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Determination of the Limiting Effects of Nitrate-Nitrogen ($N0_3^-N$) and Phosphate- Phosphorus ($P0_4^{=}P$) on the Growth of *Selenastrum Gracile* in Laboratory Culture

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Abstract: *This study on the determination of the limiting effects of Nitrate-nitrogen and Phosphate-phosphorus on algal growth, using Selenastrum gracile in laboratory culture, was conducted over a nineteen day period. Water samples were collcted from a small permanent pond in the University of Calabar Teaching and Research farm. Algal species contained in the collected water samples were identified. S. gracile which was the most dominant algae present in the pond water was used for the study. Species of the identified* S. gracile *were cultured in the laboratory using various combinations of nitrate-nitrogen ($N0_3^-N$) and phosphate-phosphorous ($P0_4^{=}P$) in triplicate cultures at concentrations of 0.00-0.40 gl^{-1} and 0.00-0.10 gl^{-1} respectively, to determine which of these nutrients is more limiting to algal growth. The study was conducted in two parts over period of 19 days. Part I (a-priori test) was to assess NO_3^-N and $PO_4^{=}P$ requirement in S. gracile. Part II (a-posteriori test), was to determine which of NO_3^-N and $PO_4^{=}P$, is more limiting to growth of S. gracile. Cell counts per ml were taken every other day for nine days in the first run of experiments while cell count the second experiment was taken every ten hours for ten days. Data collected from both experiments were analyzed using the two-way analysis of variance and means separated using New Duncan's multiple range test. Results from the a-priori test, showed significant difference ($P< 0.05$) between cell counts in the different concentrations of NO_3^-N and $PO_4^{=}P$, indicating that S. gracile require nitrate and phosphate for growth. In the second run of experiments, there was significant difference ($P< 0.05$) in cell count when the concentration of nitrate was varied, (0.00-0.10 gl^{-1}) while that of phosphate was kept constant at 0.05 gl^{-1}. When nitrate concentrations were kept constant at 0.0 gl^{-1} and 0.2 gl^{-1} and that of $PO_4^{=}P$ constant, there was no significant difference ($P> 0.05$). The result shows that nitrate and phosphate are growth limiting nutrients to algae in tropics. There is synergy between the two mineral nutrients and the effective ratio of N: P, to obtain optimum growth is 8: 1.*

Keywords: *Algal,* Selenastrum gracile**,** *phytoplankton, limiting nutrient, bloom, Nitrate-nitrogen, Phosphate-phosphorus.*

1. INTRODUCTION

The significance of phosphate, as a cause of excessive fertilization problems in fresh and marine waters has been the subject of controversy for many years. Large amount of research funds have been committed to this topic [1]. [2], in their study on the ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems, stated that it can stimulate or enhance the development, maintenance and proliferation of primary producers, resulting in eutrophication of aquatic ecosystems. [1] had reported that N:P ratio in ponds is 16:1 and that phosphorus is the key mineral nutrient that controls primary production in freshwater, while nitrogen controls primary production in the oceans. As a result of have these researches, a good understanding of the relationship between nutrient input to a water body and its effect on eutrophication-related quality problems are being understood. Phytoplankton are composed of microscopic plants which are almost exclusively autotrophic. They play a key role in the aquatic food chain, serving as primary producers. In addition they serve as food for fish and fish food organisms. Considerable efforts have been directed towards the identification of growth-limiting nutrients of phytoplankton in freshwaters [3] and [4]. Studies on plant nutrient requirement have emphasized the importance of nitrogen and phosphorus [5] whose compounds have been shown to be important in regulating the productivity of algae [6]. [7] and [8] stated that inorganic nitrogen, especially Nitrate-Nitrogen ($N0_3^-N$) is considered a limiting factor for the growth of temperate algae. Phytoplankton is important in fish food chain and in primary productivity of

aquatic ecosystems. In a bid to increase nutrient level in ponds and hence increase productivity, aquaculturists often resort to the use of fertilizers. In the process of fertilization, eutrophication occurs resulting in algal bloom. For many years, eutrophic conditions in inland water systems, have been attributed more to excessive inputs of phosphorus and nitrogen [9]. New evidence suggests that both nitrogen and phosphorus are important and that improving water quality in certain lakes and estuaries that have experienced man-made eutrophication, require reducing inputs of both nutrients [9] and [10]. This study is therefore aimed at assessing which of Nitrate-Nitrogen ($N0_3^-N$) and Phosphate-Phosphorus ($P0_4^{=}P$) is more limiting to algal growth in the tropics. For the purpose of this study *S. gracile* a phytoplankton, which is the most dominant algae in the freshwater fish pond under consideration.

2. MATERIALS AND METHODS

2.1. Study Area

This study was conducted over nineteen (19) days in the University of Calabar Fisheries Research Laboratory. Calabar is in South Eastern Nigeria, lying on longitude 8.1^0 and 8.2^0E and latitude $4,5^0$ 5.0^0N of the equator.

2.2. Collection of Water Samples and Identification of Algal Species

Water samples were collected using a 250 ml glass stoppered bottle. Sub-samples of the water were viewed under the microscope at a magnification of x40, to identify the algal species present. To aid identification, the key according to [11] was used. Five algal species viz *Selenastrum gracile, Oscillatoria lacustris, Ulothrix species, Closterium moniliferum and Diatoma vulgaris* were identified. Out of the five algal species identified, *S. gracile* and *C. moniliferum* were observed to be abundant in the pond water, while the most dominant of the two was ***S. gracile*** was used for the study. This species occurred naturally at a concentration of approximately 6,000 cells per ml.

2.3. Algal Suspension

The water sample was filtered through a sterile non-absorbent cotton wool plug, lodged in the neck of a conical flask, to remove large zooplankton and debris. A 100 ml sample of the suspension of algae was filtered through a sterile Nalgene filter unit of pore size 0.20 nm, using a vacuum pump. The algal suspension was reduced to between 1 – 2 ml and washed using distilled water, to free the algae any dissolved nutrient [12]. The washed algae was re-suspended in a 250 ml of sterile distilled water and shaken vigorously, to break up the lump.

2.4. Experimental Procedure

This study was conducted in two parts (a-priori and a-posteriori Tests).

A – Priori:

The first part was to determine the effect of Nitrate-Nitrogen ($N0_3^-N$) and Phosphate-Phosphorus ($P0_4^{=}P$) on the growth of *S. gracile.* This was achieved by preparing two sets of three stock solutions used for culturing the algae. In one set, all three solutions were without phosphate but had 0.0g/l , 0.2g/l and 0.4g/l of nitrate respectively. In the second set phosphate concentration was constant at 0.05g/l while concentrations of the nitrate were0.0g/l, 0.1g/l and 0.3g/l respectively.

A – Posteriori:

The second part of the study was conducted to confirm which of the two nutrients is more limiting to the growth of *S. gracile*. Two sets of the stock solutions were also prepared and used. In one set, nitrate concentration was kept constant at 0.0 g/l, while phosphate concentrations in the three solutions were 0.1 g/l, 0.0 g/l and 0.05 g/l respectively. The second set of solutions had nitrate concentration constant at 0.2 g/l, while phosphate concentration varied between 0.0 g/l, 0.025 g/l and 0.075 g/l. The basic algal Chu no. 10, as described by [3], [13] was used for culturing the algae. The chu 10 medium is a culture medium used in microbiology for the culture of algal species. It includes essential minerals and trace elements that are required by algae for growth.

Stock solutions were prepared at 10x concentrations of the final working solutions. Concentrations of working solutions ranged from 0 – 0.4 gl^{-1} N and 0 – 0.1 gl^{-1} P for the first part of the study. In the second part of the study, the concentration of nitrate was varied from 0.0-0.1 gl^{-1}, while that of phosphate was kept constant at 0.05 gl^{-1}. Triplicate working solutions were made from the stock solutions, by putting 10 ml into 100ml of distilled water in 150 ml conical flasks, which had been autoclaved at 1.05 $kgfcm^{-3}$ for fifteen minutes to reduce contamination.

Equal amounts of washed algal suspensions were placed in each conical flask, using a sterile pipette. The exact amount was calculated, after an approximate count of the original algal suspension, to give a concentration of between 500-1,800 cells ml^{-1} when diluted for counting. The flasks were randomly placed on a flask shaker, which had the lid removed and faced to a window vent to maximize light interception for 9 days in the first run of experiment and 240 hrs or 10 days, in the second run of experiment. The shaking speed was 130 r.p.m.

2.5. Cell Count

Algal cells were quantified using the sedgewick-rafter counting chamber, where

Algal..Samples of between 50 and 200 µml were taken, depending on density, using a micropipette and made up to 1ml with distilled water in a 1ml x 1000 counting chamber. Counting was performed under a dissecting microscope set at x100. Using laboratory culture, increase in the cell number was regarded as a measure of growth. S. *gracile* occurring as a coenobium of four cells was regarded as one individual for the purpose of counting. The number of cells in the first run of experiments were counted over a period of nine days while counting in the second part was every eight hours for ten days. Triplicate samples were compared using two-way analysis of variance and means separated using the New Duncan's multiple range test [14]; [15].

3. RESULTS

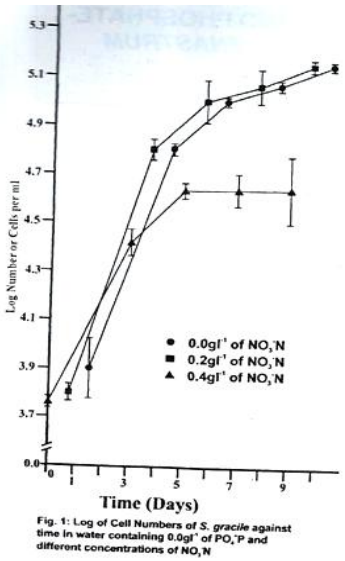

Fig. 1: Log of Cell Numbers of *S. gracile* against time in water containing 0.0gl⁻¹ of $PO_4^{=}P$ and different concentrations of NO_3N

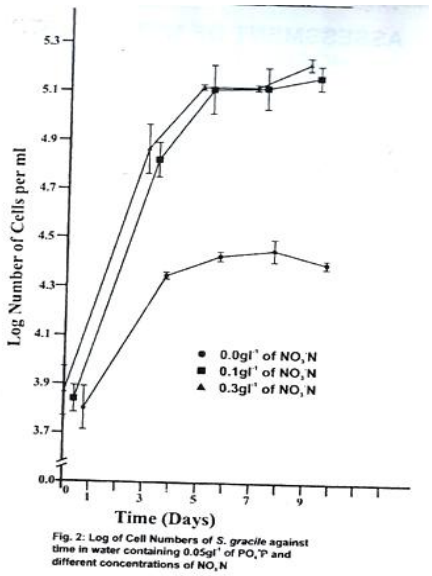

Fig. 2: Log of Cell Numbers of *S. gracile* against time in water containing 0.05gl⁻¹ of $PO_4^{=}P$ and different concentrations of NO_3N

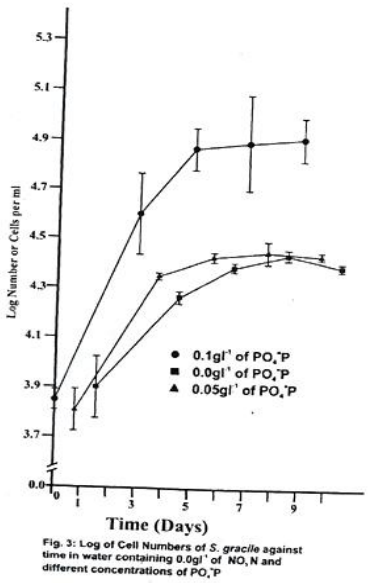

Fig. 3: Log of Cell Numbers of *S. gracile* against time in water containing 0.0gl⁻¹ of NO_3N and different concentrations of $PO_4^{=}P$

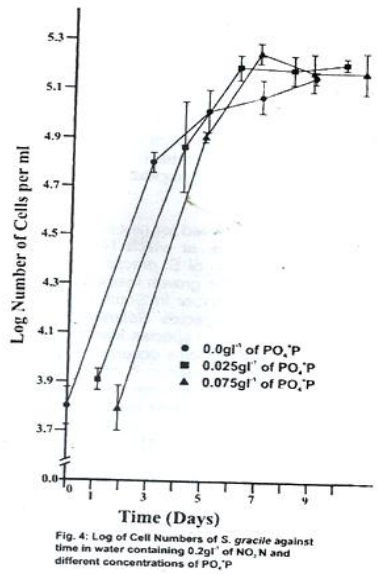

Fig. 4: Log of Cell Numbers of *S. gracile* against time in water containing 0.2gl⁻¹ of NO_3N and different concentrations of $PO_4^{=}P$

Data presented as means, with standard deviation were plotted for two phosphate concentrations (Fig 1 and 2) and two nitrate concentrations (Fig 3 and 4). Two way analysis of variance with interaction on each of the four, sets of data, indicated a significant difference ($P < 0.05$). In Fig. 1 where

phosphate concentration in culture media was kept constant at 0.0 g/l, cell counts in media with 0.0 g/l and 0.2 g/l of nitrate were not significantly different ($P > 0.05$) but were significantly different from cell counts in media containing 0.4 g/l of nitrate. At the concentration of 0.4 g/l of nitrate, cell growth reached the stationary phase of log 4.6 cells per ml by day 5 of the study period, whereas cell growth in media containing 0.0 and 0.2 g/l had the exponential phase extending as far as the day 9 of the study. Similar pattern was observed in Fig. 2 where phosphate concentration was kept constant at 0.05 g/l while concentration of nitrate was between 0.0 and 0.3 g/l. There was no significant difference ($P > 0.05$) between plots containing 0.0 g/l and 0.2 g/l of nitrate. Both plots were however significantly different ($P < 0.05$) from the plot of 0.3 g/l nitrate. Cell growth in culture media with had entered the death phase by day 9, whereas cell growth in the other two culture media temporarily showed signs of entering the stationary phase between day 7 and 8, and rose to log 5.1 cell/ml and above.

In the second run of experiment where the concentration of nitrate was kept constant, cell number was counted over a period of ten days (240hr). A plot of cell number against time (hr) gave characteristics sigmoid curves (Fig. 5 and 6), in which the following phases of growth were recognized; lag phase, exponential phase, and stationary phase in Fig 5, while in Fig 6 there was in addition the onset of the death phase. There was no significant difference between all growth curves ($P>0.05$). Plot of cell against time (hrs) in cultures containing 0.0 gl^{-1} nitrate and 0.05 gl^{-1} phosphate and only distilled water (Fig. 6) were not significantly different. However, all growth curves in Fg.5 when compared with those of Fig 6 were significantly different ($P<0.05$).

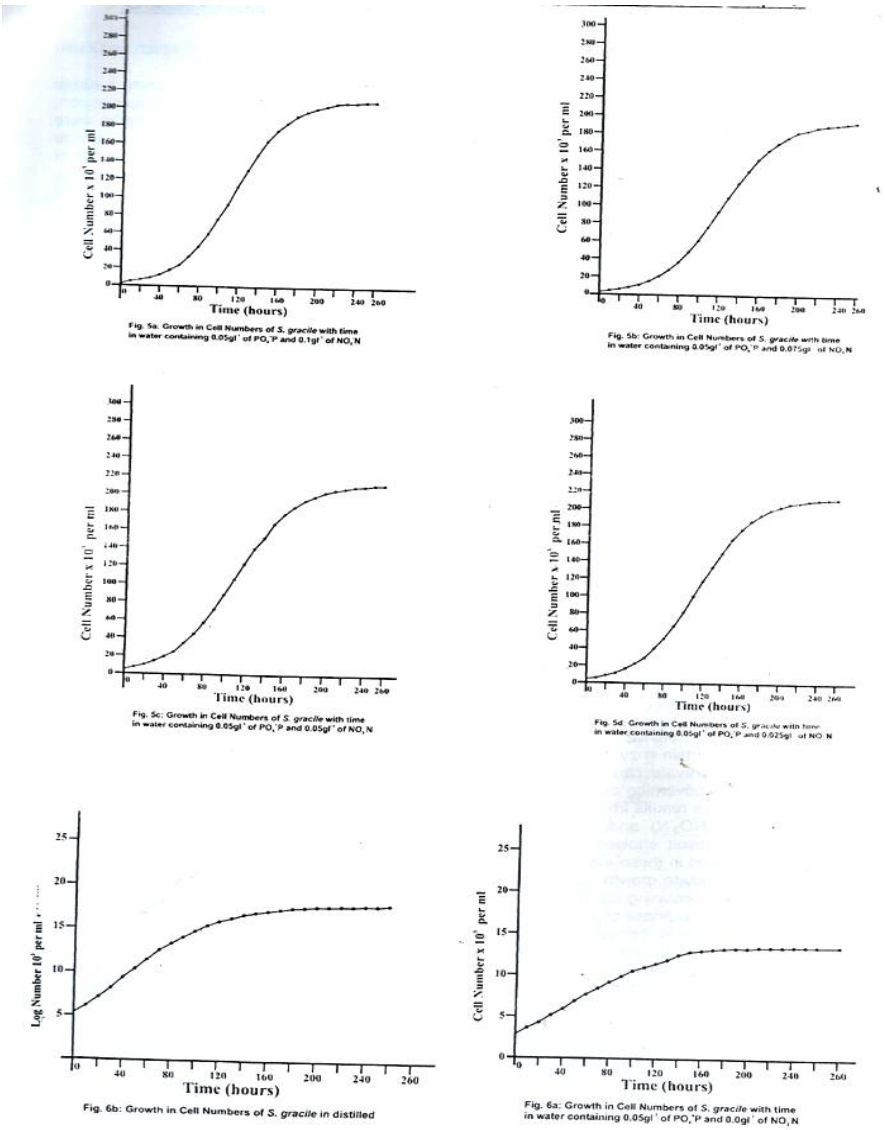

Fig. 5a: Growth in Cell Numbers of *S. gracile* with time in water containing 0.05gl⁻¹ of $PO_4^{3-}P$ and 0.1gl⁻¹ of NO_3N

Fig. 5b: Growth in Cell Numbers of *S. gracile* with time in water containing 0.05gl⁻¹ of $PO_4^{3-}P$ and 0.075gl⁻¹ of NO_3N

Fig. 5c: Growth in Cell Numbers of *S. gracile* with time in water containing 0.05gl⁻¹ of $PO_4^{3-}P$ and 0.05gl⁻¹ of NO_3N

Fig. 5d: Growth in Cell Numbers of *S. gracile* with time in water containing 0.05gl⁻¹ of $PO_4^{3-}P$ and 0.025gl⁻¹ of NO_3N

Fig. 6b: Growth in Cell Numbers of *S. gracile* in distilled

Fig. 6a: Growth in Cell Numbers of *S. gracile* with time in water containing 0.05gl⁻¹ of $PO_4^{3-}P$ and 0.0gl⁻¹ of NO_3N

4. DISCUSSION

This result shows that excess of nitrate above 0.2 g/l, suppress growth of algae. This finding is

supported by the work of [1], who stated that lower and higher ratios of Nitrate and phosphate nutrients limit algal growth.

In Figs 3 and 4, the concentration of nitrate was kept constant at 0.0 g/l and 0.2 g/l respectively while phosphate concentration was varied. In figure 3 cell count in culture media with 0.1 g/l, 0.0 g/l and 0.05 g/l phosphate were all significantly different ($P < 0.05$) from each other. However in Fig 4 where nitrate concentration was kept constant at 0.2 g/l but phosphate concentration were varied at 0.0 g/l, 0.025 g/l and 0.075 g/l, plots of cell count were not significantly difference from each other. By day 7 all three culture media were still in the exponential phase of growth.

The result shows that there is synergy between nitrate and phosphate to limit growth of algal cells. In Fig 3 maximum cell count of log 4.9 was recorded by day 9, for plot with 0.1 g/l of phosphate. Within the same period, plots with 0.0 g/l and 0.05 g/l of phosphate had entered the death phase. In Fig 4, plot with 0.0 g/l of phosphate had entered the death phase by day 9, while plots with 0.025 g/l and 0.075 g/l, were only entering the stationary phase.

This result shows that nitrate and phosphate work in synergy in the freshwater. This is supported by the view of [16], that nitrate and phosphate are basic growth limiting nutrients to algae. Their effect is the same in both tropical and temperate waters. This is contrary to the finding of [1], that phosphate is the key nutrient that controls primary production in freshwater, while nitrate controls in the ocean. Highest cell count of log 5.2 cells/ml was recorded at a N : P ratio 8 : 1. This is contrary to the ratio of 16 : 1 stated by [1] for freshwater.

This study is significant because of the strong influence which inorganic nitrogen, especially nitrate exerts on certain enzymatic activities, for example, Ribulose-1, 5-biphosphate carboxylase and phosphophenol pyruvate carboxykinase which is of basic importance for a general control of the metabolic activity, governing algal growth and development, [16].

The various results from the first run of experiment confirm the view of [17] that nitrate and phosphate are basic growth limiting factors. Analysis of the result showed nitrate to be more limiting compared to (Figs 1- 4). The concentrations used in these experiments showed that only a little amount present in solution would be enough to stimulate growth. An unusual amount of growth was recorded in flasks not containing nitrate and those containing distilled water. This may be due to samples, being from old culture, thus they may have had a phase of luxury consumption in which they may have accumulated nutrients in their cells, in excess of immediate requirements, when nutrient levels were high [18]. For example phosphate is stored in the form of volutin. The stored nutrient is then utilized during period of low nutrient concentration in natural or forced depletion as in these experiments. Death and decay of some of the cells in the culture and the subsequent release of their nutrients may also be sufficient to sustain the system for sometime at a low level.

From the sigmoid curves indicated in Figure 5 and 6, three phases of growth were recognized: (i) the lag or induction phase, in which no increase in cell number was apparent. This may be due to a large proportion of cells inoculated, not being viable. Thus cell number remained stationary until the progeny capable of dividing reached a number comparable with the total number of cells inoculated [18] or a majority of the cells inoculated may be viable but not in a condition to divide immediately, especially if the parent stock is an old one. A period of reconstitution is therefore necessary before growth can begin. (ii) Exponential phase in which cell multiplication was rapid. Numbers increased in geometric progression as indicated by the results. Cell numbers were observed to increase with the concentration of nitrate. Exponential phase ceased in the culture after about a week. This was due to exhaustion of nutrients. Nitrate was found to limit exponential growth, thus the more the quantity of the limiting nitrate, the longer was the exponential phase (Figs 5 and 6), until some other factors became more limiting. Another possible contributory factor to the ceasation of exponential growth is a reduction in light intensity by self-shading. Light absorption by algae approximately follows Beer's Law, the intensity of the penetrating light, falling off exponentially as the path length through the algal suspension increase [18]. As culture became denser, only cells at the surface received enough light for photosynthesize. Thus, growth rate became affected by light absorption. Phase of declining growth, depends on limiting factor. Nutrient exhaustion usually results in abrupt transition from exponential to the stationary phase. (iii) Stationary phase in which cell number remained more or less stationary, was observed. (iv) There appeared to be a slight decline in cell numbers in Fig. 6: which

was not significant (P>0.001). However, the slight decline in cell numbers was an indication of the onset of the death phase.

This study has shown that, as in the temperate region, nitrate-nitrogen (NO_3^-N) and phosphate-phosphorus ($P0_4^{=}P$) are also growth limiting factors to algal growth in the tropics. However of these two nutrients, nitrate is more limiting, as shown by its effect on cultured S. *gracile.* High concentrations of the nutrient in water, is a likely cause of algal bloom [19]; [20]), which becomes a serious problem to fisheries. In ponds, excess of these nutrients will lead to algal bloom causing anoxia, as a result of a decline in dissolved oxygen concentrations. This condition is deleterious to fishery and aquatic life.

5. CONCLUSION

Nitrate and phosphate are both growth limiting nutrients to algal growth in tropical freshwater. The effective ratio of these nutrients (N:P), to obtain maximum growth is 8:1

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REFERENCES

[1]. Lee, Fred G; Walter Rast and Jones Anne, R; Water Report: Eutrophication of water bodies: Insights from an age old problem. Environ.Sci. Technology., 1978. 12(8), 14 900 – 908.

[2]. Julio, A.Camargo and Alvaro Alonso: Ecological and Toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environmental International. 2006. Vol 32 (6) 831 – 849.

[3]. Twinch, A.J. and Breen, C.M. Enrichment studies using isolation columns: Aquatic Botany, 1978 Vol.4 (2) 151-161

[4]. Moss, Brain 1980. Ecology of Freshwaters. Key nutrients (Limitations of algal growth) Blackwell. Scientific Publications. London, 1980. Pp 83

[5]. Ayade, B.B. Effect of treated sewage on Aqua cultural Practice in the tropics. Nigerian Journal of Ecology, 2000. Vol 2:33-37.

[6]. Kautsky. L. Primary production and uptake kinetics of ammonium phosphate: Aquatic Botany, 1982. 12 (1)33-40.

[7]. Weidner. M and Keifer, H. Nitrate reduction in Marine Brown algae International Journal of Plant Physiology, 1981. 104 (4) 341-355.

[8]. Kumar, D. Fish culture in undrainable ponds: A manual for extension. F.A.O Fisheries Technical Paper No. 325 Rome, 1992. pp.239.,

[9]. Conley, D.J., H.W. Pearl, R.W. Howarth, D.F., Boesch, S.P., Selzinger, K.E., Havens,, S.C. Lancelot and G.E Likens. Controlling Eutrophication. Nitrogen and Phosphorus. Science. 2000. 323: 1014 – 1015

[10]. Lewis, W.M. Jr, W.A. Wurtsbough and H.W. Pearl. Rationale for control of Anthropogenic nitrogen and phosphorus, to reduce eutrophication in inland waters. Environmental Science and Technology. 2011. 45: 300 - 305.

[11]. Ward. B.H and Whipple, C.G. Freshwater Biology John Wiley and son inc. N.Y London, 1959. pp. 117-138.

[12]. Box, J.D. A simple technique for washing micro-algae in preparation for laboratory culture experiments: British Phycology Journal, 1980. 17 (1) 69-70.

[13]. Boney, A.D. Phytoplankton Edward. London, 1979. pp.29-35.

[14]. Sokal, R. and Rohlf, J.F. Biometry. W.H Freeman and Co. Sanfrancisco, 1981. pp. 324-332

[15]. SAS. Statistical Analysis Institute. The SAS system for windows. Release 801. SAS Institute, Cary USA 2000. 943.

[16]. Wilough by. L.G. Freshwater Biology Hutchinson and Co Ltd., London. 1978. Pp17.

[17]. Fogg, G.E., Algae cultures and phytoplankton ecology. The University of Wiscousin press ltd. London. 1975. pp. 12-36.

[18]. Booker, M.J and Walsby, A.E. Bloom formation and stratification by a Phytoplanktonic blue-green algae in an experimental column: British Phycology Journal, 1981. Vol 16 (4) 411 - 421.

[19]. Ogbeibu, A.E and Victor. R. Hydrological studies of water bodies in the Okomu forest reserve (Sanctuary) in Southern Nigeria (2): Physical and Chemical hydrology. Tropical Freshwater Biology. 1995. Vol. 4. 83-100.

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