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Research Article

Pure Algal Cultures Using Varying Photoperiods, Nutrients, Antibiotics, Enrichment, and Water Treatments

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Abstract

Selected freshwater microalgae (*Scenedesmus quadricauda, Chlorella vulgaris, Eudorina elegans, Closterium sp., Chlorococcum sp., Cocconeis placentula*, *Gonium sp., Anabaena sp., Chlamydomonas sp., Stephanosphaera sp., Characium sp., Tetraspora sp.,* and *Pleodorina sp.),* were artificially established axenically in illuminated culture cabinet using the gelose and dilution methods by varying culture media, antibiotics, enrichment, photoperiods, and water treatment. Most algae were selective against different antibiotics, hypochloride and U.V treatments. *Volvox sp.* defied all culture methods. Highest *Chlorella vulgaris* cell density of 244 x 10^6 cells/ml was obtained with penicillin, treatment in filtered-unautoclaved-water (FUW) followed by 154 x 10^6 cells/ml using unfiltered-unautoclaved-water (U_fUW) at 0.005%-0.01% hypochlorite solution at 15:09h L:D. Model 1 ANOVA for filtered-autoclaved-water (FAW), FUW and U_fUW, with *S. quadricuada* was significant ($p<0.05$, $^2_{26}F0.05$, 3.889) only at 24.00h photoperiod, *C. vulgaris* was significant ($^2_{31}F0.05$, 3.317) at 15:09 L:D.The Least significant difference (LSD) for *S. quadricauda* was 15:09h L:D > 24h Light >12:12h L:D while that of *C. vulgaris* was U_fUW>FUW>FAW. Thus, photoperiods and water treatments impacted significantly on algae production. The various isolated axenic phytoplankton monospecies cultures were thereafter mass produced in very high stocking densities in semi-controlled outdoor tanks of 0.6 m^3 to 10 m^3 capacities.

Keywords: phytoplankton, axenic, nutrient-media, enrichment, antibiotics, photoperiods.

Introduction

In the wild, phytoplankton abundance is easily affected by fluctuations in temperature, day length, presence of grazers, availability of nutrients, water depth and turbidity as well as the seasons of the year (Kungvankij, *et al.*, 1986). Though manipulation of the above-mentioned factors is possible under laboratory conditions or controlled rooms (Kungvanskij, *et al*, 1986), the complexity of the media or the culture of phytoplankton usually vary inversely with the scale of operation. Large outdoor tanks use agricultural fertilizers while trace metals and reagent grade nutrients (e.g. phosphates and nitrates) are used in more refined indoor operations (Yap, 1979; Pantastico, Baldia, Espegadera & Reyes, 1990) Enrichment techniques using different combinations of macro and micro-nutrients, organic and inorganic fertilizers, w-yeast, emulsified fish oil, on algae to improve the nutritional value of live foods for larval rearing have been developed (Leger, Nessens-Foucquaert & Sorgeloos, 1987, Leger, Chamorro & Sorgeloos 1987; Ajah, 2008, 2010). The production of axenic micro algae at monospecies level and subsequent mass production form the aim of this research to guarantee availability of omega fatty acids in them for effective larval rearing.

To achieve axenic monospecies cultures, a short food chain consisting of selected microalgae was artificially established. Microalgae were

Materials and Methods

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isolated from pond water and mass-produced in batch monocultures in high densities in an illuminated culture-cabinet. Their suitability as larval mono food, larval mixed food, and food for cruataaceans (calanoids, harparticoids, daphnids, larger diaptomids) and rotaroria were then established in high density cultures in semi-controlled outdoor tanks. For mass production, five large 10 m^3 / (2641.721 US liquid gallons) fertilized outdoor weaning tanks, four fibreglass tanks of 0.6 m^3 (158.503 gallons) each and four oblong concrete tanks of one cubic meter (264 us gallons) volume each were artificially inoculated with pure algal cultures.

Indoor culture Methods of phytoplankton

Several round bottom flasks of 250 ml, 500 ml, and one litre capacities were each filled to about ¾ full of 0.2 µm filtered freshwater. They were autoclaved at 120 ^{0}C and 20 psi 2016.1 sq. in for 15 minutes of small and 45 minutes of large vessel to destroy bacterial endospores (the form of life which is most resistant to heat). Culture vessels were flamed using methylated spirit during and after transfers. All tools were thoroughly disinfected during and after use. The culture cabinet was disinfected regularly. Each of them was inoculated with axenic algae species using either the dilution method that involved a series of combined dilution and transfer steps in Petri dishes of the mixed species collected from the pond using sterile Pasteur pipettes to arrive at the desired mono species. Or the gelose method that uses different nutrient media to enhance either diatomous or non-diatomous, filamentous or non-filamentous algal growth though requires repeated isolation and re-inoculation to finally achieve monospecies cultures. For ease of achieving our aim, emphasis was on the use of 'Dilution' method. Isolation and transfers were done after each identification using a light microscope. Thereafter, I ml of solution A, 0.1 ml of solution C and 6.0 ml of solution D (if diatom) using Nutrient Salt Solution (Laing & Ayala 1990) were added as the growth medium. If diatom is not involved, only 1 ml of A and 0.1 ml of solution C are required. The three axenic methods deployed included: (i). The use of hypochlorite solution: The desired alga was isolated and inoculated into the following concentrations- 0.3 ml, 0.5 ml, 1 ml, 2 ml, and 5 ml per litre of 0.005%- 0.01 % hypochlorite solution post addition of growth medium (Nutrient Salt Solution). (ii). U.V Radiation: While using u.v radiation, the batch was inoculated with mixed algae species and exposed to u.v radiation (ALLENS TYPE A409) for one hour before the growth medium was added. (iii) Antibiotics: If, antibiotics was used, 4 mg/l of 50 mg/l concentrate volume of selected antibiotics such as penicillin, chloramphenicol, and ampicillin were introduced into the flasks after adding the growth medium and the selected algae. Illumination was at 12h: 12h, 15h: 9h light: dark and 24h light.

Culture cabinet for phytoplankton

A rectangular wooden culture cabinet of 60 cm x 60 cm x 100 cm had its four walls, the top and bottom lined with reflecting aluminum foils. The cabinet was partitioned into two equal halves with the aid of a tick glass with 5 cm spaces from the rectangular walls to allow proper air circulation from the fan.

Two, three and four 36 watts daylights of two feet (2") fluorescent tubes were used to generate 3,900 lux, 5,800 lux and 7,800 lux respectively for different experimental designs and their efficiency in promoting growth, cell division and subsequent *algal* productivity were compared. A lux meter (Photometer Model 29AM 300) was used to measure the radiation in lux. The light source was connected to a time switch for regulating the illumination periods to 12:12h L:D, 15:09h L:D and 24h light.

The cabinet temperature for three fluorescent tubes was kept at 30^{o}C ± 2^{o}C by use of a small electric fan in addition to four netted circular vents each of 5-7 cm diameters on the walls of the cabinet in the absence of an air conditioner which kept the air efficiently in circulation. With two fluorescent tubes, the cabinet temperature was 28^{o}C ± 1^{o}C, while four tubes yielded 34^{o}C ± 1^{o}C. Culture vessels were kept at 15 to 20 cm from the light source to avoid excess heat.

Aeration of culture

A HYFLO pump was used to generate air dispersed through Teflon tubes into air-stones in each culture flask to keep the algae afloat.

Culture media

Two nutrient media were used to provide the needed nutrients for phytoplankton growth in the laboratory. They included: Nutrient salt solution (Laing & Ayala, 1990); and Chu No. 10 medium (Chu, 1942);

Media compositions were as follows

Nutrient salt solution for culture medium preparation

Solution A: Nutrient Salt Solution

$FeCl_3$ ….0.78g, $MnCl_2$ ….0.36g, H_3BO_3 …..33.60g, EDTA, disodium salt 45.00g, $NaH_2PO_4.2H_20$ …..20.00g, $NaNO_3$ …..100g,
Add 1-0 ml of Solution B
Make up to 1 litre with freshwater. Heat to dissolve.

Solution B

$ZnCL_2$ …2.1g, $CoCl_2.6H_2O$ …2.0g, $(NH_2)_6$ $MO_7O_{24}.4H_2O$ …. 0.9g, $CuSO_4.5H_2O$ ….2.0g,
Conc. HCl ….10.0ml.
Distilled water to heat to dissolve 100ml. Add 1.0 ml to each litre of solution A.

Solution C

Vitamin B_{12}, (cyano cobalamin) …..10.0 mg, Vitamin B_1 (Aneurine hydrochloride) -----200mg
Distilled water to 2000.0mg
Add 0.1 ml to each liter of freshwater

Prepare an iron solution by dissolving 3.35g citric acid ($C_6H_8O_7.H_2O$) in 100 ml distilled water; then add 3.35g ferric citrate ($FeC_6H_5O_3.5H_2O$), autoclave to dissolve, dispense in sterile tubes and keep sterile, refrigerated in darkness (wrapped in aluminum foil).

Prepare a trace elements solution by dissolving the salts in the amounts (milligrams) indicated together in 1 litre of distilled water, autoclave and keep sterile.

$CuSO_2.5H_2O$--------.19.6mg; $ZnSO_4.7H_2O$----------44.0mg; $CoCl_2.6H_2O$--------20.0mg; $MnCl_2.4H_2O$---------36.0mg; $NaMoO_4.2H_2O$----------.12.6mg; H_3BO_3 ----------618.4mg.

To prepare the definitive solution, add aseptically 1 ml of each of the six stock solutions in step 1 to a liter of sterile double distilled or deionized water. Then add aseptically 1 ml of stock solution in step 2 and 1 ml of the trace elements solution in step 3. Dispense aseptically into sterile containers. (Source: Bold & Wynne, 1978)

Solution D

$Na_2SiO_3.5H_2O$ ….40.0g
Distilled water to 1 litre. Shake to dissolve
For experiments, add 6 ml of Solution D to each litre of freshwater containing a culture of diatom, 1.0 ml of solution A and 0.1 ml of solution C. If no diatom is involved only 0.1 ml of solution A and 0.1 ml of solution C are required.
(Source: Laing & Ayala, 1990)

CHU No. 10 solution (Modified by Wright and Guillard; and by Van Dover)

Make stock solutions by dissolving the salts listed in the amounts indicated (in grams) each in 100 ml of distilled or deionized water; autoclave them and keep sterile.
$CaC_2.2H_2O$ -----3.67g; $MgSO_4.7H_2O$ ……3.68g; $NaHCO_3$ ……1.26g; K_2HPO_4 …..0.87g; $NaNO_3$ …..8.5g; $Na_2SiO_3.9H_2O$ (metasilicate) …….2.84g.

However, for ease of preparation and administering, the nutrient salt solution was preferred and used regularly during this research due to:

The small amount of nutrient salt solution required (1 ml of solution A and 0.1 ml of solution C) per litre used at a particular time against the 10 ml/l of Chu 10 step 1.

The relative ease of preparing nutrient salt solution.

Nutrient salt solution is more specific and has more reliable preparation steps that are algal specific compared to Chu No. 10.

Hygiene/disinfection
Sterilization

- The following precautions were taken during cultures to prevent bacterial contamination which tended to inhibit optimal development of cultures.
- Culture water was pre-filtered using a 0.20 µm membrane filter.
- Glass vessels were thoroughly cleaned and disinfected with two parts per thousand (2 ‰) of 0.005% -0.01% hypochlorite solution.
- Both the glass wares and the filtered water were autoclaved at 120 ^{o}C and 20 psi (pounds per square inch)/(1.06 kg per square cm) for 15 minutes for smaller (<1000 ml), and 45 minutes for larger (>1000 ml) vessels.
- Non-absorbent cotton wool or rubber stoppers were used to ward off bacterial contamination from the air.
- Culture vessels were also flamed using methylated spirit during and after transfers.
- All tools were thoroughly disinfected prior to use.
- The culture cabinets were disinfected regularly.

Isolation Method

Two standard isolation techniques were used, namely, the Gelose method, and the Dilution method (Harder, 1917; McVey & Moore, 1983). The gelose method used different nutrient media to enhance either diatomous or non-diatomous, filamentous or non-filamentous algal growth, but required repeated isolation and re-inoculation to finally achieve monospecies cultures. The dilution method involved a series of combined dilution and transfer steps in petri dishes of the mixed species collected from the wild using sterile Pasteur pipettes to arrive at the desired species, whereas in the gelose method, the mixed species collected from the wild were first cultured in a growth medium before repeated isolation and transfers were done after identification under a light microscope.

Axenic culture techniques

Since bacteria inhibit algal growth, the following steps were taken to inhibit bacterial growth or reduce their growth to the barest minimum.

Hypochlorite solution- the desired algae was isolated and inoculated into different concentrations (0.3ml, 0.5ml, 1ml, 2ml, and 5ml per litre) of 0.005-0.01% Hypochlorite solution post addition of growth medium.

U.V. radiation (ALLENS TYPE A409) used for bacteria screening on the algae for one hour before the growth medium was added.

Antibiotics: Four ml per litre of 50 ml-1 concentrate volume of any of the selected antibiotics (penicillin, chloramphenicol, and ampicillin) were introduced into the flask after adding the growth medium and the selected alga for culturing.

Nutrient Replenishment and Maintenance of Culture

Phytoplankton cultures were maintained in either batch cultures, semi-continuous or continuous cultures, by harvesting regularly 10 to 20 per cent of culture volume every other day. Laboratory cultures were replenished proportionately with fresh medium and fresh distilled water plus 1.0 ml of solution A, 0.1 ml of solution C, while 6.0 ml of solution D was added if diatoms were cultured, otherwise, solution D was omitted. Continuous cultures involved regular renewal of the nutrient media every other day beginning from day-2 post initial peak bloom in the laboratory. Water loss due to evaporation were also replenished. Experiments lasted for four to six weeks. All culture vessels were gently aerated via air-stones to keep microalgae afloat. Stock cultures, not for immediate use were kept for a maximum of seven days near the window where they received minimal light supply compared to the high light intensity in the culture cabinet.

Water Treatments

Three water treatments were adopted: i) Filtered autoclaved water (FAW), ii) Filtered unautoclaved water (FUW), and iii) Unfiltered unautoclaved water (U_fUW)

Cell Density Analysis

Hemocytometer Method by McVey and Moore, (1983) and Laing (1991) was followed in determining the cell density. Two to three

drops of either 3% formalin, 10% alcohol or lactophenol were used to demobilize the motile algae before loading into the chamber lens. Counting was done under x40 magnification. The mean of four such counts was taken as the cell density per ml.

The Sedgwick-Rafter (S-R) cell counter is one of the most widely used types of counting chambers of 50 mm long x 20 mm wide x 1.0 mm deep with total volume of 1.0 ml. Following this specification, an improvised S-R chamber named 'MODEL AJAH 001' was fabricated. The cell of the S-R chamber constructed locally was filled and covered with a glass slide to eliminate air bubbles and left to stand for 15 minutes to allow the plankton to settle. Counting procedures were as outlined in APHA (1980) where 10 to 15 random field counts of each species was done and the average was multiplied by the total number of fields. The fabricated S-R was 28 x 14 cells for vertical and horizontal counts, respectively.

Outdoor cultures of phytoplankton

Pure laboratory cultures were used to inoculate the semi-controlled, outdoor tanks used for the experiment.

Culture environment

- Circular concrete tanks of 10 m^3 capacity and 0.6 m depth with lateral inlet and a central drainage pipe were used. Five laterally attached airlift pumps provided vertical as well as a circular movement of otherwise stagnant culture water, providing continuous mixing of water and exposure to sunshine.
- Fibreglass tanks of 0.6 m^3 volume (100 cm length x 60 cm width x 300 cm height) and
- One cubic metre capacity oblong concrete tanks of 0.8 m depth with inlet and outlet pipes plus Teflon tubes with air-stones attached for aeration purposes were used.

Nutrient media

Technical and Agricultural grade fertilizers were used for mass-scale propagation of phytoplankton. Either 500 g dry baker's yeast (*Saccharomyces cerevisae*), 17.5 g inorganic fertilizer (NPK: 20:10:10; 5:5:5; or 5:5:6 NPK: urea: single super phosphate (ssP) etc or 1 kg organic manure (swine manure or poultry droppings) filtered through a 150 µm mesh sieve was added per day per 10 m^3 culture volume for two days to initiate algal exponential growth phase during the dry season. During the raining season, the initial doses were 1 kg baker's yeast, 35 g inorganic or 2 kg organic manure per day for two days per 10 m^3 volume. These nutrients enriched the fresh water thereby promoting faster growth of phytoplankton in shorter periods of time. The quantity of nutrients used were determined through series of experimental runs and physio-chemical analysis of results.

Nutrient Replenishment

Seven days post algal peak growth (bloom) during the dry season, 250 g baker's yeast was introduced. From day-10, only 50 g baker's yeast was needed every three days per 10 m^3 culture volume. Eight gramme of inorganic or 500 g organic was required every three days from day 7 post algal peak growth during the dry season. During the rainy season, one kilogramme organic manure was introduced every three days from day 4 post algal peak growth. The onset of algal exponential peak growth was determined by both visual observation and actual cell count. Observing a light green colouration of a culture or having from 10 x 10^6 cells per ml for smaller microalgae like *Chlorella* or 0.1 x 10^6 for larger algae like *Eudorina* marked the commencement of algal exponential growth phase. To reduce the doubling time from 10 to 3 days, introduce 10x the amount of nutrients.

Maintenance of Culture

By harvesting 10 to 20 per cent of culture volume every other day and replenishing the nutrients and water as described above, the pure culture was maintained continuously for six weeks.

Direct Count Method/Total Volume

For quantitative counts, known volumes were filtered but no filtration was carried out during qualitative counts. There were enumerated as earlier described.

Cell Division Rate (K)

$K = \text{Log}(N_1/No)(3.322/t$ (Guil1ard , 1973)

Where, N_1, No = Cells at end and beginning of t in days, respectively.

Population Growth

To calculate the population growth of animals, the formula $Nt=N_0.ert$ was used.

Where: Nt = the final number of rotifers at time t days

N_0 = initial number of rotifers.

r = the intrinsic rate of natural increase

t = time in days

e = 2.7183

$$r = \frac{2{,}3026\ (\text{Log Nt} - \text{Log N}_0)}{t} = \frac{\text{Nt} - \text{In No} + D1}{t}$$ (James and Dias, 1984)

D1= dilution rate (m^3 day 1)

tn = 0.6931/r (James and Dias, 1984)

t_D = Doubling time of the population in days.

Fixing

Plankton were preserved using 3% forrna1in or Lactophenol by adding two to three drops into the sample bottles.

Staining and Mounting

Staining for 5 to 10 minutes was done using Mayers' acid Haemalum. The Specimen was passed through series of 30%, 50%, 70% and 90% alcohol as dehydrating agent while cedar wood oil was used as the clearing agent. The mountant was either entellan or super glue. Direct temporary mount without staining using 10% glycero1 oil (Baker and Wharton; 1952), or the use of nail varnish as a permanent mount after heat shock or preservation in lactophenol was also adopted.

Plankton identification

The species earlier fixed using lactophenol were isolated from the vial tubes and transferred into staining wells composed of 7% glycerol for 12 hours to allow for complete dehydration. Thereafter they were removed and placed in few drops of absolute (100%) glycerine jelly on glass slides, covered with clean cover slips before sealing the edges with nail varnish ("Eternet"). Rotifers were drawn under x40 objective lens of a phase contrast microscope (Model euromex RM87205) by means of an attached camera lucida and photographs were also taken. Species were identified using the keys provided by Hutchinson, (1957); Ward and Whipple, (1959); Fritsch, (1975); Bold and Wynne, (1978); Koste, (1978 a,b); and Egborge and Chigbu, (1988).

Statistical Analysis

Single classification analysis of variance (anova) Model 1, two-way sand nested anova were used. The ANOVA was used for multiple comparison of the significant differences between the means of the parameters. The Least Significant Difference tests (LSD) was to find out where the significance lied (Sokal & Rohlf, 1981). The Duncan multiple range (DMR) and Duncan Newman student Keul (SNK) test (Duncan, 1955) were used to further reduce the significant differences (DMR) and arrive at a more conservative number (SNK). Correlation coefficient and the multiple regression indicated the influence of one factor on another (Sokal & Rohlf, 1981

Results

Most algae bloomed between 3 to 14 days post inoculation at 12:12h light: dark and 2 to 7 days at 15:9h light: dark regime. Under 15:09h light: dark periods, cultures containing penicillin bloomed within 48 hours. To reduce the doubling time from 10 days to 3 days, 10x the amount of food nutrients was added. Figures 1 to 3 are comparative summarization of the various algae (CV= *Chlorella vulgaris,* CP= *Cocconeis placentula,* EE= *Eudorina elegans,* SQ= *Scenedesmus quadricuada)* cell densities grown under three photoperiods using three water treatments type and various as well as varying antibiotics to produce axenic monoalgal. Batch V(V_0-V_6) was grown with filtered autoclaved water, Batch C (C_1-C_5) with filtered unautoclaved and Batch A (A_1-A_4) unfiltered unautoclaved water. In Batch V, the highest cell density of 90.76 x 10^6 cells/ml was obtained with *C. placentula* a diatom exposed to UV

radiation at 15:09h L:D followed by *Chlorella vulgaris* (Chlorophyceae) grown on chloramphenicol having 76.10 x 10^6 cells/ml at 15:09h L:D while *C. placentula* treated with UV radiation came third in Batch V with 60 x 10^6 cells/ml and overall highest under 24h light followed by 46.8 x 10^6 cells/ml for *C. vulgaris* treated with chloramphenicol then *S. quadricuada* having 38.3 x 10^6 cells/ml treated with penicillin the lowest densities were obtained under 12:12h L:D.

Batch C yielded the overall highest cell densities of 76.24 x 10^6, 158.2 x 10^6, 195.2 x10^6 and 244.5 x 10^6 *C. vulgaris* cells/ml when treated with penicillin while 55.5 x 10^6 and 62.13 x 10^6 C. *vulgaris* were obtained using chloramphenicol both at 15:09h L:D followed by chloramphenicol treated algae (*S. quadricuada)* with 48.10 x 10^6 and 51.30 x 10^6 cells/ml. The highest values of *S. quadricauda* was 12.7 x 10^6, at 12:12, 21.22 x 10^6 at 15:09, and 27.3 x 10^6 at 24h light using penicillin while 25.10 x 10^6, 35.5 x 10^6, and 38.2 x 10^6 *S. quadricauda* cells/ml were obtained using ampicillin at 12:12h L:D, 15:09h L:D and 24h light respectively.

Batch A was best at 15:09h L:D producing 96.21 x 10^6, 130.10 x 10^6, 142.47 x 10^6, and 154.47 x 10^6 *C. vulgaris* cells/ml using 1.0 ml hypochlorite followed by *S. quadricauda* treated hypochlorites yielding 51.37 x 10^6 and 55.0 x 10^6 respectively at 1.0 ml and 2.0 ml hypochlorite.

At both 12:12h L:D and 24h light, none of the three algae *C. vulgaris, E. elegans* and *S. quadricauda* treated with hypochlorite was above 30 x 10^6 cells/ml. Generally, *Chlorella, Characium, Eudorina, Pleodorina, Closterium* and *Stephanosphaera* spp were selective against U.V light. These species including *Gonium* spp were dominant in the antibiotic treatments. *Chlorococcum spp, Tetraspora spp* and *Chlamydomonas spp* were only selective against ampicillin. *Anabaena sp* and *Scenedesmus spp* were selective against penicillin and Chloramphenicol, respectively. A mixture of antibiotics gave a negative result. Attempts to culture *Volvox* spp proved abortive in all trials.

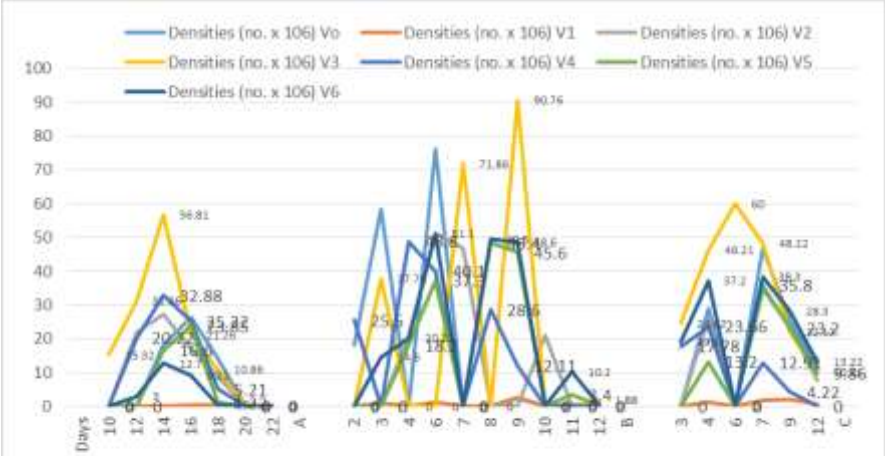

Figure 1. Changes in density of 4 microalgae-*Chlorella vulgaris, Cocconeis placentula, Eudorinaelegans* and *Scenedesmus quadricauda*(nos. x 10^6 cells/ml plus 5 axenic treatments (Vo CV on Chloram, V_1 EE on peni, V_2 CV on ampi, V_3 CP on U.V, V4 CV on ampi.

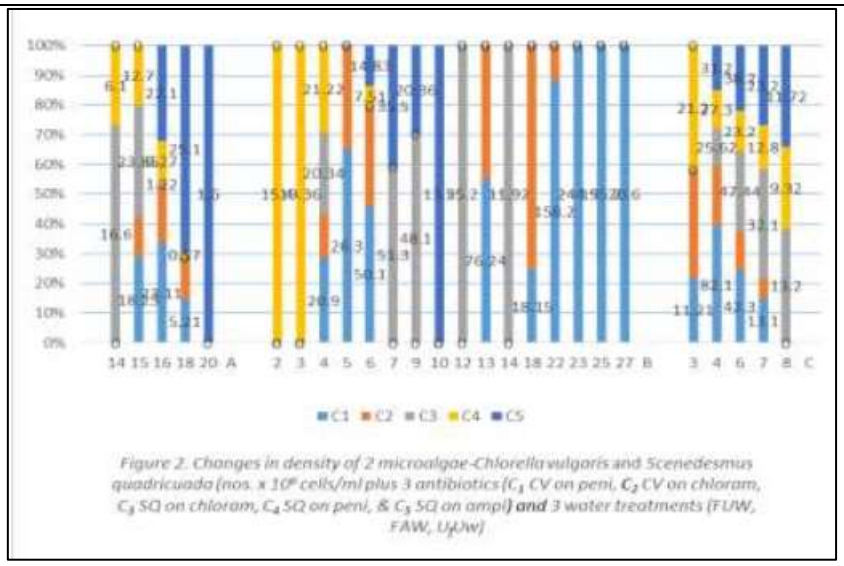

Figure 2. Changes in density of 2 microalgae-Chlorella vulgaris and Scenedesmus quadricauda (nos. x 10⁶ cells/ml plus 3 antibiotics (C_1 CV on peni, C_2 CV on chloram, C_3 SQ on chloram, C_4 SQ on peni, & C_5 SQ on ampi) and 3 water treatments (FUW, FAW, UVW)

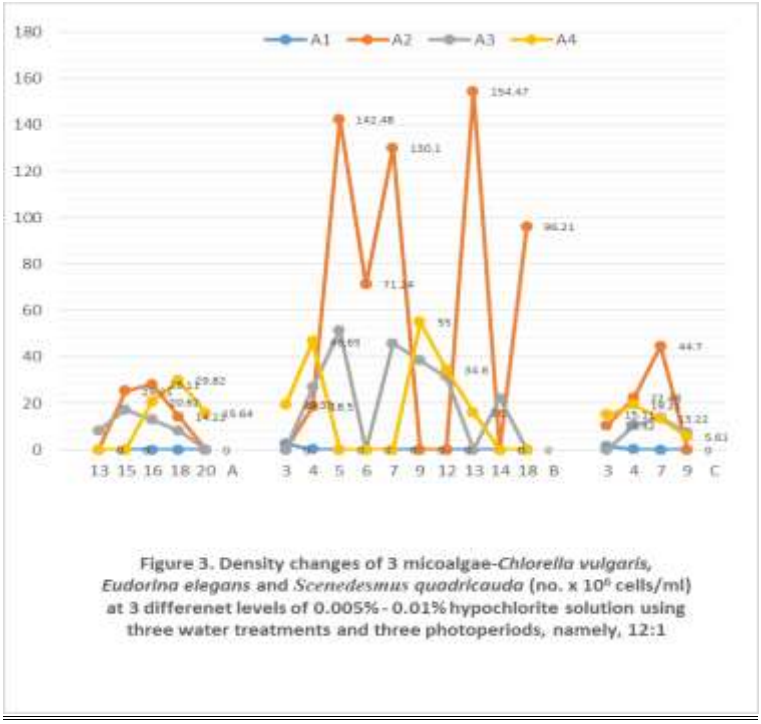

Figure 3. Density changes of 3 micoalgae-*Chlorella vulgaris*, *Eudorina elegans* and *Scenedesmus quadricauda* (no. x 10^6 cells/ml) at 3 differenet levels of 0.005% - 0.01% hypochlorite solution using three water treatments and three photoperiods, namely, 12:1

Table 1. A comparison of the cell division rates (k) of four microalgae- *Chlorella, Cocconeis, Eudorina* and *Scenedesmus* plus four axenic treatments - ampicillin, chloramphenicol, penicillin and u.v. radiation, using filtered autoclaved water (V_5 to V_6), at three photoperiods: 12:12h L:D, 15:09h L:D and 24h cycles.

Photoperiod	V_o	V_1	V_2	V_3	V_4	V_5	V_6	Av	t_D V_5	t_D V_6
12:12h L:D	0.266	0.155	-0.057	0.079	0.080	0.023	0.090	0.160	30.135	7.7
15:09h L:D	0.518	0.274	0.390	0.211	0.028	0.122	0.209	0,282	5.681	3.316
24:00h L:D	0.036	0.157	0.029	0.026	0.089	0.145	0.099	0.033	4.78	7.0
Average	0.249	0.196	0.111	0.088	0.006	0.240	0.241	0.165	-	-

Note: Nt = penultimate cell count was used in calculating k.

V_0 = *Chlorella vulgaris* treated with chloramphenicol, V_1 = *Eudorina elegans* treated with penicillin V_2 = *C. vulgaris* treated with ampicillin, V_3 = *Cocconeis placentula* treated with u.v radiation

V_4 = *C. vulgaris* treated with ampicillin, V_5 = *Scenedesmus quadricauda* treated with chloramphenicol
V_6 = *S. quadricauda* treated with penicillin

Cocconeis placentula and *E. elegans* exponential growth started at day three post inoculation and maintained a steady increase up to the peak of 90.76 x 10^6 cells per ml (overall) highest initial peak and 2.63 x 10^6 cells per ml respectively at day 9. The rest attained their peak cell densities before day-9 and started declining. The shortest time of two days for the lag phase to commence was observed with *C. vulgaris* using chloramphenicol (18.12 x 10^6 cells per ml) and 25.5 x 10^6 cells per ml using ampicillin. Bloom take-off time was on the average three days and lasted for average of 11.1 days. Overall ranking in descending orders is V1 as highest followed by V_0, V_4, V_5, V_2, V_6 and V_1.

Eudorina elegans maintained same steady increase as in 12:12h and 15:09h L:D up to day-9 while *C. placentula* was steady up to day-6, attaining a peak of 60.0 x 10^6 cells per ml. On the average, it took 3.56 days for bloom to be attained, 12 days for decline. Generally, growth was steady, and a gradual decrease was observed. A uniform pattern of growth was observed for all V_0 to V_6. Ranking in descending pattern gave V_3 the first position followed by V_6, V_0, V_2, V_5, V_4 and V_1. Thus, displacing V_0 from 6^{th} to 2^{nd} position. The increase of the illumination hours must have been in favour of *Scenedesmus* (V_6) and *Chlorella* (V_0, V_2) but a decrease in *Chlorella* treated with ampicillin (V_6). peak cell densities for *Chlorella* ranged from 23.66 x 10^6 to 46.8 x 10^6, 2.12 x 10^6 cells per ml for *E. elegans*, and *Cocconeis* 58.21 x 10^6 cells per ml.

An average of 15 days was required for initiation of the log phase of cell growth (Table 4) compared to the 13 days required using filtered autoclaved water (Table 1), under the same 12:12h L:D photoperiod. A generally lower cell growth and sharp fall in cell densities was also observed. Microalgal growth in the filtered autoclaved water performed better than the filtered un-autoclaved water of Table 6 at the 12:12h L:D photoperiods.

An average of 4.0 days was required for the onset of cell log phase (Table 5) compared to three days needed for the same 15:09h L:D photoperiod with filtered autoclaved water (Table 2). However, the cell growth rate was very similar to each other. When *Chlorella* was divided into four portions at peak cell density of 76.24 x 10^6 cells per ml, attained (in day-13, and re cultured in four -one-liter flasks, maximum average peak cell density of 244.50 x 10^6 cells per ml within 10 days was obtained.

While commencement of log phase of cell took 3.4 days on the average using filtered unautoclavd water, 3.14 days was required under the same 24h photoperiod with autoclaved water. Table 6 shows that cell decline in filtered un-autoclaved water took nine days as against 12 days with filtered autoclaved water (Table 3). However, a significant edge in cell growth with filtered un-autoclaved water, over those of filtered autoclaved water was observed. The highest initial cell density peak of *Chlorella* (82.10 x 10^6 cells per ml) was observed with un-autoclaved water plus penicillin (C_1) at day-6 and day-13 respectively as against four days for C recorded under continuous light cycle.

Model 11 analysis of variance (anova) for *S. quadricauda* was significant at α= 0.05 level of test in either FAW, FUW or U_fUW at the three photoperiodic regimes combined. Least significant difference test (LSD) indicated that 15:09h L:D > 24h > 12:12h; 24h >15:09h >12:12h >24h; and 15:09h >12:12h > 24h photoperiods in FAW, FUW and U_fUW respectively. When FAW, FUW and U_fUW were pooled together at three separate photoperiods, *S. quadricauda* was not significantly different at 112:2h L:D and 15:09h L:D photoperiods but significantly different at each treatment under 24h photoperiod ($p < 0.05$). LSD showed that FAW > FUW > U_fUW at α = 0.05 level test.

Anova for *C. vulgaris* was significant at $p<0.05$ level test using FAW and U_fUW but not significant using FUW ($p>0.05$). LSD showed that both at FAW and U_fUW; .15:09h > 12:12h. When *C. vulgaris* was tested at a combination of FAW, FUW and U_fUW, using three separate

photoperiods. Only 15:09h photoperiods indicated significance ($p<0.05$) . At 15;09h, LSD indicated that U_fUW was significantly greater than FUW and FAW, and FUW significantly greater than FAW ($p<0.05$).

On bacterial-microalgal interactions, the interrelationship between the two using the single - classification model 1 anova, a highly positive significant difference ($P<0.001$, F ratio 7.090, F expected α = 0.05 = 4.05) was observed in group 1, with the microalgae significantly greater than the bacteria. Student t - test at α = 0.05 level test, equally indicated positive significance (tscal =2.662, ts α=0.05 = 2.069). Group 2 in which monitoring commenced when the algae population was collapsing, showed a non-significant F test at α = 0.05, though the mean of bacteria was greater than that of microalgae, the correlation values (0.563) were non-significant in both groups at α= 0.05 level test.

Table 2. A comparison of the cell division rates (k) of four microalgae- *Chlorella, Cocconeis, Eudorina* and *Scenedesmus* plus four axenic treatments - ampicillin, chloramphenicol, penicillin and u.v. radiation, using filtered unautoclaved water (C_1 to C_5), at three photoperiods: 12:12h L:D, 15:09h L:D and 24h cycles.

Photoperiod	C_1	C_2	C_3	C_4	C_5	Average
12:12h L:D	0.342	0.672	0.523	0.302	0.092	0.386
15:09h L:D	0.381	0.173	0.039	0.231	0.114	0.187
24:00h L:D	0.639	0.093	0.081	-.146	-.107	0.112
Average	0.454	0.312	0.214	0.129	0.033	0.228

C_1 = *C. vulgaris* treated with penicillin, C_2 = *C. vulgaris* treated with chloramphenicol
C_3 = *Scenedesmus* quadricauda treated with chloramphenicol, C_4 = *S. quadricauda* treated with penicillin
C_5 = *S. quadricauda* treated with ampicillin

Table 3. A comparison of the cell division rates (k) of four microalgae- *Chlorella, Cocconeis, Eudorina* and *Scenedesmus* plus four axenic treatments - ampicillin, chloramphenicol, penicillin and u.v. radiation, using filtered unautoclaved water (C_1 to C_5), at three photoperiods: 12:12h L:D, 15:09h L:D and 24h cycles

Photoperiod	A_2	A_3	A_4	Average
12:12h L:D	0.157	0.403	2.452	1.004
15:09h L:D	0.922	0.028	0.093	0.348
24:00h L:D	1.109	0.124	-0.048	0.395
Average	0.729	0.185	0.832	0.592

A_2 = *C. vulgaris* using 1.0 ml hypochlorite, A_3 = *S. quadricauda* using 1.0 ml hypochlorite
A_4 = *S. quadricauda* using 2.0 ml hypochlorite

Table 4. Intrinsic rate of natural increase (r) and the mean doubling tine (t_D) in days of microalgae at the three photoperiods: 12;12h L;D, 15:09h L:D, and 24:00h L:D cycles

Microalga	Symbol	12:12h		15:09h		24:00h	
		r	t_D	r	t_D	r	t_D
Chlorella	V_0	0.185	3.758	0.359	1.031	-0.025	-27.78
	V_1	-0.039	-17.59	0.270	2.563	-0.020	-34.10
	C_1	0.237	2.922	0.264	2.626	0.443	1.566
	C_2	0.466	1.4898	0.119	5.838	0.064	10.776
	A_2	0.109	6.365	0.111	6.267	0.364	1.904
Scenedesmus	V_5	0.181	3.925	0.184	3.773	0.093	7.474*
	V_6	0.235	2,951	0.200	3.458*	0.066	10.577
	C3	0.362	1.913*	0.027	25.88	0.056	12.295

	C4	0.105	6.625	0.160	4.324	0.101	-6.868
	C5	0.064	10.89	0.079	8.748	-0.074	-9.358
	A1	0.232	2.988	0.019	35.723	0.086	8.094
	A4	0.185	3.758	0.065	10.714	-0.033	-20.747
Cocconeis	V1	0.055	12.691	0.146	4.742*	-0.018	-38.837
Eudorina	V1	0.247	2.803	0.342	2.027*	0.109	6.366

Table 5. Bacterial-microalgal interaction at commencement of bloom (1) and from the collapse of microalgae (2) under semi-controlled condition.

Day	Bacteria (no. X 10^6 cells/ml)	Phytoplankton: *C. vulgaris* (no. x 10^6 cells/ml)	Bacteria (no. X 10^6 cells/ml)	Phytoplankton: *C. vulgaris* (no. x 10^6 cells/ml)
03	3.67	37.85	46.81	9.35
06	5.78	25.15	55.30	8.03
07	6.35	16.52	15.30	7.18
08	9.13	19.60	9.58	10.88
-09	12.40	17.60	-	-
12	37.35	13.23	6.03	7.68
15	13.78	6.16	12.88	6.75
16	3.10	13.85	4.68	7.63
17	7.08	7.88	7.68	7.60
19	3.00	6.88	3.73	10.28
20	1.53	11.73	3.28	5.23
22	5.40	13.13	2.41	8.13
23	3.88	6.65	3.95	10.75
24	2.88	7.15	-	-
26	5.00	3.65	-	-
29	20.33	3.05	3.80	9.3
31	1.25	10.01	-	-
32	1.80	6.80	1.53	11.73
34	1.48	8.33	-	-
35	0.98	10.90	9.63	7.43
36	0.83	11.85	-	-
38	2.28	11.20	2.90	0.9
40	7.80	43.40	1.80	6.80
46	1.53	11.73	1.48	8.33

The interrelationship between bacteria and microalgae is shown in table 5. using the single classification model 1 anova, a highly positive significant difference ($p<0.001$) was observed in group 1 with the microalgae significantly greater than the bacteria. Student t-test at $\alpha = 0.05$ level test, also indicated positive significance. Group 2 in which monitoring commenced when the algae population was collapsing, showed non-significant F-test at $\alpha = 0.05$ level test, though the mean of bacteria was greater than that of microalgae, the correlation values were non-significant in both groups at $\alpha = 0.05$ level test

Discussion

Microalgal Growth

Growth, measured in terms of number of cells per volume, cell division rate and mean doubling in days of *Chlorella vulgaris* Beijerinck; *Cocconeis placentula var. euglypta* (Ehr.) CL, *Eudorina elegans* Ehrenberg and *Scenedesmus quadricauda* Meyen were best at 15: 09h light (L): dark (D) illumination period, followed by 24h L and 12:12h L:D cycles. Similar to the findings in

this work, Toro (1989) had higher mean doublings per day for exponentially growing cells at 12:12h L:D cycles as 1.47 and 1.56 for *Chaetoceros gracilis* Schult and *Isochrysis galbana* clone T iso respectively, corresponding to the present values of 1.93 and 2.03 for *Chlorella vulgaris* Beij, and *Eudorina elegans* Ehr. At continuous light, Toro (1989) had 1.37 and 1.49 for *Chaetoceros* and *Isochrysis* respectively, while a better t_D of 1.90 for *Chlorella* and higher t_D of 6.37 for *Eudorina* was obtained in this report. The lowest value of 1.48 was obtained under 12:12h for *Chlorella. Scenedesmus quadricauda* Meyen had its lowest mean doublings per day as 1.91, 3.46 and 7.47 under 12:12h L:D, 15:09h L·D and 24h cycles, respectively. *Cocconeis placentula var euglypta* (Ehr.) CL had 12.69, 4.74 and -38.84 for 12:12h L:D, 15:09h L:D and 24h cycles in that order. Other values are as given in Table 5. Castenholz (1964) proved the growth of two littoral diatoms *Fragilaria striatula* and *Synedra tabulata* depend upon day length. With a significantly lower cell short day periods which agrees with the result of 12:12 L:D cycles shown in Tables 1, 2 and 3. Cell division rate was found to be best at 12:12h (Av. k=0.517) cycles with average values of 0.16, 0.39 and 1.00 for rnicroalgae in filtered autoclaved water, filtered un-autoclaved water and unfiltered un-autoclaved water respectively. At 24:00h L: D the second highest division rate of 1.11 was realized for *Eudorina* using 0.5 ml hypochlorite solution while the overall highest value of 2.45 was at the 12:12h L:D photoperiod for *Scenedesmus* using 2.0 ml hypochlorites solution as the antibiotics. Vitamin B1, B6 and B12 enhanced algae growth.

Light intensity of 5,800 lux and cabinet temperature of 30°C ± 2°C proved to be the best condition for the growth of four micro-algae. This work agrees with that of Jeong-yeol and Jong-hwa, (1985) who had better growth at 6000 lux and poor growth at l000 lux, and instant death of cells at 10,000 lux. Rapid reduction in growth and photosynthetic rate observed at 12: 12h L: D cycle with a correspondingly maximum growth and photosynthetic rate at 15:09h L: D photoperiods confirm the findings of Castenholz (1964). Castenholz, Bauld and Jorgenson (1990). Toro (1989), and Su, Lei and Liao (1990) observed low cell densities to low temperature, illuminance, salinity, and their interactions in the incubators like the lowest temperature of 28 °C observed in this report under 12:12h L:D cycle. Day-length was not a variable and temperature increase were within a certain range which generally resulted in an increment in the maximum growth and photosynthesis. Curl and Mcleod (1961); Jitts, McAllister, Stephen and Strickland (1964) agree with this result.

Subjecting microalgae (*Coccolithus huxley, Dictylum brightwellii* and *Nitzschia turgidula*) to continuous light resulted in an inhibition in growth (Pirson, 1957; Tamiya 1957; Sorokin & Krauss, 1959; Erben, 1962). It was observed that cell growth at 24h light lasted for less than 24h in some species with maximum of nine days, seven days, and six days respectively, for filtered autoclaved water (FAW), filtered un-autoclaved water:-(FUW), and un-filtered un-autoclaved water (U_fUW) treatments, far below 27 days under 15·09h L: D cycle. On the contrary, Sorokin and Krauss, (1959) observed - a nonsignificant difference between the continuous light and 16:8h L:D cycle, whereas the F-test (Sokal & Rohlf, 1981) carried out here indicated a positively significant difference, that is, at $p<0\ 05$. In both the FAW and U_fUW the 15:09h L:D photoperiod was significantly greater than the 24h photoperiod. The continuous light also had significantly greater cell densities than the 15·09h L:D cycles using FUW at $p<0.05$ level test with reference to *Scenedesmus.* The 12:12h L:D was least significant among the three photoperiods. With *Chlorella*, the 15:09h L:D photoperiods always gave a significantly better growth performance over the 24h light cycle at $p<0.05$. Least cell densities were obtained under 12·12h L:D photoperiod over the 24h photoperiod indicating that certain amount of the dark phase is needed in the tricarboxylic acid (TCA) cycle during photosynthesis of *C. vulgaris; C. placentula; E. elegans* and *S. quadricauda* as pointed out by Pirson (1957) on *Hydrodyction* spp and by Bountry, Barbier and Richard (1976) on *Chaetoceros simplex var calcitrans* but not as much as 12 hours. Continuous light supply resulted in massive production of carbohydrates

(Castenholz, 1964; Paasche, 1968; Vander & Bonotto,1972).

Sequel to a dark phase, the carbohydrate either disappears in some species like *Dictylum* or are further converted to protein or to other rnonosaccharides and amino acids (Werner, 1966; Eppley, Holmes & Paasche, 1967; Hama, Matsunaga, Handa & Takahashi 1988). Light: dark cycles on the other hand may affect the growth rate of algae positively or negatively, depending on the species and other environmental factors (Brand & Guillard, 1981). In this report a positive effect of light: dark phase was observed at 15:9h L:D, while negative effect was seen under the 12:12h L:D photoperiod.

The effect of isolation procedure of axenic cultures by washing and by using u.v. radiation for *Oscillatoria rubescens* and *O. redekei* proved that the mechanical method by washing and culturing of a subsequent series of dilutions was the most suitable (Meffert & Chang, 1978). Treatment with u.v radiation was selective to some algae such as *Chlorella, Characim, Closterium* and *Cocconeis placentula* etc as observed in this report, though more attention was paid to *C. placentula*. The high dosage must have made it intolerable to most algae (Meffert, 1972). Best growth (244. 5 x 10^6 cell per ml) of *Chlorella* was observed with penicillin using filtered un-autoclaved water post cell division. This value far exceeded the 175 x 10^6 *Chlorella vulgaris* cells per ml realized after CO_2 addition (De Pauw & De Leenheer, 1979). Thus, indicating that penicillin effectively axenized the culture thereby yielding such an impressive growth. The next highest value of 154.47 x 10^6 *C. vulgaris* cells per ml was observed using 1.0 ml hypochlorite solution in U_fUW, while the 3rd (82.10 x 10^6 *C. vulgaris* cells per ml) with penicillin, the 4th highest (76.10 x l0^6 cells per ml) was observed with chloramphenicol in FAW. Investigations revealed that autoclaving is not too crucial in attaining axenic cultures. Filtration and autoclaving without appropriate antibiotic treatments in most cases yielded no positive result. Algal growth is seen to be dependent on its selectivity to specific antibiotic treatment. Most algae were not selective to ampicillin treatment, hence, in-efficient in inhibiting bacterial growth, often leading to low yield.

Growth inhibition and lysis contributed to washing procedures, such as washing of individual trichomes (Pringsheim, 1965), of colonies (Gorham, Mclachlan, Hammer & Kim 1964) washing with chlorine water (Fogg, 1942), or with filtered tap water, was not observed in this work, rather, treatment with chlorine proved second best to penicillin and produced axenic algae that lasted for 18 days. Lysis only occurred at very high dose (5.0 ml per litre) while using 0.005% to 0.01% hypochlorite solution. While inhibition in growth was recorded using ampicillin in most cases, U.V. radiation yielded very high cell density of 90.76 x 10^6 per ml at 15:09h L:D and up to 56.81 and 60 x 10^6 cells/ml at 12:12h L:D and 24h photoperiods, respectively. Thus, growth was not inhibited at any of the three photoperiods.

Subdividing algal cells into smaller units at initial cell density peaks have been proven to be very rewarding. Peak cell densities of 154.47 x 10^6 and 244.5 x 10^6 *Chlorella vulgaris* cells per ml were realized using hypochlorite and penicillin after dividing them into two and four portions respectively. These new peaks must have been due to reduction in the accumulated metabolic load, enhancement of growth resulting from the addition of fresh nutrient medium, increased space for further cell divisions, and reduced pH via the inclusion of more CO_2 produced by the nutrient medium and fresh borehole water.

The results from the present study are quantitative (number of cells per volume), and show that these four microalgae under three lighting regimes produced varying concentrations of cells per ml. However, further studies are required to assess the biochemical compositions of these algae, though the importance of their being tested during nutritional status have been tested during feeding in combination with zooplankton on catfish larvae.

Bacterial -Microalgal Interaction:

Both the model 1 anova and the student t-test indicated high positive significance ($p<0·001$) between bacteria and algae in group 1 whereas group 2 did not statistically show any edge of

one over the other, although bacteria had a higher mean than the microalgae.

Bacteria population tended.to increase or decrease with that of microalgae to an extent. As soon as either the bacteria or microalgae gained advantage over the other, inhibition in growth of the other starts. This could result in total decline or near death. The ratio of bacterial growth to algal growth and vis-a-vis averaged 1:10 from this report. This is in consonance with the findings by Hirata, Ushiro and Hirata (1982) who observed increase in bacterial density after *Nannochloropsis* sp had been consumed by rotifers. As soon as microalga reaches a point of no return as in group 2, it becomes almost impossible to resuscitate it. Momentarily, the bacterial population explodes, reaching a peak and subsequently, follow the decline curve.

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