, 1970 and Lange, 1970) or 4.90 ppm LAS and 3.05 mg/l LAE, then membrane filtered and added to the media. Glucose 0.5% v/v solution (Pelroy et al., 1972) was also membrane filtered and added to the media, media plus surfactant and media plus 10^{-5} M (3,4 dichlorophenyl)-1, 1 dimethyl urea (DCMU). Eight sets of 3 flasks each were cultured to test for the effects of added carbon.

Growth Measurements

The optical density of each of the bacteria-associated cultures was measured at 650, 550 and 443 nm on a daily basis. Photometric determinations were made using a Gilford 240 spectophotometer.

Bacteria-free cultures were measured using a Bausch and Lomb Spectronic 20. Two instruments were used for determining the optical density at the three wavelengths due to the volume of cultures (Guillard, 1973). A dilution curve of a dense culture was used to estimate population increases (Guillard, 1973). The maximum optical density at each wavelength was equated to maximum standing crop and compared with cell count data (Sorokin, 1973).

Semilog plots of the increase in optical density versus time were used to identify the log phase of growth and thus determine exponential growth rates during the growth period. Growth rates of cell counts were also used to equate doubling time. Optical density growth rates and cell count growth rates were compared using a Fortran computer program. Statistical analysis of data was performed using Duncan's multiple range at the 95% confidence level.

A dry weight biomass was performed on the 20th day of culture and 10 days after the maximum optical density was reached. Duplicate aliquots of 3 ml - 5 ml of cell suspension were placed on preweighed GF/A Whatman glass fibre filters. These were then washed with distilled water to remove salts and exogenous material, then filtered under vacuum for 15 min. The filter was then oven dried at 102 C in an aluminum drying pan. After the filters reached room temperature they were weighed to the closest 0.1 mg; data were converted to mg/l of dry weight of algae.

Initial and final cell counts were made using a Sedwick-Rafter phytoplankton counting chamber. A 10 ml aliquot of cell solution was diluted to 100 ml with distilled water. The 100 ml solution was then

sonicated for about 5 min. to further break up the clumps of cells (USEPA, 1971 and Guillard, 1973). From this solution 1 ml was placed into the counting chamber and the cells were counted according to McAlice (1971). Table III lists the growth parameters used to assay algal production.

Morphological variation was monitored by photographing cells at various stages of growth and during different time periods. Color plates were analyzed for various forms of deviation in growth from the control by methods discussed by Green (1973).

TABLE III

GROWTH PARAMETERS

I. Dry Weight Biomass at the 20th day of culture and 10 days after maximum optical density.

II. Initial and Final Cell Counts.

III. Growth Rate analysis of logarithmic growth period.

IV. Maximum optical density (OD_{max}) obtained during growth period at three wavelengths.

V. Optical density obtained during stationary phase of growth on the 20th day of culture (OD_{20}).

RESULTS

Toxicity Bioassay

Growth Studies with LAS. LAS at 5.0 ppm reduced the dry weight slightly. LAS at 10.0 ppm however significantly reduced the dry weight of Gloeocapsa sp. by 36.55% of the control. When 20.0 ppm of LAS was added to the media the dry weight was reduced by 58.60%. Since the dry weight procedure was performed during the 20th day of culture, could a longer growing period allow cultures subjected to surfactant to obtain a biomass comparable to the control? A second group of cultures were allowed to grow until peak population density occurred as determined photometrically. By taking dry weight measurements at the peak, any lag time could be accounted for and overcome if only simple inhibition had occurred. However, the percentage of inhibition was proportional to the dry weight at the 20th day of culture. Table IV illustrates the dry weight biomass of LAS and LAE for two growth periods and Figure 2 shows the toxicity of surfactant on dry weight biomass.

Final cell counts of cultures plus LAS were lower at concentrations of surfactant at or greater than 5.0 ppm; however, 10.0 ppm was needed to significantly reduce the final cell count. The final cell count of the control was 7.76×10^6 cells/ml while the cell count was reduced to 6.85×10^6 cells/ml when 10.0 ppm of LAS was added to the media (Table V). Figure 3 and 4 demonstrate the toxic effect of the

TABLE IV

DRY WEIGHT BIOMASS

Surfactant Concentration	Dry Weight Biomass mg/l	Percentage Reduction*
0.1 ppm LAS	1486.4	1.77
LAE	1320.0	12.76
1.0 ppm LAS	1440.0	4.83
LAE	1273.3	15.85
5.0 ppm LAS	1220.0	19.37
LAE	1180.0	22.01
10.0 ppm LAS	960.0	36.55
LAE	993.3	34.35
20.0 ppm LAS	626.4	58.60
LAE	660.0	56.38

* Percentage reduction based on the final dry weight biomass of the control (1513.2 mg/l).

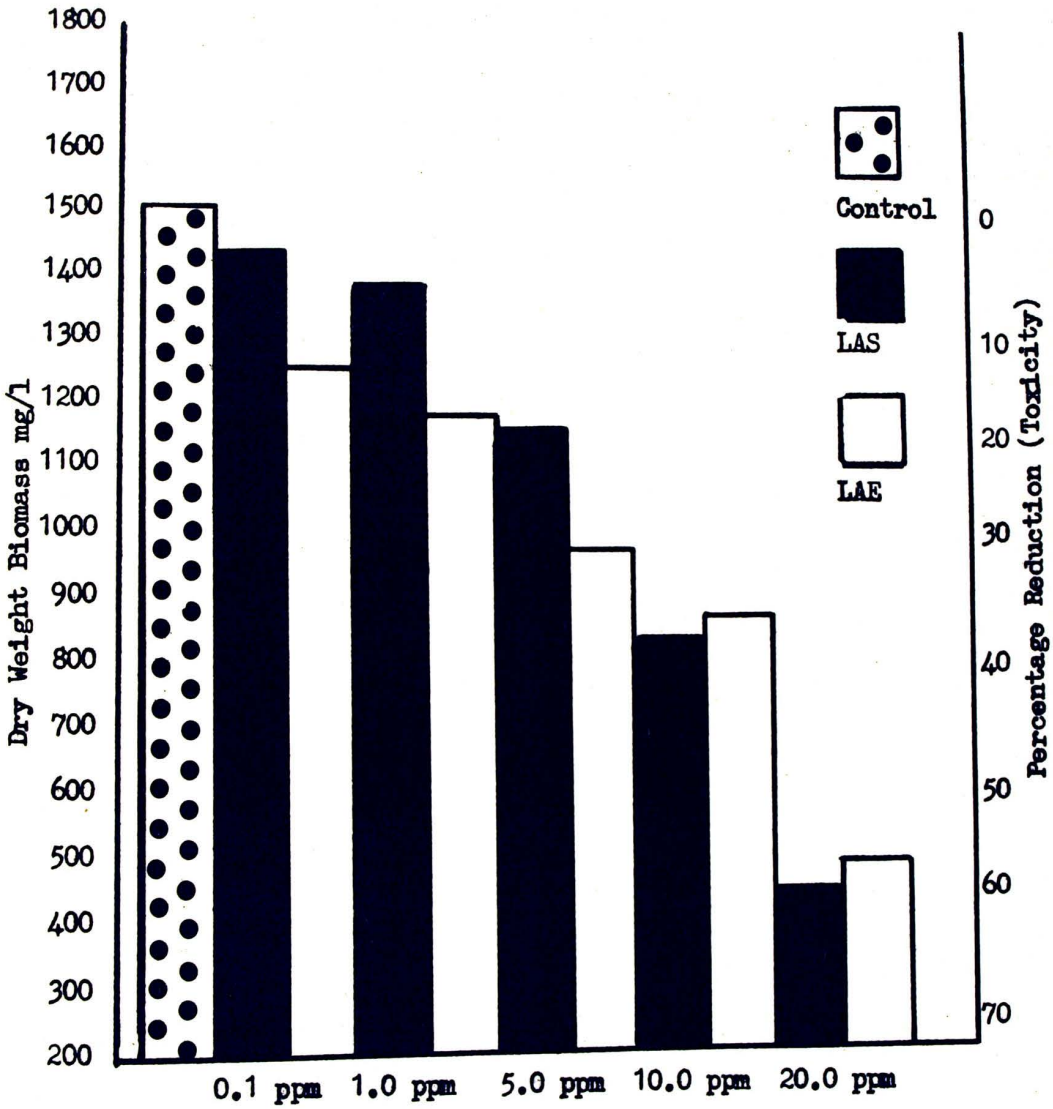


Figure 2. The Percent Reduction in Biomass (Dry Weight) When LAS and LAE were Added to the Media

TABLE V

FINAL CELL COUNTS

Surfactant Concentration	Cell Counts*	Percentage Reduction**
0.1 ppm LAS	8.45**	8.89
LAE	7.33	5.54
1.0 ppm LAS	7.67	1.15
LAE	7.53	2.96
5.0 ppm LAS	7.26	6.44
LAE	6.49	16.36
10.0 ppm LAS	6.85	11.72
LAE	6.12	21.13
20.0 ppm LAS	5.03	35.18
LAE	5.30	31.70

* Cell counts expressed as 10^6 cells/ml.

** Percentage reduction based on the final cell count of the control (7.76 x 10^6 cells/ml).

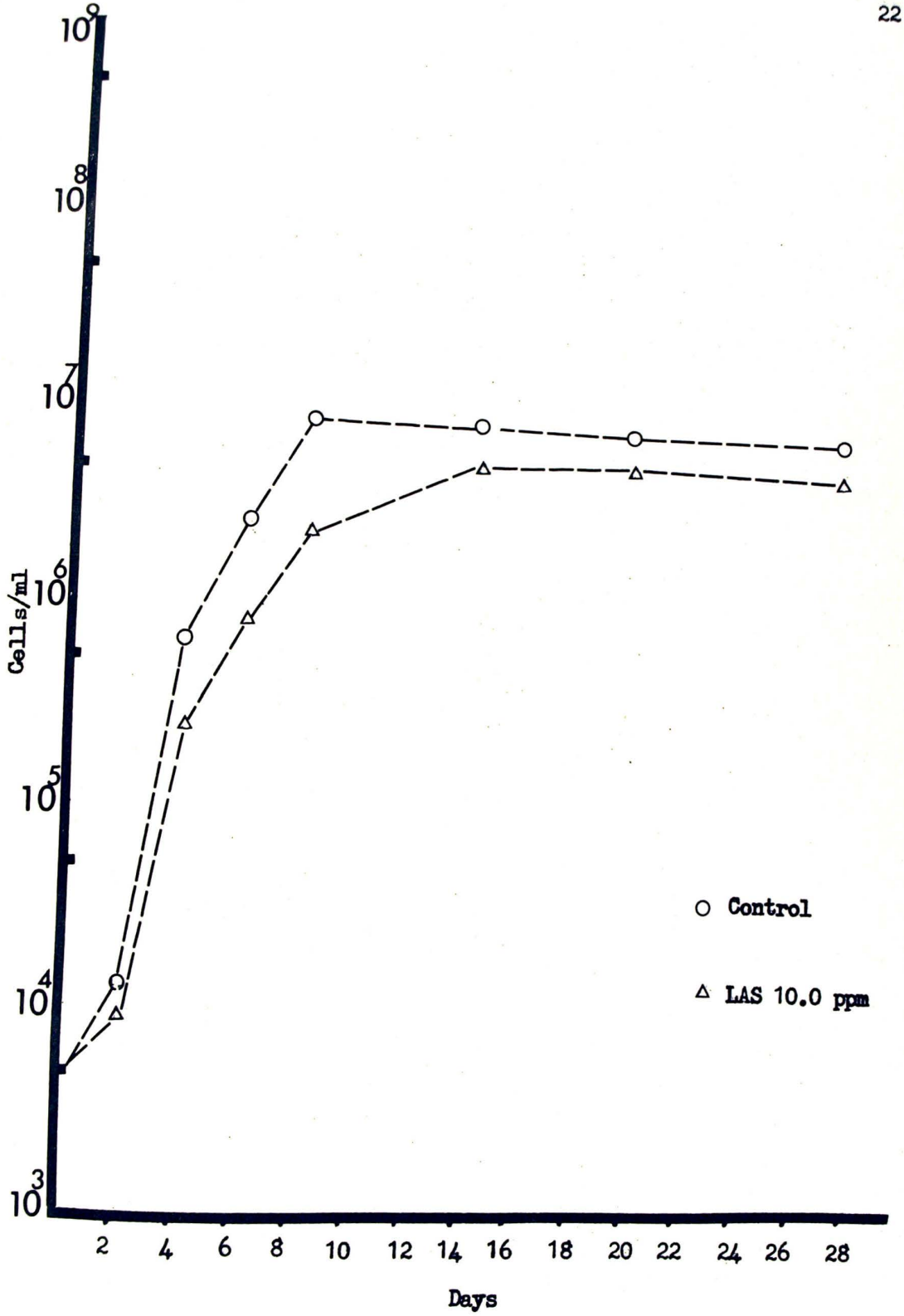


Figure 3. The Toxicity of 10.0 ppm LAS on Gloeocapsa sp. LB 795 Cell Yields

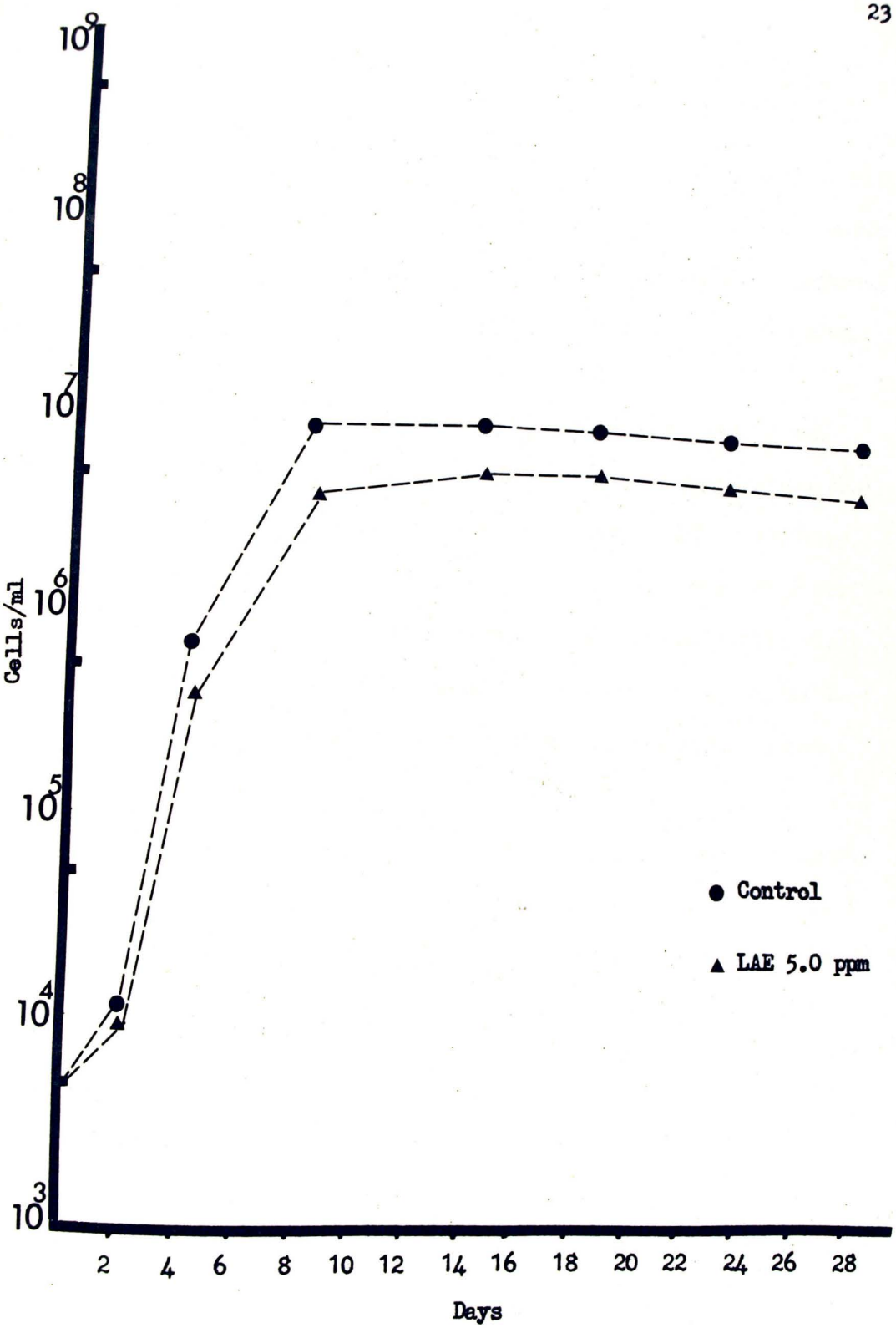


Figure 4. The Toxicity of 5.0 ppm LAE on Gloeocapsa sp. LB 795 Cell Yields

surfactants on cell yield when comparing the control, and 1.0 ppm and 5.0 ppm of LAS or LAE.

Growth rates were determined by the increase in optical density from the third to the seventh day of culture (Figure 5 and 6). Growth rates were similar at 0.1 ppm, 1.0 ppm, and 5.0 ppm LAS when compared to the control (Table VI). LAS at 10.0 ppm reduced the growth rate significantly and inhibited the rate by 29.53%.

The maximum optical density (OD_{max}), a measurement of the maximum standing crop biomass, was obtained during approximately the 29th day of culture. LAS inhibited the OD_{max} by 15.83% at 10.0 ppm. The OD_{max} resembled the other parameters in that 10.0 ppm LAS significantly altered the biomass when compared to the control (Table VII). The same results occurred at all wavelengths recorded and Figure 7 indicates the relative inhibition in optical density at three wavelengths.

Since all of the cultures obtained stationary phase on approximately the 20th day of culture the optical density at day 20 (OD_{20}) of each was compared to the OD_{max}. The results indicated that the percent reduction in biomass at the 20th day of culture remained proportional to the OD_{max} and differences among groups were also unchanged (Figure 8).

Growth Studies with LAE. When LAE was added to the media a greater inhibition occurred at lower concentrations. LAE reduced dry weight significantly at 5.0 ppm. Dry weight was inhibited to 25.58% of the control at 5.0 ppm. Larger concentrations also significantly lowered the dry weight and 20.0 ppm reduced the dry weight by 56.38% (Table IV).

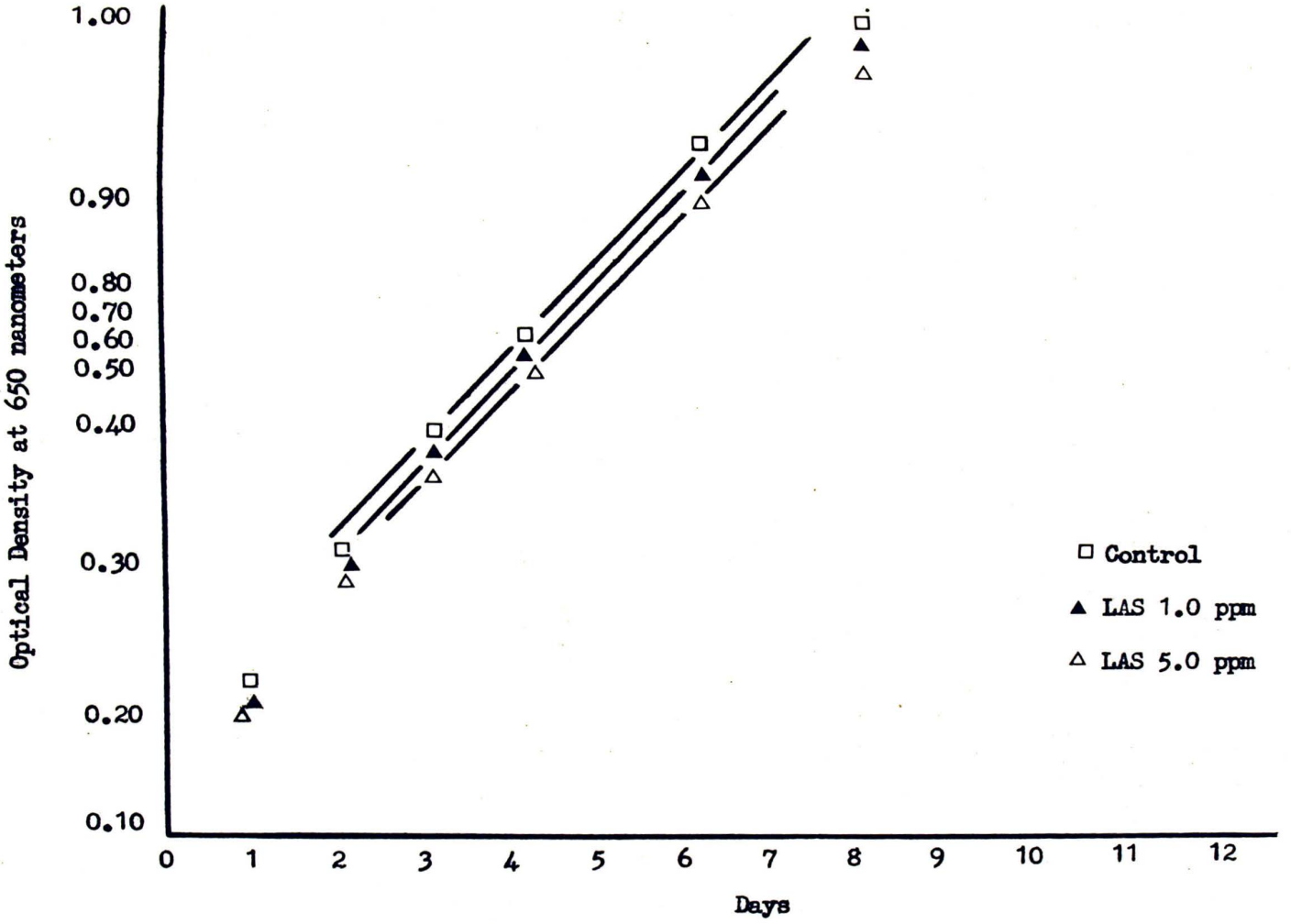


Figure 5. Logarithmic Growth of Control, 1.0 ppm LAS and 5.0 ppm LAS

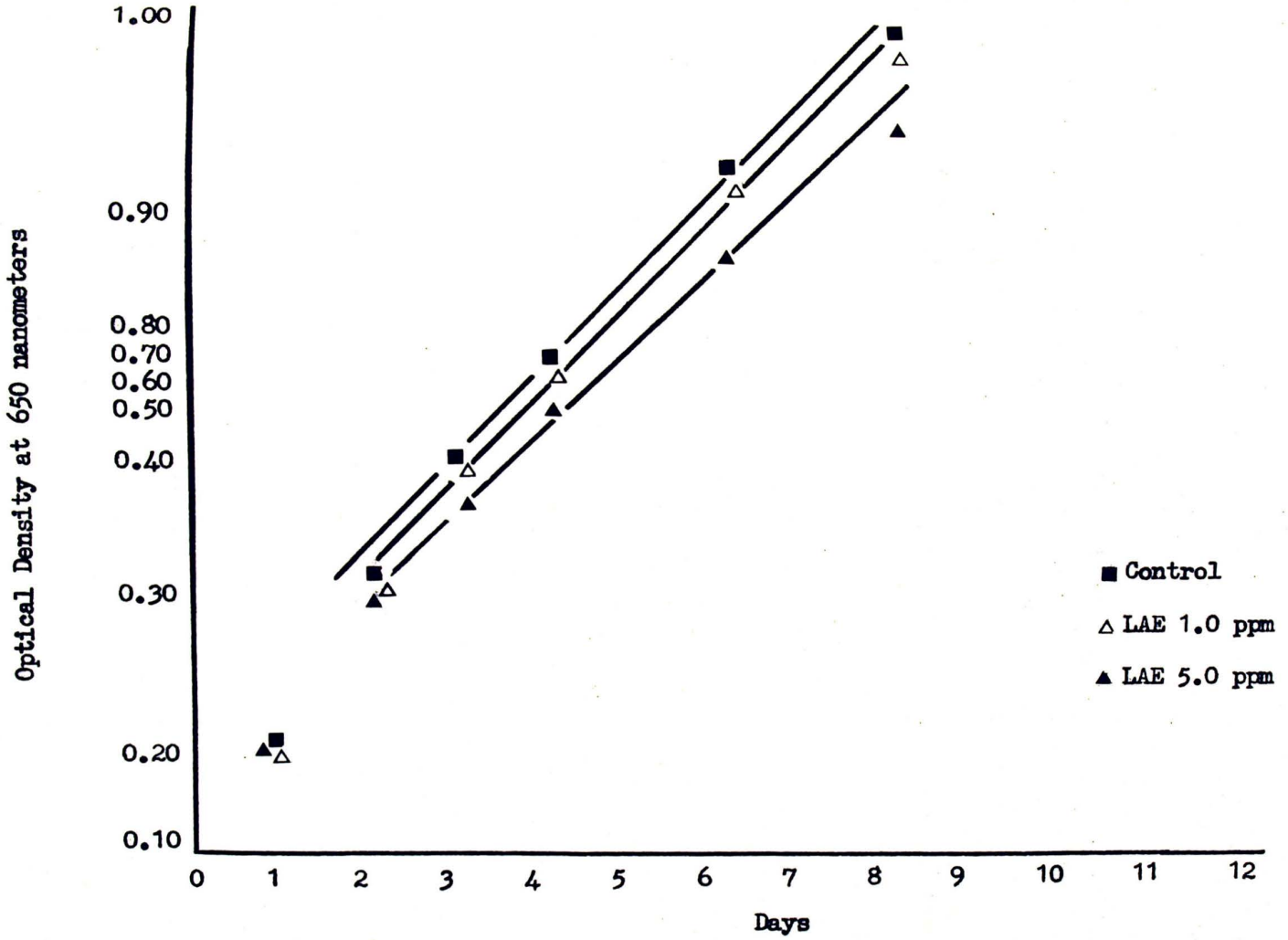


Figure 6. Logarithmic Growth of Control, 1.0 ppm LAE and 5.0 ppm LAE

TABLE VI

GROWTH RATES

Surfactant Concentration	Rate $\log_e$ units*	Percentage Reduction**
0.1 ppm LAS	0.0138	7.04
LAE	0.0134	10.06
1.0 ppm LAS	0.0123	17.44
LAE	0.0115	22.81
5.0 ppm LAS	0.0115	22.81
LAE	0.0107	28.18
10.0 ppm LAS	0.0105	29.53
LAE	0.0088	40.93
20.0 ppm LAS	0.0045	69.79
LAE	0.0071	52.34

* Rate of logarithmic growth from day 3 to day 7, culture period from 29 to 35 days.

** Percentage reduction based on growth rate of the control (0.0149 $\log_e$ units/day).

TABLE VII

OD_{max} FOR TOXICITY BIOASSAY

Surfactant Concentration	OD_{max}	Percentage Reduction*
0.1 ppm LAS	1.497**	14.08
LAE	1.548	11.13
1.0 ppm LAS	1.499	13.94
LAE	1.445	17.04
5.0 ppm LAS	1.446	17.00
LAE	1.379	20.83
10.0 ppm LAS	1.275	26.78
LAE	1.274	26.84
20.0 ppm LAS	0.806	53.74
LAE	1.100	36.84

* Percentage reduction based on the maximum optical density obtained by the control during a 29 to 35 day growth period (1.742 at 443 nanometers).

** Optical density measured at 443 nanometers.

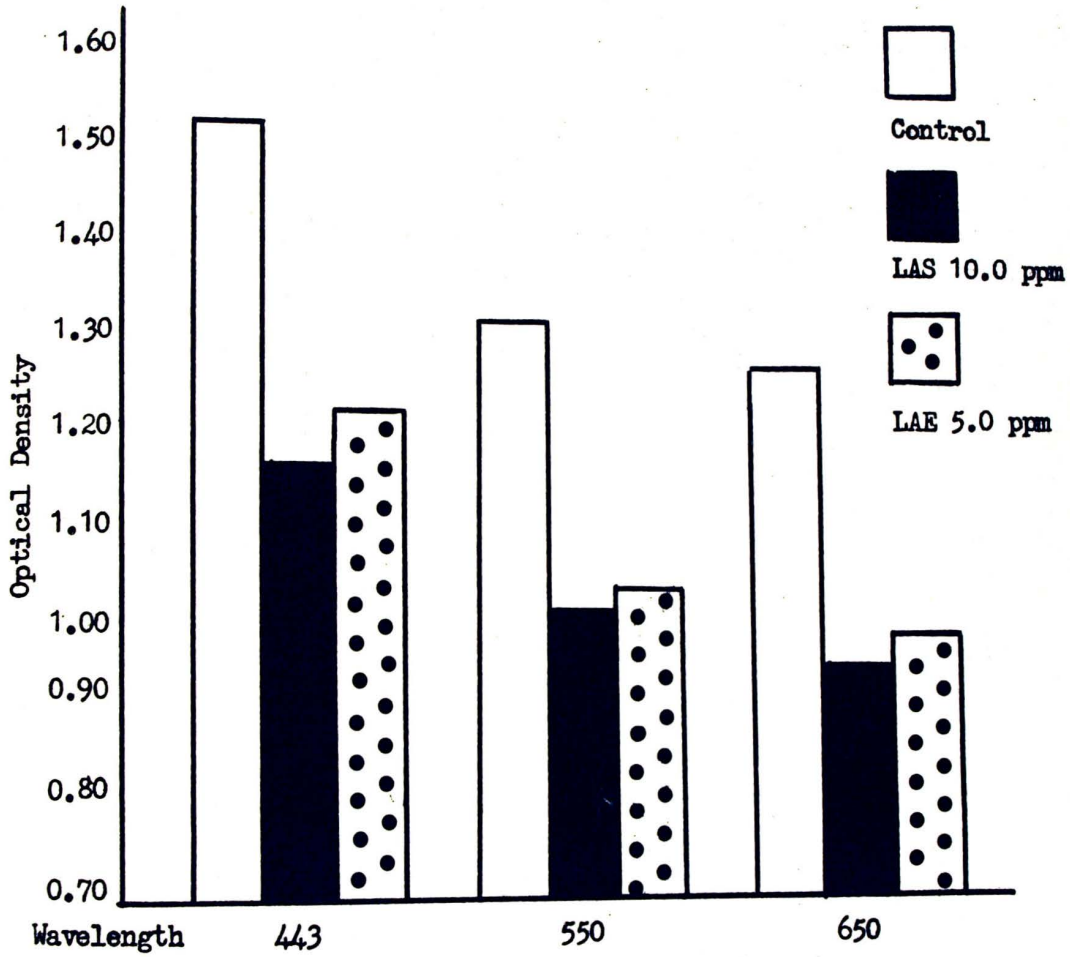


Figure 7. Comparison of Biomass Yields at Three Wavelengths for Control, LAS 10.0 ppm and LAE 5.0 ppm

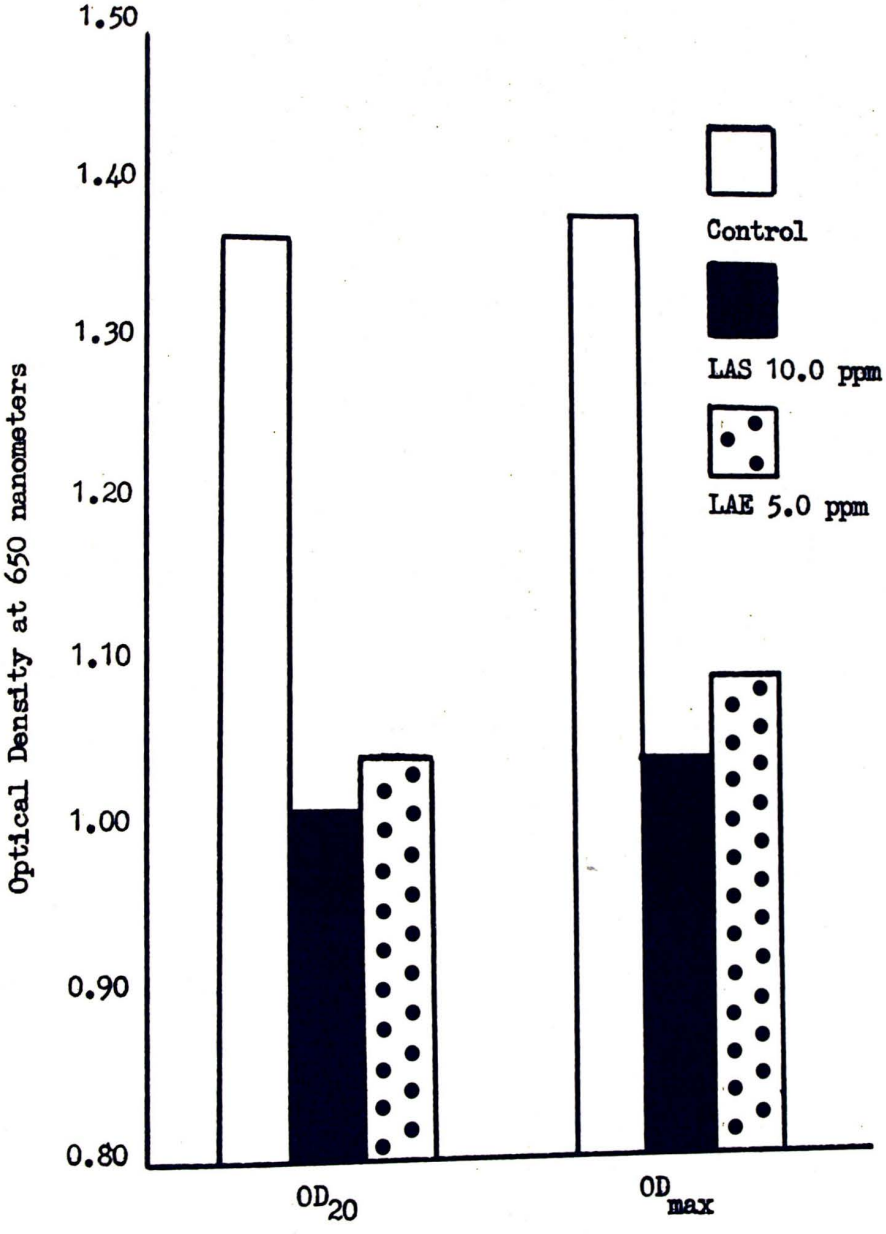


Figure 8. The Inhibition of Optical Density (650 nanometers) at Both OD_{20} and OD_{max} for Control, LAS 10.0 ppm and LAE 5.0 ppm

LAE reduced cell numbers significantly at 10.0 ppm and greater. Although 5.0 ppm of LAE reduced cell numbers no significant difference was detected among groups when compared to the control (Table V, page 21). Cell numbers were lowered from 7.76×10^6 of the control to 6.49×10^6 at 5.0 ppm and 5.30×10^6 at 20.0 ppm.

LAE 1.0 ppm suppressed the growth rate by 22.81%; however, there was no significant difference between the control, 0.1 ppm and 1.0 ppm. However, 5.0 ppm of LAE significantly lowered the growth rate and 20.0 ppm reduced the rate by 52.34. Table VI, page 27, indicates the inhibition of logarithmic growth at various concentrations of the surfactants.

The OD_{max} and OD_{20} of LAE showed a significant reduction when 5.0 ppm of surfactant was added. Media with 5.0 ppm lowered the OD_{max} by 15.83%. LAE at 10.0 ppm reduced the OD_{max} by 22.64% of the control. There was a 50.49% decline in production at 20.0 ppm. The similarity in the inhibition of production caused by the surfactants in both the OD_{max} and OD_{20} is illustrated in Figure 8, while Figure 9 demostrates the toxic effect of the surfactants on OD_{max}.

Bacteria-free cultures of the toxicity assay showed little if any difference when compared to the bacteria-associated cultures. There was no significant difference among groups of the bacteria-associated and bacteria-free cultures.

Both LAS and LAE, comparing axenic and bacteria-associated cultures, inhibited growth. Figure 10 compares the reduction in dry weight at 1.0 ppm of LAE and 5.0 ppm LAE in bacteria-associated and bacteria-free cultures.

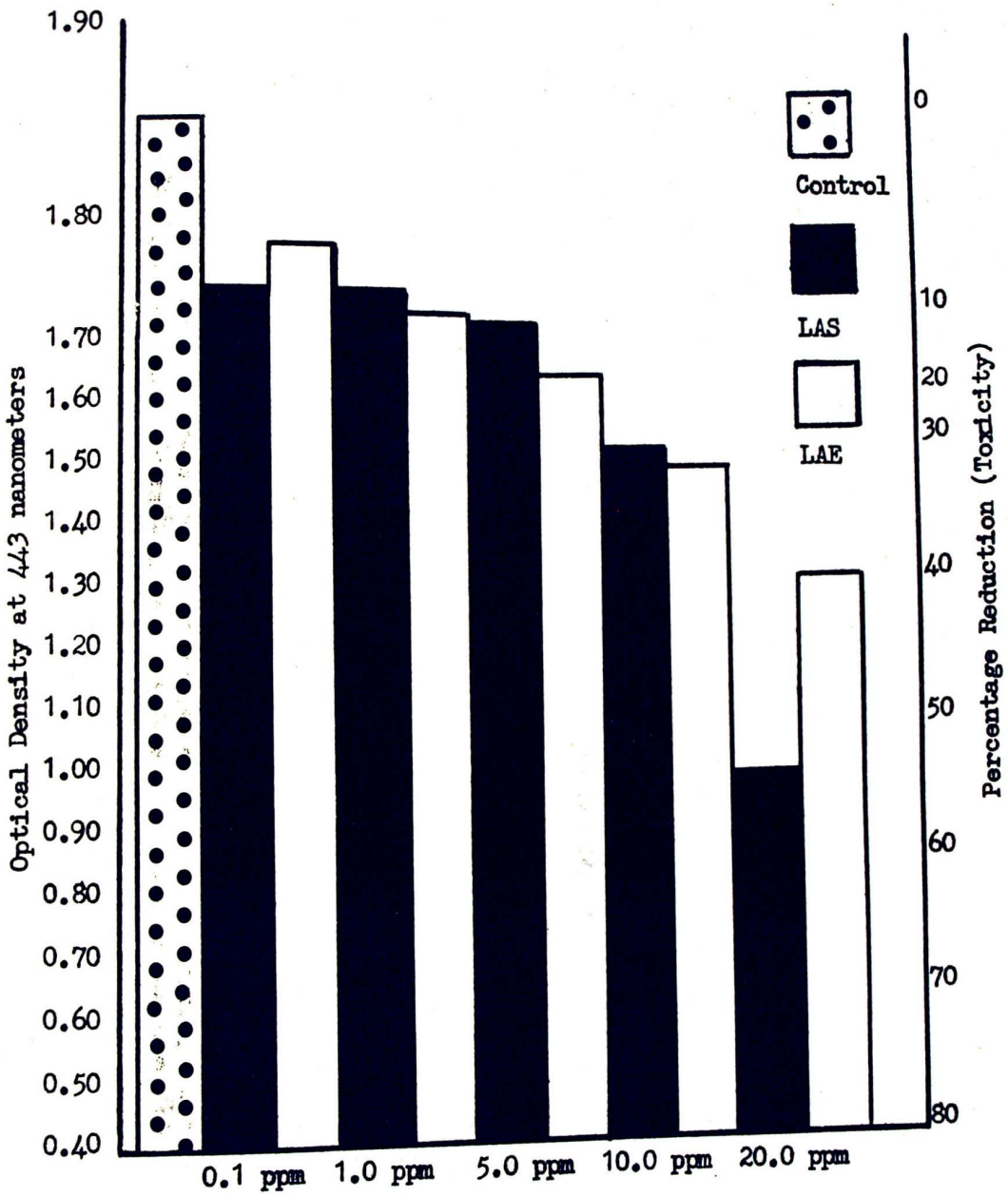


Figure 9. The Percent Reduction in Biomass (OD_{max} at 443 nanometers) Resulting from the Addition of LAS and LAE Surfactant to the Media

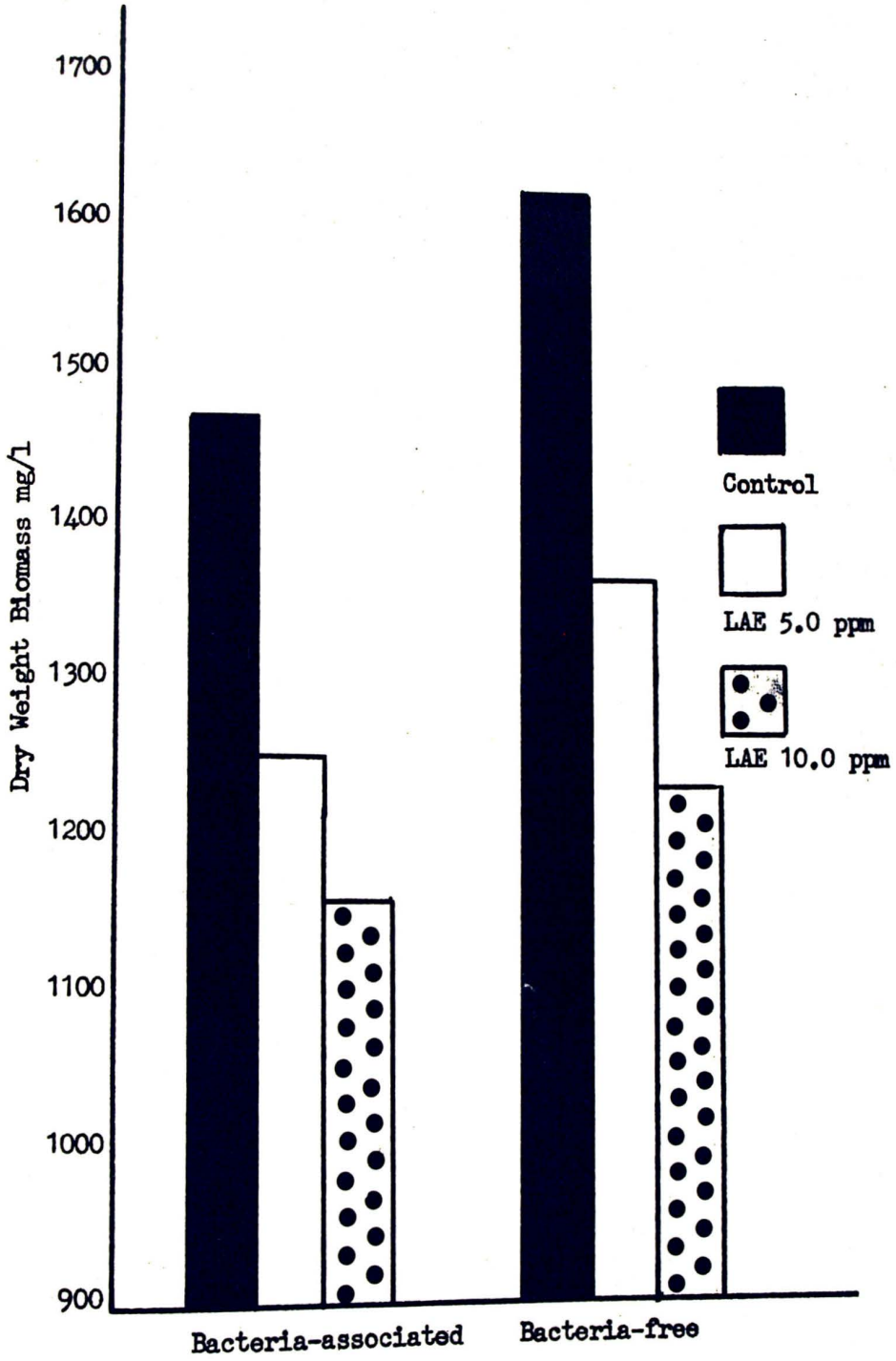


Figure 10. Comparison of Bacteria-free and Bacteria-associated Cultures with Added LAE Surfactant

Observations indicated that in all cultures with surfactant added to the liquid media there was a tendency for cells to clump in larger than normal aggregates. Also extracellular materials appeared in increasing quantities as the level of surfactants increased. Plate I shows extracellular material associated with a cell clump from a culture with 20.0 ppm LAE added to the media.

Aeration with CO_2 also did not change the percentage reductions which occurred in the bacteria-associated cultures in both the LAS and LAE cultures. The pH also remained unchanged in the cultures with bacteria, and neither axenic cultures nor CO_2 aerated cultures affected pH changes. The initial pH of all cultures was approximately 7.2, final pH ranged from 7.8 to 8.5 although, this change in pH did not affect culture groups.

Carbon Assimilation Bioassay

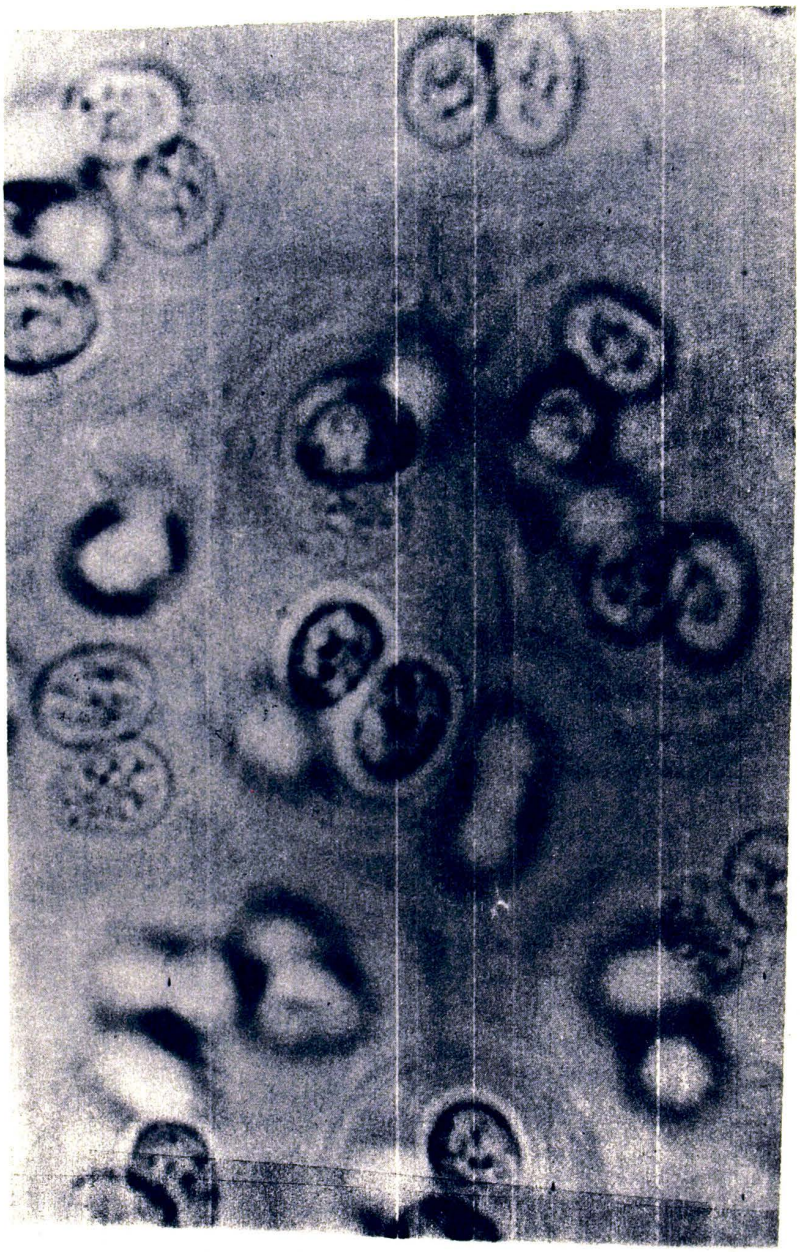
Growth with Glucose. Final dry weight biomass of the control and media plus glucose were similar with only a 9.10% reduction in biomass occurring in the cultures with added glucose.

Although a minimal amount of growth was detected in cultures with glucose and DCMU added to the media, an increase in biomass was noted. However, only 16.28% of the dry weight of the control was obtained and no significant increase from the initial dry weight of the inoculum was observed.

Dry weight biomass was reduced in cultures with DCMU by 83.72% when compared to the control. Table VIII indicates the relative dry weight biomass yields for each of the sets used to assay for carbon assimilation.

PLATE I

EXOGENOUS MATERIAL ASSOCIATED WITH CELL CLUMP IN A CULTURE WITH 20.0 PPM OF LAE ADDED TO THE MEDIA



6 microns

TABLE VIII

DRY WEIGHT BIOMASS YIELDS FOR CARBON ASSIMILATION BIOASSAY

Composition of Media	Dry Weight Biomass mg/l	Percentage Reduction
EPA only (control)	942.03	—
EPA + glucose	888.66	5.66
EPA + glucose + DCMU	153.33**	83.72
EPA + LAS*	683.33	27.46
EPA + LAS + DCMU	no growth	—
EPA + LAS + DCMU + glucose	no growth	—
EPA + LAE*	636.34	32.45
EPA + LAE + DCMU	66.60	92.93
EPA + LAE + DCMU + glucose	59.58	93.67

* Surfactant concentration equated to 2.0 mg/l of carbon.

** Initial dry weight 99.59 mg/l.

Individual cell counts were not performed due to the risk of contamination with bacteria. Final cell counts were not performed in the carbon assimilation bioassay due to the increased clumping of cells in the larger Fernbach flasks as well as the increased clumping due to the surfactant or glucose. Cell count biomass was correlated proportionally with an optical density dilution curve (Figure 11).

Growth rates of the cultures with added glucose were similar to the control. Growth rates of cultures with added glucose however did not exceed that of the control. Growth rates with glucose plus DCMU were greatly reduced and never exceeded 10.0% of the control or 10.0% of media plus glucose cultures. Table IX shows growth rates and percentage reduction for cultures used in the carbon assimilation bioassay.

Since previous cultures indicated that the stationary phase of growth was obtained prior to the 20th day of culture, cultures were only grown for 20 days. The OD_{20} was used as in the toxicity bioassay to indicate biomass of the final stage of growth. The OD_{20} of the media plus glucose when compared with the control indicated a slight, but insignificant, increase in optical density (Table X).

The optical density of cultures with glucose plus DCMU did not show any significant increase from the initial optical density recorded after the cultures were inoculated with Gloeocapsa sp.

Growth with Surfactant. When LAS was added to the media at 2.0 mg/l or 4.90 ppm the dry weight was inhibited by 27.46% of the control. Dry weight was significantly reduced from 942.03 mg/l of the control to 683.33 mg/l of the control plus LAS (Table VIII). If LAS

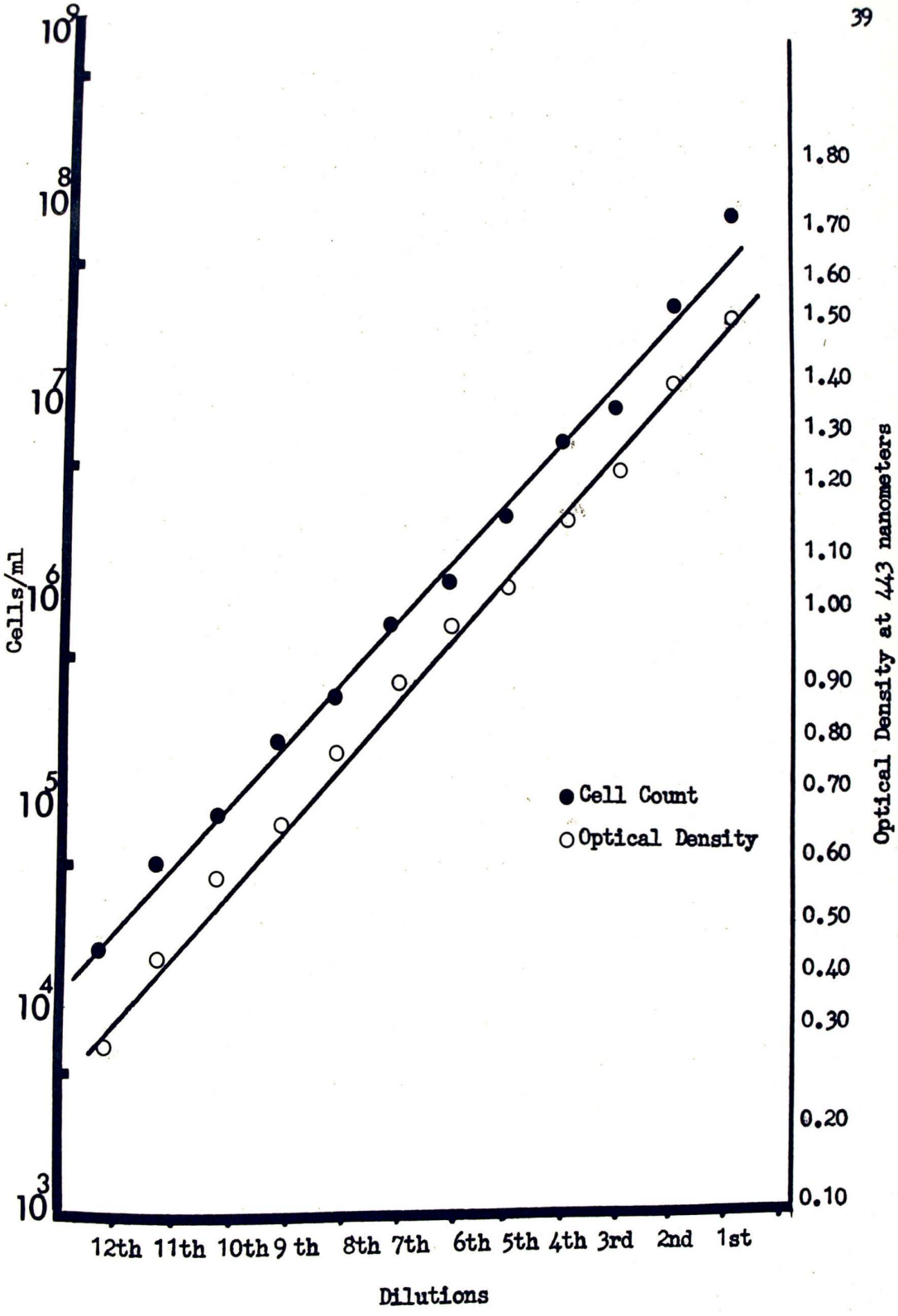


Figure 11. Dilution Curve Equating Biomass (Cell Count) Increases to Optical Density

TABLE IX

GROWTH RATES FOR CARBON ASSIMILATION BIOASSAY

Composition of Media	Rate $\log_e$ units*	Percentage Reduction
EPA only (control)	0.0121	—
EPA + glucose	0.0119	1.65
EPA + glucose + DCMU	no growth	—
EPA + LAS**	0.0115	4.95
EPA + LAS + DCMU	no growth	—
EPA + LAS + DCMU + glucose	no growth	—
EPA + LAE**	0.0118	2.47
EPA + LAE + DCMU	no growth	—
EPA + LAE + DCMU + glucose	no growth	—

* Rate of logarithmic growth from day 5 to day 8, culture period from 29 to 35 days.

** Surfactant concentration equated to 2.0 mg/l of carbon.

TABLE X

OD_{20} FOR CARBON ASSIMILATION BIOASSAY

Composition of Media	OD_{20}*	Percentage Reduction
EPA only (control)	0.343*	—
EPA + glucose	0.330	3.87
EPA + glucose + DCMU	0.008	97.66
EPA + LAS**	0.287	16.05
EPA + LAS + DCMU	0.008	97.66
EPA + LAS + DCMU + glucose	0.008	97.66
EPA + LAE**	0.265	22.54
EPA + LAE + DCMU	0.008	97.66
EPA + LAE + DCMU + glucose	0.008	97.66

* Optical density at the 20th day of culture at 650 nanometers.

** Surfactant concentration equated to 2.0 mg/l of carbon.

was added to the media plus DCMU or media plus glucose plus DCMU, no detectable growth occurred and dry weight yields were lower than the initial dry weights of the inoculum.

Growth rates of cultures with added LAS were significantly lower than the rate of the control. Logarithmic growth in cultures with LAS were reduced by 4.95% of the control (Table IX).

The OD_{20} of cultures with LAS were also lower. The OD_{20} of the control was reduced by 16.05% with LAS added to the media.

LAE reduced the dry weight by 32.45% of the control when added to the media at 2.0 mg/l or 3.05 ppm. LAE inhibited dry weight to 636.34 mg/l as compared to the control which obtained 942.03 mg/l of biomass.

When DCMU was added to the media plus LAE or media plus LAE plus glucose, only minimal growth occurred. Dry weight biomass of both the media plus LAE plus DCMU and media plus LAE plus DCMU plus glucose was similar to the initial inoculum dry weight. Neither set was significantly higher than the initial dry weight of 99.59 mg/l.

Growth rates were also lower in cultures with LAE added and no stimulation or enhancement was noted. With LAE added logarithmic growth was inhibited to 2.47% of the control.

Growth rates of the control were significantly higher than the rates of cultures which contained media plus LAE. In either set of cultures with DCMU and LAE added to the media, no recorded increase in the rate of growth occurred during the 20 day exposure period.

The OD_{20} of cultures with DCMU added to the media also did not change significantly from the optical density of the inoculum

(approximately .008 at 650 nanometers) and, in some cultures, the only change occurring was a decrease in optical density. The optical density of the cultures with LAE added was reduced by 22.54% at 2.0 mg/l or 3.05 ppm. Inhibition in cultures with DCMU added were reduced to only a small percentage of the control (Table X).

In both sets of cultures with added LAS and LAE several flasks were aerated with 5% CO_2 in air. Growth yields in all parameters were similar and no significant difference occurred.

DISCUSSION

The bioassay was designed to test for possible toxicity of the surfactants LAS and LAE during a 20-40 day exposure period as well as to assay the potential for utilization of carbon in the form of surfactant by photoassimilation. Both bacteria-free and associated bacteria cultures were used in studying the toxicity of the surfactants. However, only bacteria-free cultures were used in studying the effects of added carbon. The two bioassays were conducted with and without CO_2 aeration.

Toxic Inhibition in Cultures with LAS and LAE

Bacteria-associated cultures of Gloeocapsa sp. showed little if any difference from the control when 0.1 ppm and 1.0 ppm of either surfactant was added to the media; however, at 5.0 ppm LAE significantly reduced biomass. There was no significant difference at any growth parameter between groups of the control, and 0.1 ppm, 1.0 ppm, and 5.0 ppm when LAS was added to the media.

Inhibition of growth by both LAS at 10.0 ppm and LAE at 5.0 ppm, as measured by all five growth parameters, indicated a toxic effect of the chemical on all growth phases. The irreversible inhibition caused by the surfactants occurred prior to the log phases of growth. Although in the toxicity bioassay concentrations of surfactants used in the media may have exceeded the normal environmental levels (Hall, 1973), data acquired over a two year period from the effluent of Frosty Morn

Meats Co. suggest that concentrations of LAS type detergent is similar to those used during the experiment. Therefore, levels of detergents may exist in other industrial waste lagoons. Figure 12 indicates the mean detergent concentrations for several months of sampling from the Frosty Morn lagoon (data used with permission).

Sakaguchi et al. (1975) found that 10.0 ppm of LAS did not affect the purification of sewage by an activated sludge process. However, their data indicated complete inhibition of the removal of both proteins and fatty acids when 50.0 ppm of LAS was present in sewage effluent.

The inability of cultures of Gloeocapsa sp. exposed to 10.0 ppm of LAS and 5.0 ppm of LAE to reach the growth potential of the control clearly demostrates the toxicity caused by the surfactants (Blankley, 1973). The toxicity was not due to bacterial degradation of surfactant since proportional inhibition occurred in bacteria-free cultures of both LAS and LAE surfactants.

The toxicity of LAE appears to be more pronounced at lower concentrations than that of LAS. LAE 5.0 ppm causes significant reductions in dry weight, growth rate, OD_{max} and OD_{20} while LAS exhibits comparable effects at the higher concentration of 10.0 ppm.

The degree of toxicity displayed by LAS was analogous in all growth parameters measured in that 10.0 ppm or greater was needed to significantly reduce biomass. LAE also reduced the growth potential proportionally in all growth parameters at 5.0 ppm and greater concentrations. However, 10.0 ppm of LAE was required to reduce the cell count significantly from the control. This difference may be due to the method of counting the cells since the presence of cell clumps

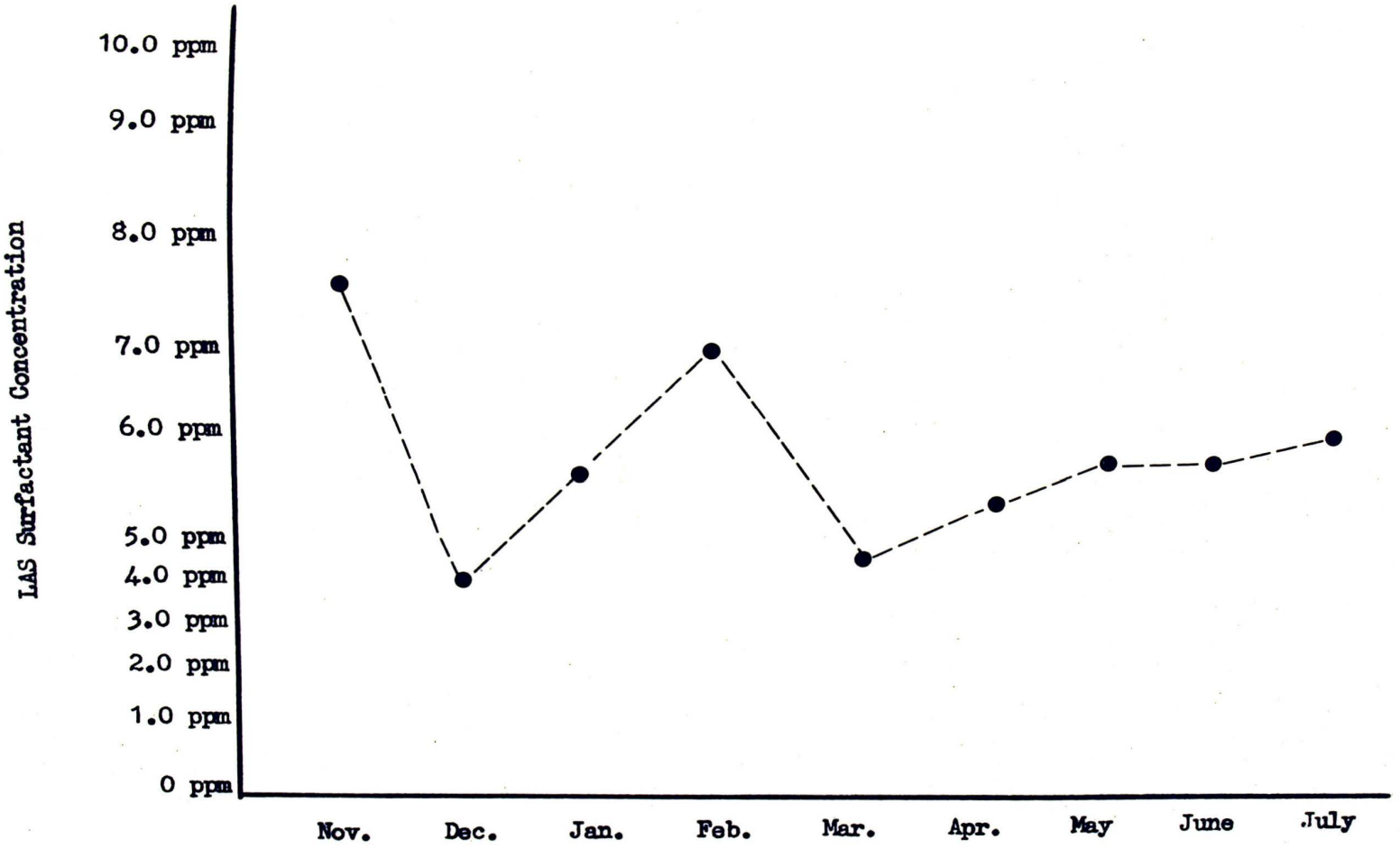


Figure 12. LAE Surfactant Concentration (Measured by Methylene Blue and Toluene Extraction Method) from Waste Lagoon Effluent

may have interfered with effective enumeration. There also was no significantly difference cell counts of 5.0 ppm and 10.0 ppm of LAE.

Growth rates of LAS and LAE were significantly reduced with the addition of 10.0 ppm and 5.0 ppm of surfactant respectfully. Although regardless of the surfactant concentration, the cultures of Gloeocapsa sp. remained in logarithmic growth for approximately the same length of time (96±6 hours). In addition the length of log phase of growth was extended from 18 to 36 hours in all cultures with surfactants added; whereas, cultures of the control began logarithmic growth almost immediately after inoculation.

The increase in cells/ml for both LAS and LAE from 20 days to approximately 30 days show no distinct variation in biomass increases when surfactant was added to the media (Figures 3 and 4, page 22 and 23). These plots when compared with optical density and dry weight biomass again indicate the toxicity of 5.0 ppm LAE and 10.0 ppm LAS (Figures 2 and 9, page 20 and 32).

The depression of growth rate in log phase and lower cell numbers, as indicated photometrically, at log phase initiation could be the causative factor in biomass reduction (Fogg, 1971 and Sorokin, 1973). Therefore, reduction in biomass as indicated by final cell count, dry weight biomass and OD_{max} would be due to some inherent factor associated with metabolism or cell division prior to the beginning of log phase or during the log phase of growth.

When surfactant, either LAS or LAE, was added to the media a distinct difference in the method of growth occurred. In the 20.0 ppm solution cells clumped together into aggregates of 16-24 cells; whereas, in the control and lower concentrations of surfactant cells were

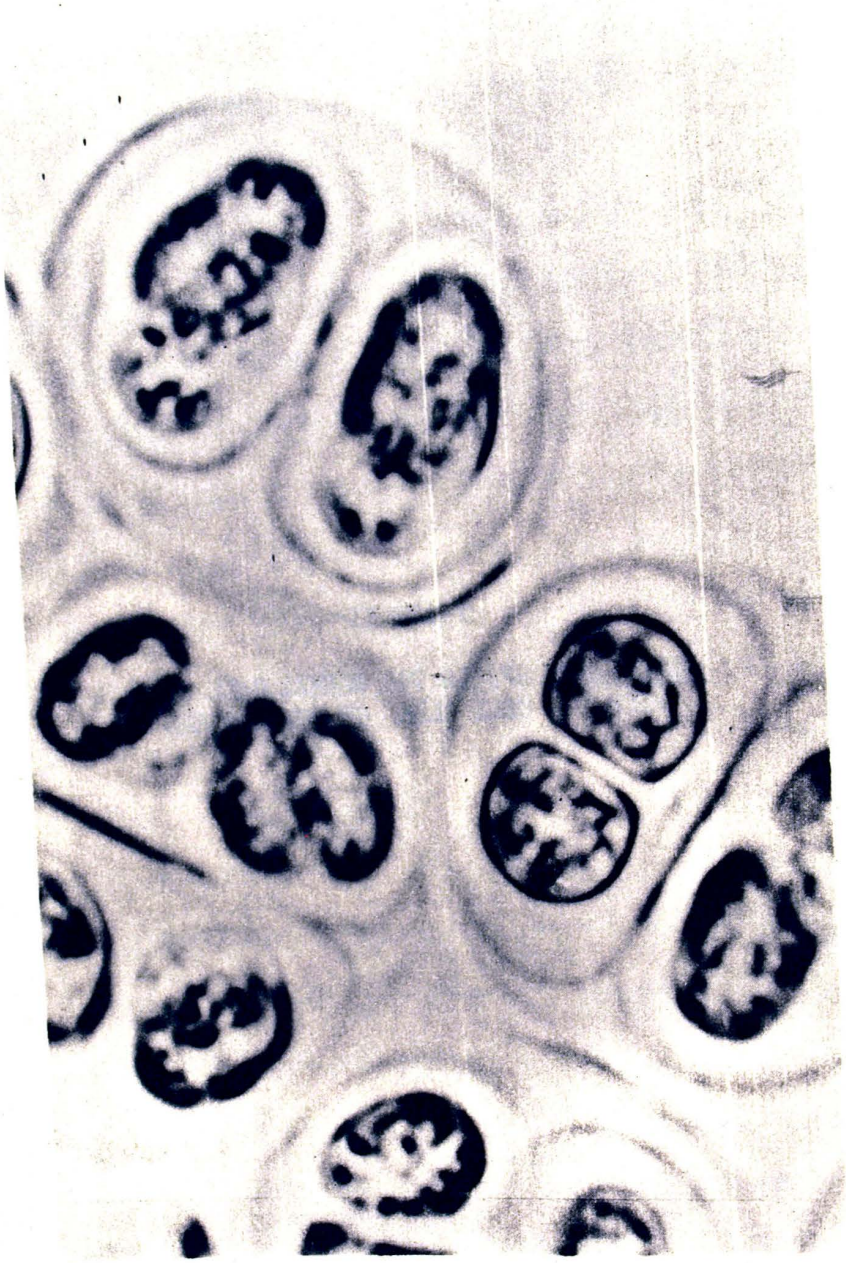
usually arranged in smaller clumps of 4-8 cells (Plate II) or in the normal 2-4 cell aggregate (Allen, 1973 and Stanier et al., 1971). There also appeared to be a larger amount of extracellular gelatious material surrounding the cell in the 10.0 ppm and 20.0 ppm. However, no distinct intracellular morphological changes were detected (Green, 1973).

The observed difference in biomass could be attributed to reduction in cell numbers prior to the log phase of growth, or reduction in the relative rates of cell division, or to metabolic changes, each of which may have been caused by LAS or LAE toxicity. Formation of intracellular or extracellular exogenous material is known to occur in the blue-green and other algae. Hellebust (1974) thoroughly discusses exogenous cell material and gives a detailed review of the current literature related to extracellular products. Blue-green algae have been found to excrete extracellular products to favorably chelate metals (Lange, 1970 and 1974). The production of gluculate products were reported in response to carbon compound in Anabeana flos-aquae by Wang and Tischer (1973). Moore and Tischer (1964 and 1965) also reported on the process of excretion of this polysaccharide in the same species. Fogg (1962) discusses the ecological aspects of the excretion of growth inhibiting substances as a form of autoregulation.

High light intensities have resulted in the increase of a soluable photosynthate in phytoplankton (Fogg, 1965 and Hellebust, 1965). In addition, under conditions in which CO_2 may be limited or the presence of high pH, Watt and Fogg (1966) found that glycolic acid was a major extracellular product in Chlorella pyrenoidosa. However,

PLATE II

GLOEOCAPSA SP. LB 795 NORMAL CELL SIZE
WITH 2-4 CELL CLUMP OF THE CONTROL



8 microns

when cultures of LAS and LAE were aerated with a 5% CO_2, CO_2/air mixture, the reduction in biomass was proportional to cultures without aeration (Figure 13).

The production of extracellular products in response to the technical grade surfactants LAS and LAE has not been reported. This exogenous material, although present early in the growth period, seemed to increase during the later stages of the growth period. This would seem to confirm similar results of Guillard and Wangersky (1958) and Guillard and Hellebust (1971) that found increase in exogenous material in the stationary phase of growth and also Rippka (1972) found that Aphanocapsa sp. excreted a brown pigment as the stationary phase was approached. Data indicate that exogenous material associated with cell clumping occurred when surfactant was added to the media (Plate I, page 36 and Plates III and IV). In Gloeocapsa sp. this material seemed to be more prevalent during the later stages of growth.

The presence of exogenous material therefore suggests that the extracellular product is being excreted in response to the presence of LAS and LAE surfactant. Beale (1970) found that amino acids were excreted in response to the foreign substance levulinic acid which inhibits chlorophyll synthesis.

Gloeocapsa sp. LB 795 is a known nitrogen fixing species (Wyatt and Silvey, 1969); the details of which are presented in length by Rippka et al. (1971) and Gallon et al. (1975). The release of several nitrogenous compounds in response to various growth conditions has been reported by several researcher (Fogg, 1952; Whitton, 1965 and Jones and Stewart, 1969).

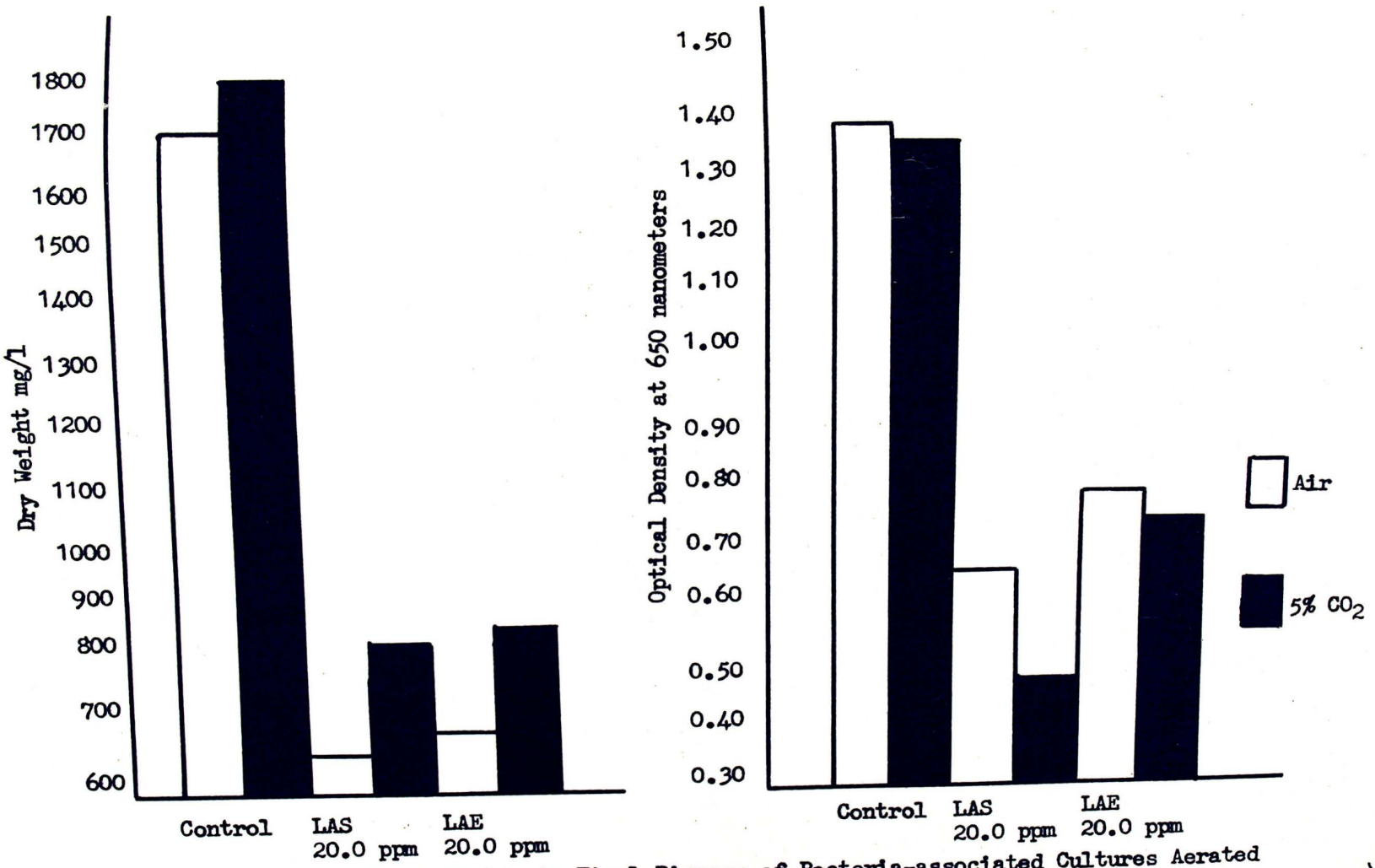
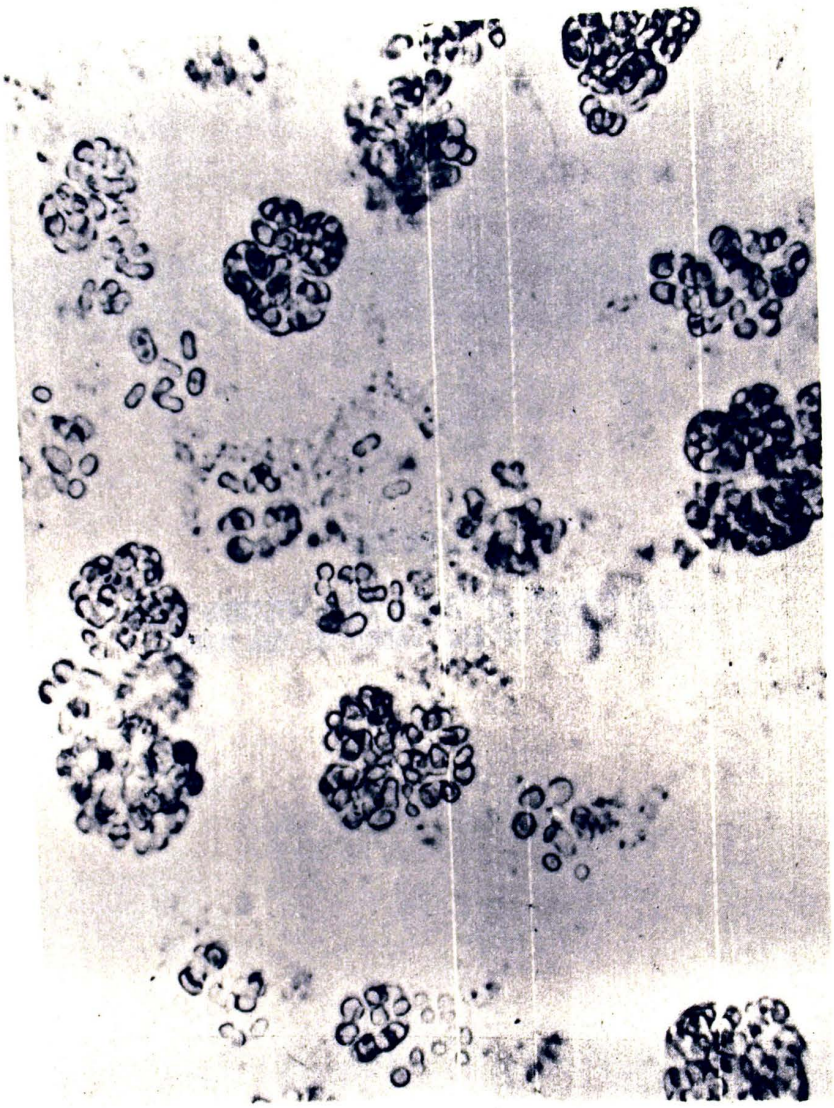


Figure 13. The Proportional Yields in Final Biomass of Bacteria-associated Cultures Aerated with and without 5% CO_2

PLATE III

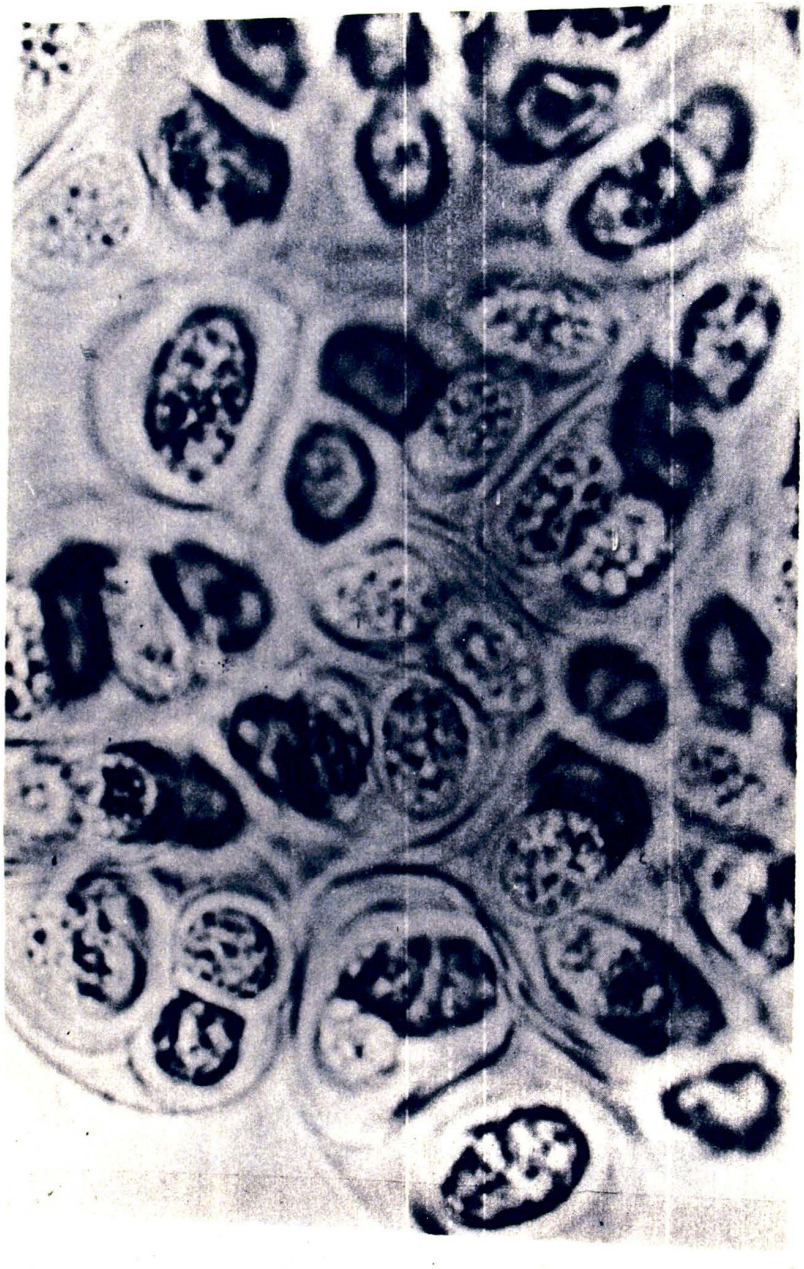
LARGE CLUMPS OF CELLS IN A CULTURE WITH
20.0 PPM OF LAS ADDED TO THE MEDIA



80 microns

PLATE IV

CELL CLUMP IN A CULTURE WITH 10.0 PPM
OF LAE ADDED TO THE MEDIA



6 microns

The presence of this extracellular material in association with decline in biomass may indicate that nutrient availability is reduced either by the substance or due to its formation. Although chelation by this exogenous material of micronutrients is also a possibility (Lange, 1970 and 1974).

However, if nutrients were being absorbed in association with this material, then the length of log phase of growth would be at a ratio equivalent to the nutrients available (Fogg, 1971). Although the equal length of log phase at all concentrations of surfactant, as mentioned before, should rule out this possibility. Should the nutrients be absorbed by this material or their availability hindered, then the changes in production would be proportional to the concentration of surfactant. Data indicate that as the concentration of surfactant increases, exogenous material also increases and that biomass decreases as measured by dry weight, cell count and optical density. Data would possibly suggest that in Gloeocapsa sp. cells are producing this extracellular material in response to the surfactant; therefore, then the decline in biomass may be a result of the utilization of nutrients by the cells to metabolize this material instead of synthesizing new cellular materials (Kunisawa and Cohen-Bazire, 1970).

Obligate Photoautotrophy in Gloeocapsa sp. LB 795

Gloeocapsa sp. LB 795 failed to increase biomass in the presence of glucose therefore, data confirms that of Rippka (1972) and Stanier et al. (1971). Further evidence of the role of photoautotrophy

in Gloeocapsa sp. LB 795 is the complete inhibition of growth in cultures with added DCMU, which inhibited electron flow and thus blocked CO_2 reduction (Bishop, 1958). It would appear that no usable substrate could be acquired from glucose or glucose intermediates by Gloeocapsa sp. in bacteria-free cultures.

Gloeocapsa sp. failed also to grow in the presence of DCMU when either surfactant was added to the media or media plus glucose. When LAS or LAE was added to the media at 2.0 mg/l, inhibition of growth occurred in dry weight, growth rate and OD_{20}. Inhibition of growth without DCMU added would seem to imply that neither surfactant could be photoassimilated as a source of carbon or that if the surfactant were incorporated that toxicity from some portion of the surfactant molecule may occur. Rippka (1972) found that when the chemotroph Aphanocapsa sp. 6714 was grown in the presence of glucose that the mean generation time was shortened from the normal 15 hours to 11 hours. However, she found when DCMU was added to the media plus glucose, the generation time was increased to 30 hours. She also found that low molar concentrations (2.5×10^{-4}) or high molar concentrations (5×10^{-2}) of glucose increased the time required to double biomass (genration time) from 100 hours to 110 hours.

Experiments have shown that when organic substrates were added to the media several species of blue-green algae were capable of growth in low light intensities which would normally inhibit growth (Hoare et al., 1971 and Van Baalen et al., 1971). However, light intensities used to grow Gloeocapsa sp. would support healthy growth as evidence by the control. Therefore, if Gloeocapsa sp. could utilize

either surfactant as a carbon source then growth most probably should have been increased in cultures with added surfactant.

However, the toxicity bioassay indicated that concentrations higher than those used in the carbon assimilation experiments resulted in an irreversible toxic effect of the surfactant. Also, concentrations lower than the 2.0 mg/l in these experiments had no effect on final biomass. Unlike Aphanocapsa sp. 6714 (Rippka, 1972) added glucose decreased the growth rate of Gloeocapsa sp. LB 795 when compared to the control. However, data was similar to that of Rippka (1972) when she used two other strains of Aphanocapsa sp. that were grown in the presence of glucose.

Rippka (1972) found that Aphanocapsa sp. 6714, formerly called Gloeocapsa alpicola (Smith et al., 1967), as well as, several other isolates could readily utilize glucose as a substrate by photoassimilation.

However, it would appear as a result of the toxicity bioassay and experiments with DCMU (Stanier, 1973) that Gloeocapsa sp. LB 795 could not utilize either glucose or the surfactants LAS and LAE as a source of carbon by photoassimilation.

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