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Full Length Research Paper

Response of microalgae from mud-flats to petroleum hydrocarbons in the presence of nitrogenous fertilizer effluents

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Untreated industrial waste (effluent) from out-fall of the National fertilizer company of Nigeria (NAFCON) was found to be weakly alkaline with a pH of 7.8. Total dissolved solids and sulphate were relatively high with levels of 2052 and 1599 mg/l, respectively. Nitrogen as ammonia was high in comparison to nitrate nitrogen. Phosphate was also found to be low in concentration. Using indigenous microalgae and aeration, there was a reduction in nutrients; NH_4^+, NO_3^-, PO_4^{3-} and SO_4^{2-}. However, in the presence of the hydrocarbon, there was delayed nutrient uptake. The consequence of this response was the observed reductions in chlorophyll content, biomass, etc. There was however a quick recovery in the oiled systems.

Key words: Industrial, effluent, petroleum, mudflat, microalgae, nitrogenous, fertilizer, response.

INTRODUCTION

The increasing deterioration of the environment and the need for energy and food is forcing the exploration of the feasibility of wastewater recycling and resource recovery. Within this context, bio-treatment with microalgae has been particularly attractive because of their photosynthetic capabilities converting solar energy into useful biomass and incorporating nutrients such as nitrogen and phosphorus which cause pollution and eutrophication (De la Noue and De Pauw, 1988). They play a major role as producers in waste stabilization ponds and other polluted aquatic environments. Microalgae thus have been used extensively for domestic as well as industrial waste water treatment. Emphasis may be put on wastewater treatment and/or algae biomass production. Processes involved in the removal of nutrients are precipitation, stripping and uptake by algal biomass. The efficiency of removal of a particular nutrient (for example N or P) will also depend on whether or not these nutrients are limiting in the wastewater to be treated. In practice, with adequate stirring, more than 90% nitrogen and/or phosphorus can be removed (Shelef et al., 1980).

During the past years, various systems and species, mainly unicellular species have been investigated in order to improve the efficacy and economic feasibility of such bio-treatment systems. Soler et al. (1991) studied temporal changes in physico-chemical parameters and photosynthetic microorganisms in a self-depuration waste water lagoon. In the study, some phytoplanktons played vital roles in the bio-treatment or stabilization. These microalgae include *Chlamydomonas* sp., chlorophytes mainly of the genera *Golenkia, Chlorella* and *Scenedesmus* and the cyanophyte of the genus *Synechococcus.* These microalgae were characterized by proliferation with corresponding reduction of nutrients levels such as PO_4^{3-} and NH_4^+.

A large number of nitrogenous fertilizer industries refining, coking and organic chemical industries generate high concentrations of ammonia and urea. Effluent discharges from these sources are the main cause of eutrophication – the fertilization of lakes and waterways by nitrogen and phosphorus compounds. Eutrophication occurs naturally as nutrients move from field to inland waters, but the use of inorganic of organic nitrogen in agriculture and release from industries undoubtedly enhance the enrichment of those bodies of water in which these elements limit photosynthetic populations. Only a low concentration of nitrate is necessary, and lakes with 0.3 ppm nitrate nitrogen sometimes may support unwantted algal blooms, provided other nutrients are in adequate supply (Alexander, 1977).

Excessive blooms result in high cost of water treatment, fish kills as oxygen is depleted, offensive odour and bad taste of water and navigational problems for ships. Because of toxic and other effects on aquatic life, in addition to deterioration of water quality, the limit of discharging of waste water containing ammoniacal nitrogen in aquatic environments was fixed at 50 mg N/L (Gupta and Sharma, 1996) in India. Thus there is need to remove ammoniacal nitrogen prior to discharge. Biological nitrification-denitrification is the process commonly used to accomplish this objective. The use of aquatic plants as nutrient removal agents has also been effective. The water hyacinth, *Eichhornia crassipes*, is the most studied in this aspect. It has been shown to remove 40 – 45% of the orthophosphates in water effluents in 25 – 30 days and also 94% removal of the nitrate–nitrogen (NO_3-N) and NH_3-N in 10 days (Cornwell et al., 1977).

Microalgae have been identified in environments polluted with oil. An estimated 6.1 million metric tons of hydrocarbons enter the marine environment annually (Alexander and Schwarz, 1980). While some oil seepage may occur naturally, the current pollution of the oceans by oil at the estimated rate of 5 million metric tons annually is clearly a consequence of man's activities (Atlas and Bartha, 1973). The local impact of major, or even small oil spills is well documented (Blumer et al., 1971; Atlas and Bartha, 1973) but such accidents contribute only about 4% of the total oil pollution load of the oceans (Atlas and Bartha, 1973). The remaining 96% are contributed by 'normal' operations in the production, transportation and disposal of petroleum and its refined fractions

A considerable number of studies have been carried out on the biodegradation of these hydrocarbons on terrestrial soils (Jobson et al., 1974; Westlake et al., 1978) and in marine and estuarine waters and sediments (Blumer et al., 1971; Atlas and Bartha, 1973; Hollaway et al., 1980; Colwell and Walker, 1977; Gibson et al., 1975; Schwartz et al., 1974).

There have also been studies on eutrophication, waste water treatment and biomass production using microalgae cultures (De la Noue and De Pauw, 1988; Soler et al., 1991; Azov and Shelef, 1982; Fallowfield and Garret, 1985; Shelef et al., 1980). These studies have been necessitated by the need for environmental protection and preservation, domestic and industrial wastewater treatment and also resource recovery.

However, studies on the response of the microalgae which are the primary producers in these environments, to crude pollution in the presence of nitrogenous fertilizer effluents has not been given much attention.

The objective of this study, therefore, was: 1. To study the various aspects of microalgae composition and dynamics i.e. species diversity (distribution and succession) that are involved in the treatability of the NAFCON effluent. 2. Evaluate the nutrient levels and other physicochemical parameters that affect self-depuration or biotreatability of the NAFCON effluent and 3. Study the response of the microalgae to different levels of crude pollution in the presence of the NAFCON effluent.

MATERIALS AND METHODS

Effluent waste water and sediment

The industrial wastewater (effluent) and sediment were obtained from the outfall point of the National Fertilizer Company of Nigeria, (NAFCON), Onne, Rivers State. Samples of Bonny light crude used in this study were obtained from the Shell Petroleum Development Company SPDC) flow station at Agbada near Port Harcourt.

Collection of samples

The NAFCON industrial wastewater (effluent) was collected in clean 25 L plastic containers at the out-fall of the NAFCON plant. The sediment samples were collected at low tide near the out-fall in open plastic containers while crude petroleum was supplied in a four-litre metal container.

Chemical and reagents

All the chemicals and reagents used in this study were of analytical grade and obtained from the Institute of Pollution Studies (I.P.S.) Laboratory (1986), the Chemistry and the Biology Department Laboratories, all of the Rivers State University of Science and Technology, Nkpolu, Rivers State.

Reactor vessels

The Institute of Pollution Studies (I.P.S.) Laboratory of the Rivers State University of Science and Technology, Port Harcourt provided eight Coca-Cola concentrate glass bottles of 3.5- L capacity.

Light source

The light source was artificial illumination from two parallel 4 ft white fluorescent lamps emitting about 15 μ E m^{-2}/s of light each and mounted in a box-like chamber behind the reactor vessels.

Aeration and agitation

Aeration and agitation were by means of three aquarium aerators /air pumps (Tecax AP 1500 models). They were linked to one another by means of plastic Teflon tubes of about 6 mm diameter.

Experimental design

2 L of the NAFCON effluent collected was introduced into each of the eight Coca-Cola concentrate bottles (3.5 L capacity). The bottles were aerated by means of three aquarium air pumps and illuminated by two parallel 4 ft fluorescent lamps emitting about 15 μE m^{-2} S^{-1} light and left at ambient temperature in the laboratory (plate 1). Different levels of crude 0.2, 0.6, 0.8 and 1.0% were introduced into the vessels whilst another four without crude served

Table 1. Physico-chemical characteristics of untreated fertilizer factory effluent.

Parameter	Value
pH	7.80
Temperature	34ºC
Conductivity	3537 µmhos/cm
Total dissolved solids (T.D.S.)	2052 mg/L
Ammonia	2.98 mg/L
Nitrate	0.35 mg/L
Phosphate	0.25 mg/L
Sulphate	1599 mg/L

as control. 20 g of sediment was introduced into each vessel. The vessels were set up as follows:

A medium contained only sediment
B medium contained only sediment
C medium contained only sediment
D medium contained only sediment
AA medium contained only sediment and 0.2% crude petroleum
BB medium contained sediment and 0.6% crude petroleum
CC medium contained sediment and 0.8% crude petroleum
DD medium contained sediment and 1.0% crude petroleum

Analysis

The NAFCON effluent and sediment were analysed on the day of collection, a day after that and subsequently, weekly for nine weeks for each of the oil concentrations. The following parameters were monitored, using modified methods from the standard methods for water and waste water (APHA, 1985): pH, temperature, conductivity, total dissolved solids (T.D.S.), Ammonia, (NH_3), nitrate (NO_3^-), sulphate (SO_4^{2-}), Phosphate (PO_4^{3-}) and biomass estimations.

Biomass determination and cell numbers

The biomass (growth) was determined as dry weight, chlorophyll a estimation and the number of cells (cell count).

Viable cell counts

The counting of cells was done using the improved Neubauer cytometer (counting chamber).

Dry weight

50 ml of sample was harvested by centrifugation at 3,000 r.p.m in a bench-top centrifuge for 15 m. The cells were washed three times with distilled water, transferred to a pre-weighed filter paper and dried to a constant weight in a hot air oven at 70°C.

Chlorophyll estimation

The chlorophyll content was measured according to the standard method of water and wastewater analysis (APHA, 1985).

Identification of microalgal cells- algal diversity

Cells were identified, using the x 10 and x 40 objectives of the microscope (HM-Lux Leitx Wetzlar, Germany), on the basis of cell morphology and colonial characteristics (Round, 1973; APHA, 1985).

RESULTS AND DISCUSSION

Physico-chemical parameters

Untreated industrial waste (effluent) from out-fall of the National fertilizer company of Nigeria (NAFCON), had the characteristics outlined in Table 1. The effluent at collection was found to be weakly alkaline with a pH of 7.8. Total dissolved solids and sulphate were relatively high with levels of 2052 and 1599 mg/l, respectively. Nitrogen as ammonia was high in comparison to nitrate nitrogen. Phosphate was also found to be low in concentration.

Nutrient uptake studies

In solutions containing NH_4NO_3, ammonia is usually preferentially absorbed and the pH of the medium falls. Assimilation of nitrate ions on the other hand tends to raise the pH. Marine phytoplankton has been shown to contain nitrogen in proportion to carbon and phosphurus in the ratio of 7:4:2 (Round, 1973). Apart from ammonium and nitrate ions, elemental nitrogen is also utilized by some species of cyanophyta e.g. *Phormidium, Oscillatoria* etc. during nitrogen fixation (Gallon, 1989). Nitrogen is considered to be the most important macronutrient in the control of growth of marine phytoplankton (Gilbert, 1988; McCarthy, 1980; Ietswaart et al., 1994). Many species of marine algae have also been shown to use amino acids and other organic nitrogen sources (Antia et al., 1991), as they possess an uptake system for amino acids through amino acid oxidases (Palenik and Morel, 1990). In this study, there was a significant difference in the uptake and utilization of nitrate and NH_4 ions between the stressed and non-stressed samples. The non-stressed samples experienced a rapid decline in the concentration of ammonia mostly after 2 weeks signifying a rapid on uninhibited uptake system in contrast to the stressed samples which still had high concentrations of ammonia till 7 weeks after which decreases were noticed. This could be due to a delayed or inhibited uptake system occasioned by the presence of the hydrocarbon. There were increases in concentration of ammonia after 7 weeks for most samples probably due to the death of some microalgae releasing free ammonia with a consequent rise in pH (Figures 1 and 2).

The utilization of nitrate had a similar profile to that of ammonia probably because it is a mixed microalgal community and both macronutrients were used up simultaneously though the ammonium was preferred (De la Noue and De Pauw, 1988). There was equally a de-

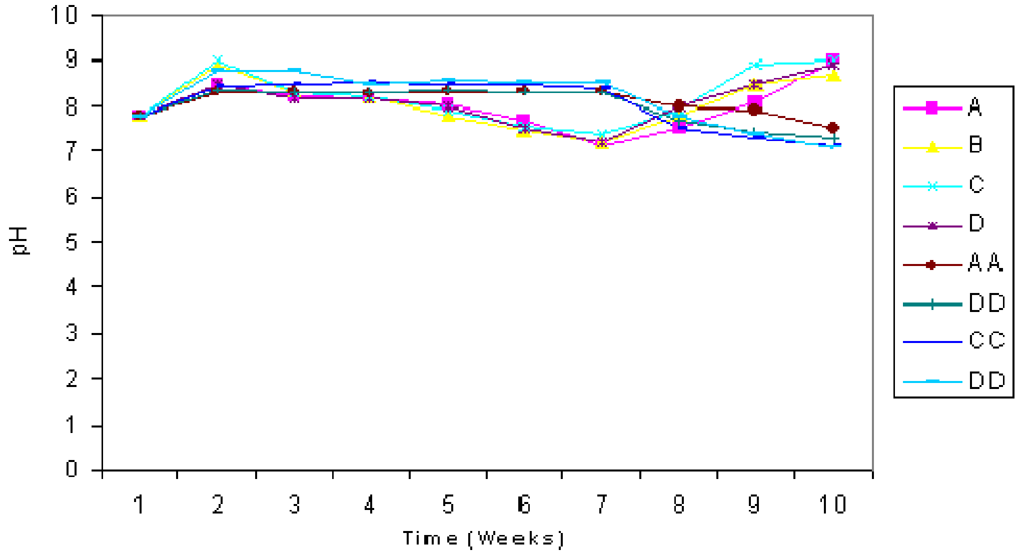

Figure 1. Changes in pH levels of fertilizer factory effluent exposed to crude petroleum hydrocarbon.

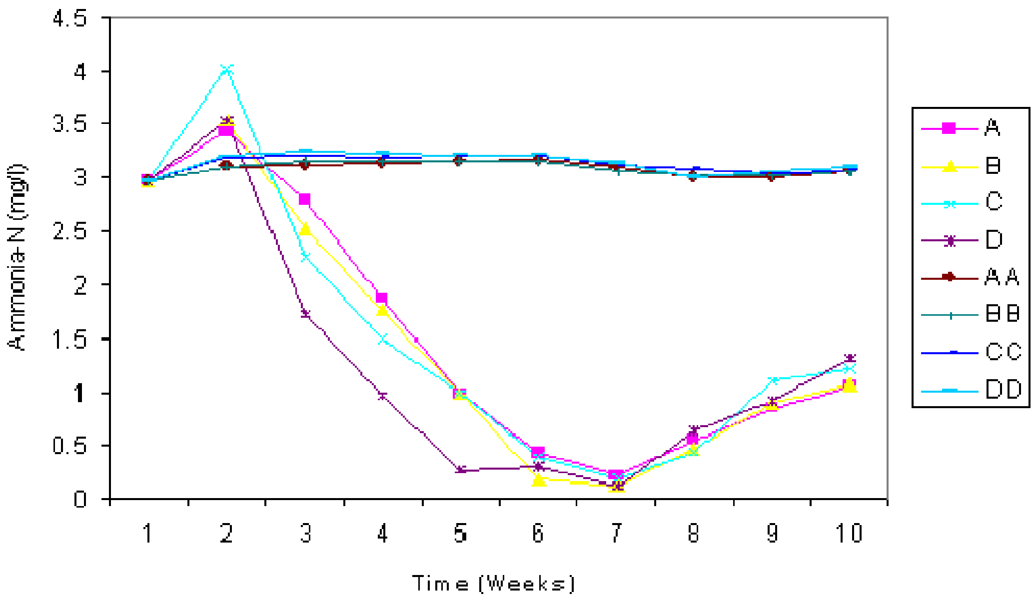

Figure 2. Changes in ammonoacal nitrogen in nitrogenous fertilizer plant effluent exposed to crude petroleum hydrocarbon

layed utilization of the nitrate ions till after 7 weeks and this was probably due to the presence of the hydrocarbon (Figure 3). The rise and fall of pH in this study could be indicative of the utilization dynamics of these ions. The hydrocarbon in the various samples could have stressed the microalgae causing a nutrient- dependent lag period of microalgal growth leading to increased excretion (Letswaart et al., 1994) and interferred nutrient supply. Kinetic studies have indicated that algae generally have low specific affinities for ions (Button, 1985) which in this case could have been compounded by the presence of the hydrocarbon. It is however well known that under nitrogen-limiting conditions, algae are capable of utilizing alternative nitrogen sources (Flynn and Byrett, 1986; North and Stephens, 1972). Recent studies have also indicated that inorganic nitrogen and phosphorus can directly limit phyto planktonic growth. In system supplemented with NH_4N0_3, phosphorus addition resulted in up to two to five fold increases in total phytoplankton biomass (Jianhua Le et al., 1994).

In this study, there was no significant difference between the means of most of the samples suggesting that

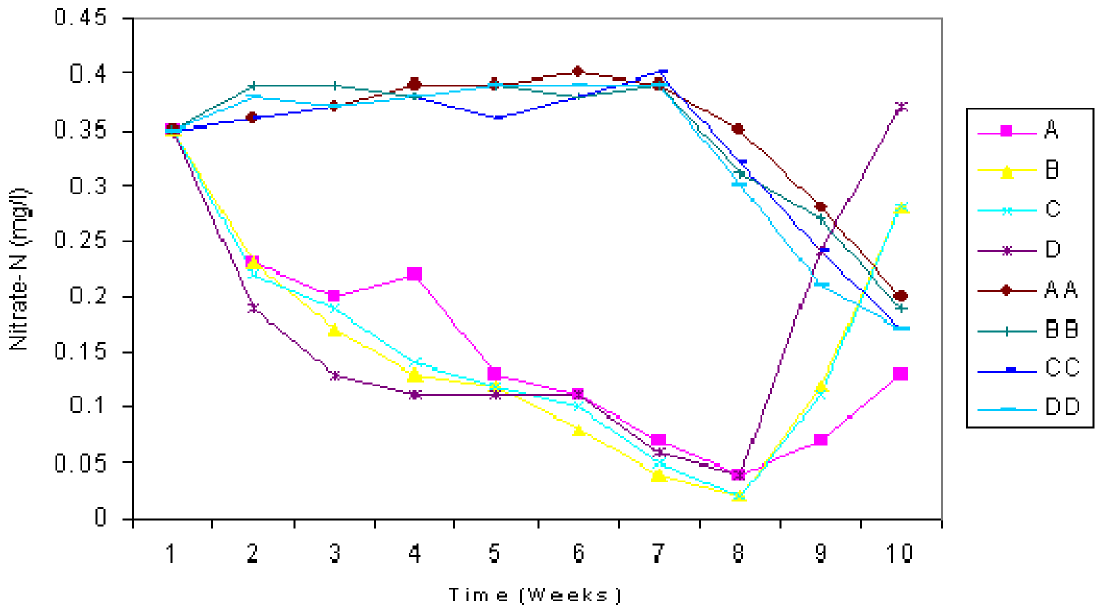

Figure 3. Changes in nitrate nitrogen levels of fertilizer factory effluent exposed to crude petroleum hydrocarbon.

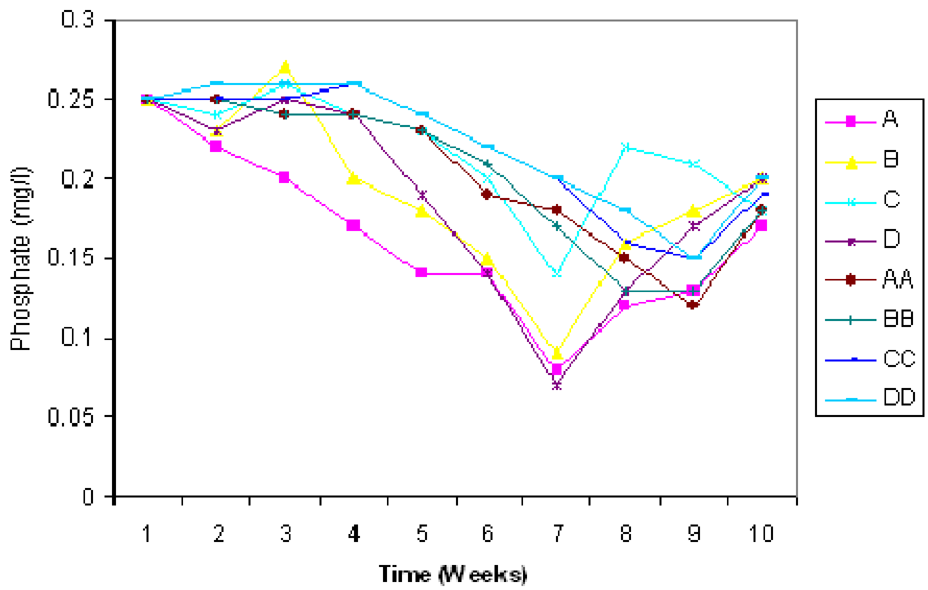

Figure 4. Changes in phosphate levels of fertilizer factory effluent exposed to crude petroleum hydrocarbon.

the $P0_4^{3-}$ was probably not a limiting factor in the experiment. The phosphate levels were also not constant in the two sample groups. The increases observed beyond 7 to 9 weeks (Figure 4) and could be attributed to death of certain taxa particularly the diatoms. Sulphur is present in small quantities in all microalgal cells (Round, 1973). The $S0_4^{2-}$ concentration showed a gradual decline in both oil and non-oil samples till the last week of study when equally, there were increases possibly due to microalgal deaths through putrefaction leading to a slightly higher mineral water (Figure 5). The diatoms particularly require the $S0_4^{2}$-for silica uptake which is required in soluble form for wall silification. Utilization of silica by diatoms is linked with their sulphur metabolism, so that shortages of this element could indirectly be growth limiting. The decreases in $S0_4^{2-}$ level could therefore be linked to utilization by diatoms. The decline in the population of the diatoms probably by death through putrefaction could have led to

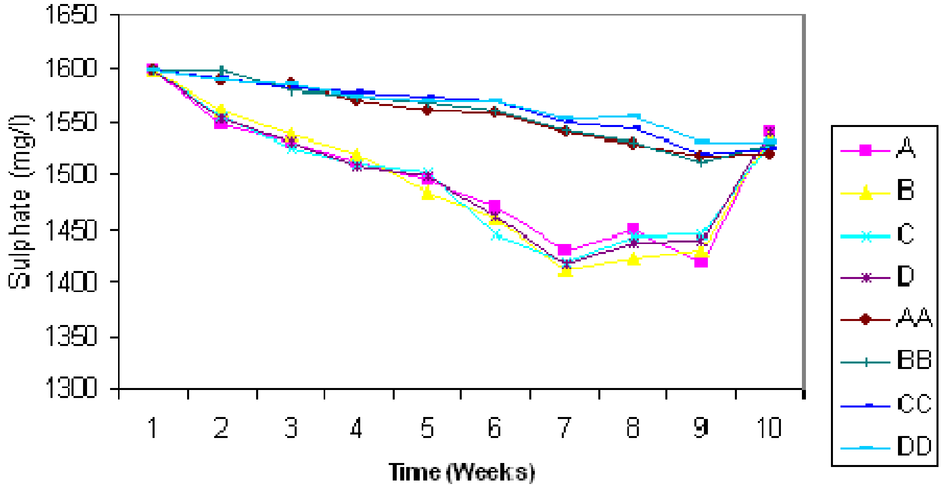

Figure 5. Sulphate levels in fertilizer factory effluent exposed to crude petroleum hydrocarbon.

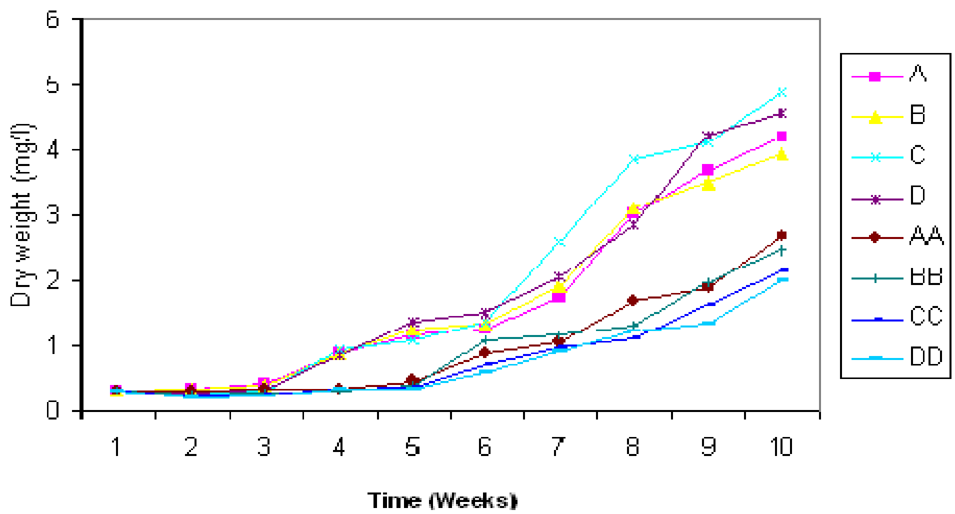

Figure 6. Biomass (dry weight) production by microalgae in fertilizer factory effluent exposed to crude petroleum hydrocarbon.

the release of the $S0_4^{2-}$ into the medium.

This succession pattern and nutrient uptake were almost similar to that reported by Soler et al. (1991). The biomass equally was in line with the work of Jianhua Le et al. (1994) where two to five fold increases in total phytoplankton biomass were reported in response to N and P fertilizations (Figure 6). However, since chlorophyll a content is affected by the different abilities of the organisms to obtain PO_4^{3-} (Jianhua Le et al., 1994), the non-stressed samples showed greater increases in chlorophyll a content at each given time in the study (Figure 7). Stressed phytoplankton communities also increased in abundance and biomass. This suggested obvious recovery of the systems. The changes in conductivity of fertilizer factory effluent exposed to crude petroleum hydrocarbon is presented in Figure 8.

In this study overall, there was a reduction in nutrients; NH_4^+, NO_3^-, PO_4^{3-} and SO_4^{2-} between 2 days to 7 weeks for the non-stressed samples and between 5 days to 9 weeks for the stressed samples, leading to microalgae proliferation. However, due to the presence of the hydrocarbon (which showed no significant difference between samples with the different crude concentrations), there was delayed nutrient uptake. This is in line with the findings of Baker (1970) and Amakiri and Onofeghara (1983) for higher plants. The consequence of this response is the observed reductions in chlorophyll content, biomass etc. There seemed however to be a quick recovery in the oiled systems as in the case of the Oshika oil spill where there was reported cases of blue-green

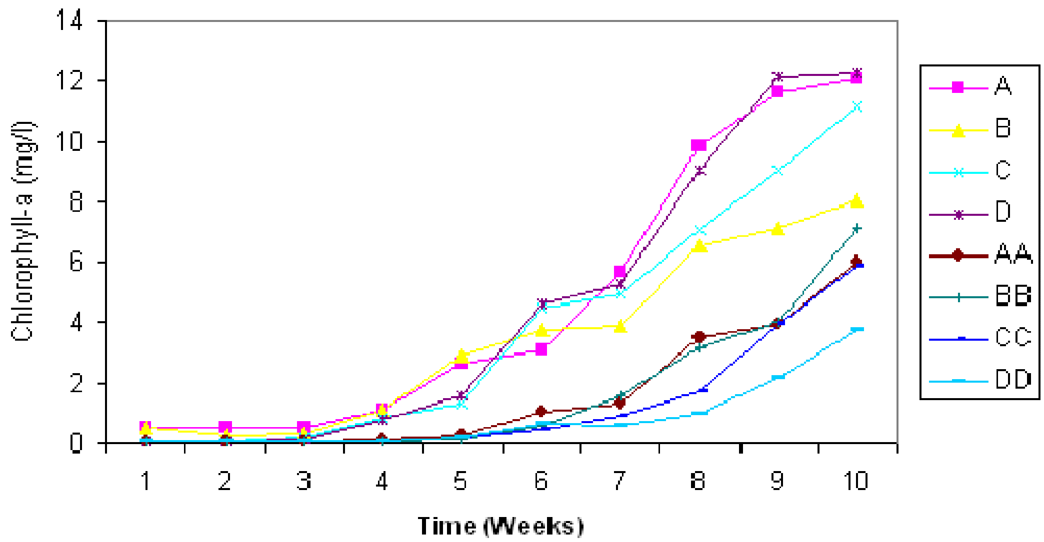

Figure 7. Chlorophyll-a production (mg/l) by microalgae in fertilizer factory effluent exposed to crude petroleum hydrocarbon.

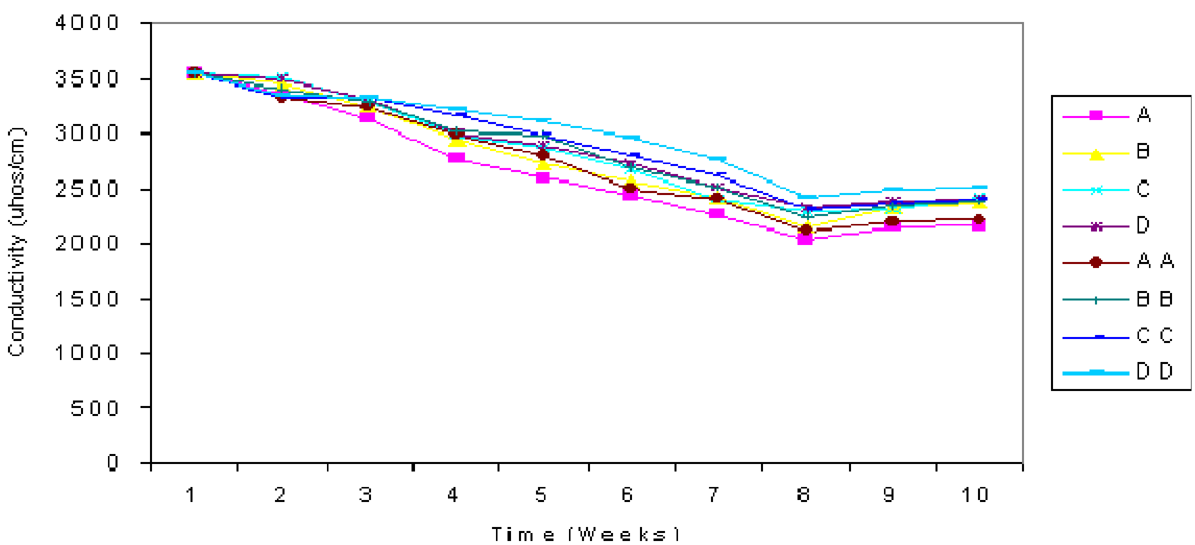

Figure 8. Changes in conductivity of fertilizer factory effluent exposed to crude petroleum hydrocarbon.

algae bloom a few weeks after the spill (IPS, 1986).

Biomass measurements

Dried biomass measurements of microalgae cultures indicated increases in dry weight and chlorophyll a content particularly after one week when full bloom was attained in the samples without crude additions. Significant increases in biomass and chlorophyll a content was observed in the samples with crude additions only after 4 weeks (Figure 7). The original NAFCON effluent/ sediment culture on the day of collection had high numbers and diverse species of microalgae probably because of the high nutrient concentrations of the effluent that is discharged into the river.

Taxonomic studies showed that three main groups were present during the course of the experiments. The microalgae profile included genera of cyanobacteria (blue-green algae), Bacillariophycaea (the diatoms) and the chlorophyceae (green algae). In the raw effluent, the diatoms had the least percentage distribution of 29.08% followed by the green algae with 35.29%. The blue-green algae had the highest percentage distribution of 35.62%. (Table 2) The Bacillarophyceae include species of *Amphiprora, Cyclotella, Navicula pinnularia* and *Nitzschia.*

Table 2. Microalgal diversity, species characterization and percentage occurrence.

Organism	Untreated sample	A	B	C	D	AA	BB	CC	DD
Diatoms									
Amphiprora sp.	20	48	69	21	11	9	18	71	14
Cyclotella striata	33	22	23	28	-	2	35	36	28
Coscinodiscus radiata	41	13	18	55	24	-	28	22	33
Dictylum brighwelli	65	39	27	34	17	8	12	14	21
Diatoma liemale	36	10	94	-	28	10	16	21	26
Melosiru granulata	9	66	15	46	11	14	-	10	20
Navicula cuspidata	59	37	7	109	61	-	65	39	19
Navicula minima	80	24	3	42	5	54	22	17	20
Pinnularia gibba	3	-	3	28	19	18	4	11	24
Nitzschia closterium	7	10	2	39	36	55	9	9	10
Naviculabacillum	3	10	-	65	81	28	11	-	3
Total	356	370	261	467	293	198	220	250	218
%	29.08	39.15	32.22	41.92	34.03	38.82	35.43	34.29	46.09
Blue-green algae									
Anacystis Geroginosa	68	55	-	-	52	11	10	-	4
Anabaena flos-aguae	45	36	25	94	35	19	-	36	13
Oscillatoria Sancta	20	-	39	58	-	28	27	20	1
Oscillatoria Chadybaca	37	57	41	24	-	9	15	6	16
Phormidium Uncinatum	42	21	11	-	21	14	20	3	5
Pleurosyna donyatum	20	13	28	33	28	6	5	12	6
Oscillatoria sp.	204	26	26	64	27	6	9	19	10
Total	436	208	170	273	163	93	86	96	55
%	35.62	22.01	20.99	24.51	18.93	18.24	13.85	13.17	11.63
Green algae									
Schiothrix Pulvinata	261	154	96	112	143	112	106	149	130
Scenedesmus Quadricauda	44	135	140	195	140	67	160	97	37
Chlorella Sp.	127	78	143	67	122	40	49	137	33
Total	432	367	379	374	405	219	315	383	200
%	35.29	38.84	46.79	33.57	47.04	42.94	50.72	52.54	42.28
Overall Total	1224	945	810	1114	861	510	621	729	473

The cyanobacteria included species of *Anacystis, Anabaena, Oscillatoria phormidium* and *pleurosyna*, while the chlorophytes were made up of three *genera Schizothriz, Scenedesmus* and *Chlorella*. From the 8th day of study, when samples with no crude additions attained full bloom, the chlorophytes dominated the other groups till the end of the study attaining percentages that ranged from 33.57% on day 8 to 89.99% after 9 weeks. In this, the *Chlorella* followed by *Scenedesmus quadricauda* were particularly dominant from week 1 up till week 5 when the species *Schizothriz pulinata* appreciated in number and from 6 to 9 weeks, all three species dominated in the culture. The chlorophytes in the samples with hydrocarbon were dominant at day zero till day 8 when the cyanobacteria gained control of the culture. By week 5, the chlorophytes once again became the dominant species till end followed by the cyanobacteria.

The NAFCON industrial waste water or effluent was found from this study suitable to support the blooming of microalgae including *Chlorella, Sscenedesmus* etc. as was previously done by Anaga and Abu (1996), Abu and Epegu (1992) suggesting use for the waste in microalgae biomass production and effluent biotreatment. This is in consonance with the work of Chapman and Gellenbeck (1989) which showed that some species of microalgae such as *Porphyridium, Scenedesmus, Chlorella* and *Spirulina* could actually be cultivated in the laboratory.

The biomass measurements as illustrated by the dry weight and chlorophyll contents and also the number of cells indicated that there were increases in biomass indicating growth. However, this was much more in the non-stressed than in the stressed samples where a much longer lag phase of growth was noticed. In the non-stressed samples, nutrient uptake was much more rapid and immediate. Cross-system analysis of results however, showed that phytoplankton chlorophyll-a, dry weight and total number of cells were non-significant in all systems. In microalgal diversity, microalgal populations showed 3 main taxa with the cyanobacteria dominating at the commencement of study. The profile later showed the chlorophyta particularly, *Chlorella* sp. and S*cenedesmus quadricauda* dominating till end of the study during which time the diatoms had almost become extinct (Table 2).

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