GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL 14, NO. 1, 2008: 97 - 102
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A LABORATORY ASSESSMENT OF ANAEROBIC BIODEGRADATION OF PETROLEUM HYDROCARBONS IN A TYPICAL NIGER DELTA WETLAND

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(Received 28 February 2007; Revision Accepted 14 August 2007)

ABSTRACT

Anaerobic degradation of hydrocarbons in sediment samples from a wetland environment that had been impacted by crude oil for more than thirty years was investigated in the laboratory. The experiment comprised sterile and non-sterile treatments of the sediment samples that were incubated either aerobically or anaerobically in flasks designated A, B, C, D and E. Some treatments were amended with inorganic fertilizer. The experiment was monitored by the measurement of heterotrophic bacterial activities, hydrocarbon utilizing bacterial activities, sulphate reducing bacterial activities, gravimetric loss of oil with time. The pH, Dissolved Oxygen (DO), conductivity, nitrate, sulphate and phosphate of the treatment conditions were monitored. The Dissolved Oxygen (DO) of various options tended towards zero while flask B recorded a net (DO) value of 0.3 mg/l. The pH of most treatment options was moderate with flask C giving the highest value of 8.50. There was total hydrocarbon loss of 18 %, 60 %, 57 %, 49.5 %, 43.7 %, for the test conditions A, B, C, D and E respectively. Counts of aerobic heterotrophic bacteria ranged between 1.8×10^5 and 2.5×10^5 cfu/g. Counts of anaerobic heterotrophic bacteria ranged between 8.5×10^4 and 9.5×10^4 cfu/g. Counts of sulphate reducing bacteria ranged between $3.4 \times 10^4 - 1.6 \times 10^5$ cells/ml. The levels of nitrate, phosphate and sulphate decreased with time during the study period except in flask A. The results indicate that there is a significant difference ($P<0.05$) between total hydrocarbon level and number of days of incubation. Biostimulating the sediment under anaerobic conditions has almost the same effect on hydrocarbon degradation as under aerobic conditions. It is recommended that these conditions be optimized under field situation to enhance anaerobic bioremediation of anoxic environments in the Niger Delta.

KEY WORDS: Anaerobic biodegradation, Sulphate reducing bacteria, petroleum hydrocarbons, terminal electron acceptors, laboratory investigation

INTRODUCTION

The arrival of oil prospecting and exploitation in Nigeria has no doubt impacted both positively on development of the area and negatively on the environment (Abu and Ogiji,1996., Ayalogu, *et al.*, 2001). Crude oil samples vary greatly in composition but consist quantitatively of hydrocarbon and non-hydrocarbon compounds. Different crude oils vary in their composition depending on their source. Even crude oil from the same field may show considerable variation. Hydrocarbon contamination in sediments from shipping activity, fuel oil spills and runoff has been the subject of continuous environmental and human health concern over the last few decades. Components of petroleum waste, airborne combustion particulates and creosote are particularly persistent in sedimentary subsurface environment due to their relatively low aqueous solubility, low volatility and high affinity for sediment particles (Coates *et al.*, 1997; Rockne *et al.*, 2000).

Polyaromatic hydrocarbon (PAH) contaminated sediment is a concern to human health because of the risk through the consumption of contaminated seafood stocks. Fish and shellfish frequently bioaccumulate PAHs to concentrations that are several orders of magnitude greater than their aqueous solubility. The human health effects of PAH exposure are well documented. Acute exposure effects range from skin and lung irritation to cyanosis. Exposure to some PAHs has been implicated as carcinogenic and tumorigenic to both human and wildlife (Rockne *et al* ., 2000).

Some practices such as the use of gasoline and kerosene in washing and rinsing of hands by automobile mechanics without properly washing their hands before eating is often a source of health hazard. Furthermore, the drinking of gasoline, kerosene and crude petroleum as antidotes to snake poisoning also pose health risk (Ayalogu *et al.*, 2001; Dede *et al.*, 2001). Because of these human health concerns, acurrate estimates of the persistence of PAHs in the environment is important in assessing the risk presented by PAH contamination.

Biodegradation is the partial simplification or complete destruction of the molecular structure of an organic compound by physiological reaction catalyzed by microorganisms (Madsen, 1997). This appears to be the most important of the processes which account for the ultimate fate of oil in both terrestrial and marine environments (Atuanya and Obuekwe, 2000; Abu and Ofurum, 2006). Aerobic degradation of petroleum hydrocarbon at the surface is well documented (Huesemann,1993; Abu and Ogiji,1996; Odokuma and Dickson, 2003). However, the extent of anaerobic hydrocarbon biodegradation remains strongly contested (Aitken *et al.*, 2004) and little is known about the microorganisms responsible for anaerobic hydrocarbon degradation (Rockne *et al.*, 2000). The objectives of this study were: to enumerate microorganisms involved in the metabolism of hydrocarbons under anaerobic conditions; to find out whether anaerobic degradation of hydrocarbons is obtainable under conditions tested in the laboratory and to determine the role of non-oxygen terminal electron acceptors (bioenergetics) in the metabolism of hydrocarbons in a typical Niger delta wetland.

MATERIALS AND METHODS

Sample Source, Collection and Processing

Sediment samples were collected from Egbara stream (Ewenta Egbara) in Ejamah Ebubu, Rivers State, Nigeria. The stream is a swampy wetland which was chosen for sampling because of the heavy crude oil it received from a damaged Trans Niger pipeline belonging to Shell Petroleum Development Company (SPDC) Limited more than 30 years ago. This has been left unattended to all this while. Using a manual sediment grab duplicate samples were collected from below the water surface and transferred into two sterile 30 liter plastic buckets. These were transported to the laboratory and analysed within 24 h.

Microcosm Set-Up

Five 250 ml Erlenmeyer flasks were used to set up microcosms of the sediment sample to simulate various conditions. The sediment sample (200 g) was put into each of

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the flasks (A, B, C, D, and E). The sample was amended with 65 ml of fresh crude oil before distribution into the flasks. Since the site of collection of the sample was polluted over 30 years ago, abiotic weathering processes would have reduced appreciably. In order to monitor these processes along side biodegradation, the sample was amended with crude oil and thoroughly mixed and allowed to settle before distribution into the flasks. The conditions simulated are as follows:

Flask A(SS) contained 200 g of sterile(autoclaved) sediment sample kept under anaerobic condition. This would prevent activity of microorganism.

Flask B(AS) contained 200 g of sediment sample kept under aerobic condition. This simulated natural attenuation processes.

Flask C(NS) contained 200 g of sediment sample plus 2 g of fertilizer (NPK 15:15:15).This was kept under anaerobic condition. This simulated biostimulation under anaerobic conditions.

Flask D(ANS) contained 200 g of sediment samples kept under anaerobic conditions. This is to evaluate anaerobic degradation of hydrocarbons.

Flask E(NAS) contained 200 g of sediment sample plus 0.15 g of biocide (sodium azide) to prevent growth of microorganisms thereby ruling out biodegradation activities. Other processes like photoxidation, volatilization and evaporation would proceed here. To evaluate the effectiveness of the treatment, viable plate counts were done every fourteen days.

Total Heterotrophic Bacterial (THB) Count, Enumeration of Hydrocarbon–Utilizing Bacteria and Sulphate Reducing Bacteria (SRB).

The mean total aerobic heterotrophic bacterial populations present in the samples at the beginning of the experiment (zero day) and at two weeks intervals for each of the treatment options were estimated using nutrient agar (Oxoid). The anaerobic heterotrophic bacterial population were also estimated using nutrient agar (Oxoid) plates incubated in the anaerobic chamber at 28 ^{o}C.

The Modified mineral salts medium of Zajic and Supplison (1972)was used for the enumeration of hydrocarbon-utilizing bacteria. It contained the following in g/l: NaCl 10.0; $MgSO_4.7H_2O$ 0.42; KCl 0.29; KH_2PO_4 1.25; NH_4NO_3 0.42; Agar 15 and distilled water 1000ml. Serial dilutions of the sediment samples were carried out and aliquots were inoculated onto the agar plates in duplicates. A sterile filter paper (Whatman No.1) saturated with 1ml crude oil was placed on the inside cover of each agar plate and the plates incubated in an inverted position. The filter paper situated on the lower side supplied the hydrocarbons by vapour-phase transfer to the inoculum. After incubation at 28^{0}C for 7 days, colonies were counted from duplicate plates and the mean counts recorded. Anaerobic hydrocarbon-utilizing bacteria were also enumerated by placing plate in the anaerobic chamber. Postgate medium was used for the enumeration of sulphate reducing bacteria. It contained the following in g/l: KH_2PO_4 0.5; NH_4Cl 1.0, $CaSO_4$ 1.0; $MgSO_4.7H_2O$ 2.0; sodium lactate (60 %) 3.5ml; Yeast extract 1.0; Ascorbic acid 0.1; Thioglycollate 0.1; $FeSO_4.7H_2O$ 0.5; NaCl 1.0; Agar 1.5 and Tapwater 1 litre.

The five-tube mean probable number (MPN) procedure was used for enumeration of sulphate-reducing bacteria (SRB) was used. A total of 20 test-tubes containing 9ml Postgate medium were used. Each set of five test-tubes were inoculated with 0.1ml of 10^{-3}, 10^{-4}, 10^{-5} dilution of the test sample. A set of five tubes were used as control. The test-tubes were plugged with sterile cotton wool and incubated at 37 ^{o}C for 21 days in the anaerobic chamber. Black precipitates indicated the presence of sulphate reducing bacteria (SRB).

The pH of the contents of each flask was taken with a digital pH meter (Jenway 015 model) at zero day and then every fortnight. The temperature of the sample was measured with a standard mercury-in-glass thermometer.

Physicochemical Analysis

The dissolved oxygen of the samples was determined using the unmodified Winkler procedure. Here 0.025N thiosulphate was used to titrate the sample while freshly prepared starch solution was used as indicator, this was done at day zero and then every forth night. The conductivity of the samples was taken with a digital conductivity meter at day 0 and then every fortnight for each of the treatment options throughout the period of the study.

The nitrate level of the various treatment options was determined using the Brucine method as described by Allen *et al.* (1984) at day zero and every fortnight.

The phosphate level of the various treatment options was determined using the Ascorbic acid method (APHA, 1985) at day 0 and every fortnight.

The sulphate level of the various treatment options was determined using turbidometric method (APHA, 1985), at day 0 and every fortnight.

Extraction of Crude Oil from Sediment

Cold extraction was used for the day 0 and at the end of the experiment for each of the treatment options. The sediment samples were dried at ambient temperature (25-29 ^{0}C) to constant weight. Ten grams of the sediment samples were placed in labeled, sterilized and chemically rinsed flasks. To these, 20 ml of toluene was added, shakened and the residual crude oil was extracted using a separatory funnel. The procedure was repeated. Each extract was filtered through cotton wool. The extracts were pooled together and stored at 4-5 ^{0}C.

Measurement of Crude Oil Degradation Using a Gravimetric Method

The method described by Gundlach and Hayes (1979) was adopted. A standard curve of
the crude oil and absorbance was read using filter-photo colorimeter (Model A T-135) set at 420 nm wavelength. The hydrocarbon concentration at day 0 and the residual hydrocarbon concentrations in the various treatment conditions were calculated from the standard curve after multiplying by the appropriate dilution factor.

Maintenance of Anaerobiosis

An anaerobic chamber (Coy Laboratory: P.H. 735475-22100) was used to maintain anaerobiosis. The chamber contains a gas-tight glove box connected to a vacuum pump and two cylinder containing highly purified nitrogen and hydrogen. A palladium catalyst is included within the chamber and is flushed through several times with the anaerobic gas mixture. An oxygen detector within the chamber indicates if there is any trace of oxygen. Anaerobiosis is normally attained after 12-18 h.

Statistical Methods

The analysis of variance (ANOVA) and correlation analysis were applied to data to establish levels of difference and orrelations among the treatment options.

RESULTS

From the five different treatment options used to demonstrate anaerobic biodegradation of petroleum hydrocarbons as shown in Fig. 4, the original THC value of 1000 mg/kg in flask A witnessed a reduction to 820 mg/kg. The 18 % loss recorded could be due to heat applied to the sample during sterilization of the sample.

Flask B simulated aerobic natural attenuation conditions. It served as a positive control. This set up recorded a residual THC value of 400 mg/kg, representing 60 % removal. Flask C contained nutrient amended sample (NS), simulating biostimulation under anaerobic conditions and this demonstrated THC loss through enhanced condition (nutrient addition), recording a residual THC value of 430 mg/kg representing 57 % removal.

Flask D which contained the sediment sample only (ANS), was used to compare aerobic and anaerobic biodegradation and recorded a residual THC value of 505 mg/kg, representing 49.5 % removal. Flask E(NAS) contained the biocide and from the original value of 1000 mg/kg it witnessed a reduction to 563 mg/kg, representing 43.7 % removal.

The response of the indigenous hydrocarbon degrading microorganisms to the treatment under anoxic laboratory condition was positive. All counts are presented as mean of duplicates. The total aerobic heterotrophic bacterial count and percentage hydrocarbon utilizing bacterial population for treatment option B(AS) are presented in Fig.1. It increased steadily for treatment options B(AS), C(NS) and D(ANS). Options A(SS) and E(NAS) recorded no colonies

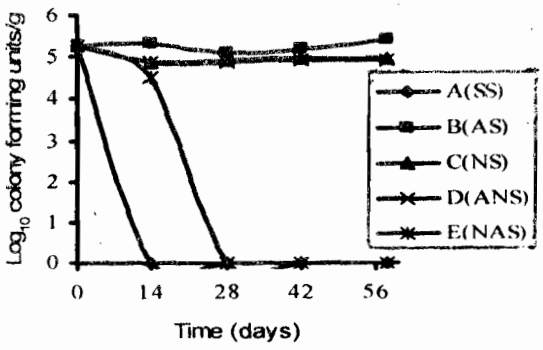

Fig. 1. Aerobic heterotrophic bacterial counts (cfu/g) obtained from various treatment options

after days zero and fourteen respectively. Fig.2 shows anaerobic heterotrophic bacterial count which also increased steadily except for treatment option A(SS). The sulphate reducing bacterial count (SRB) of the various treatment options is presented in Fig.3; it increased steadily for all options except A(SS).

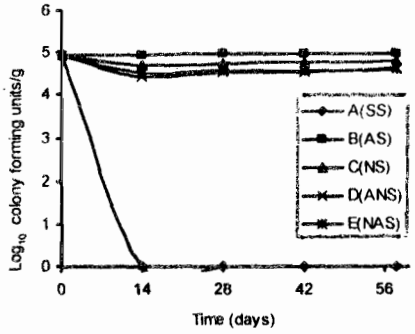

Fig. 2. Anaerobic heterotrophic bacterial counts (cfu/g) obtained from various treatment options

The total hydrocarbon (Oil and Grease) remaining in the sediment as the experiment progressed is graphically represented in Fig. 4. The result show losses in the order B > C > D > E > A.

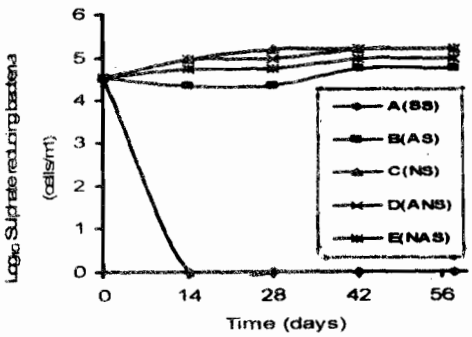

Fig. 3 Sulphate Reducing Bacteria counts (cells/ml) obtained from various treatment options

The dissolved Oxygen of the treatment options tended to zero during the course of the experiment but in flask B it increased and remained at 0.3mg/l mark (Fig. 5). This shows that anaerobiosis was created and maintained in all the set-ups except flask B. Generally the pH values changed from acidic range to alkaline range with treatment option C(NS) giving 8.50 (Fig. 6). The conductivity measurement (Fig.7) indicates removal of the ions possibly by the microorganisms since the sterile set–up showed little or no change in conductivity.

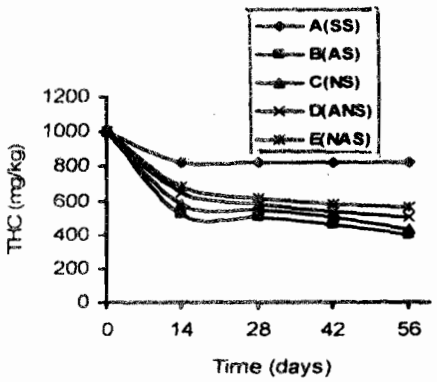

Fig.4 Time series analysis of Total Hydrocarbon (THC) of the treatment options

The phosphate, sulphate and nitrate concentration of the sediment in the various treatment options are represented graphically in Figs. 8, 9, and 10 respectively.

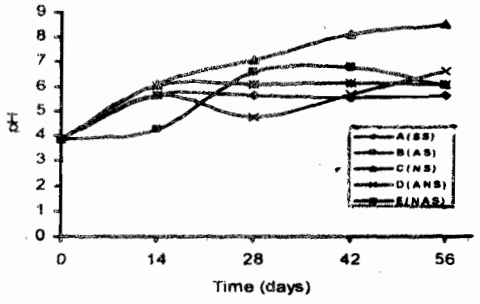

Fig 6. Time series analysis of pH of the treament options

Phosphate level in flask A(SS) remained constant for the study period but reduced drastically for other flasks, with flask D(ANS) tending to zero. It is clear that after nitrogen, phosphorus is the next limiting nutrient apart from oxygen. There was no nitrate in the sediment sample.

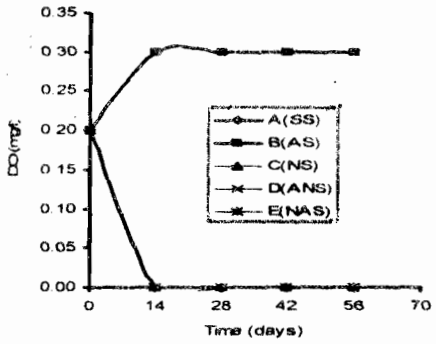

Fig 5. Time series analysis of Dissolved Oxygen (DO) of the treament options

The sharp rise in nitrate and phosphate are due to the fertilizer amendment in flask C. This could be attributed to heat. Flasks B, C, D and E recorded a reduction in sulphate level as the experiment progressed but flask A witnessed a reduction from 82.01 mg/kg to 8.27 mg/kg and remained constant.

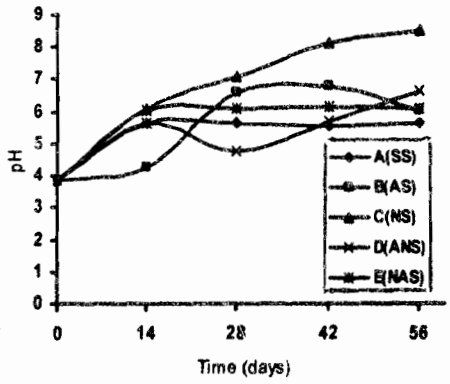

Fig 6. Time series analysis of pH of the treament options

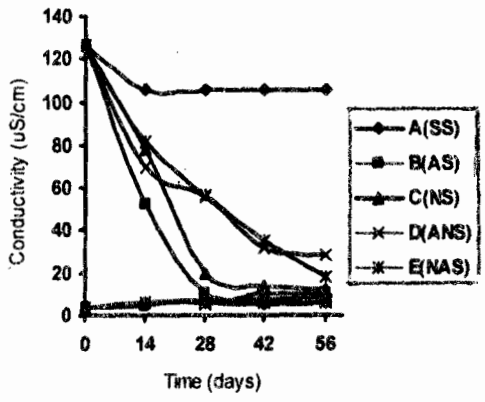

Fig. 7. Time series analysis of conductivity of the treatment options

DISCUSSION

Anaerobic bioremediation technology holds a lot of promise not only for developed but also for developing nations, such as Nigeria. The eiciency of the laboratory bioremediation test was monitored in different ways. The high level of the aerobic hydrocarbon utilizing bacteria at day 0 (25 %) of the total aerobic heterotrophic counts in the environment is a reflection of the degree of oil contamination in Egbara stream. In an unpolluted environment the oil degraders will generally constitute less than 0.1 % of the total heterotrophic population (Atlas, 1981) and would rise as they become acclimatized to the impacted environment. The conditions in option B (AS) led to a rise in the percentage of hydrocarbon utilizing bacterial count from the first day through the 60 days of the test period (Fig1). The records for the anaerobic heterotrophic bacteria and the sulphate reducing bacteria (Figs 2 and 3) are a confirmation that anaerobiosis was achieved and maintained using the anaerobic chamber. Sulphate reducing bacteria are strict anaerobes (Abu, 1992). The percentage hydrocarbon reduction for the various treatment options (Fig.4) shows statistically that at (P=0.05) 95% level of confidence, there is a significant difference between total hydrocarbon level and number of days. The higher level of hydrocarbon loss in options B(AS) and C(NS) could be attributed to the nutrient requirement of the microorganisms. C (NS) received 2 g of 15:15:15 NPK fertilizer while B(AS) was kept under aerobic conditions having a supply of oxygen. Similar conditions have been reported (Abu and Ogiji, 1996) favourable for aerobic hydrocarbon degradation.

The dissolved oxygen level in the various treatment options (Fig.5) shows anaerobic condition except in B(AS) which recorded a net DO value of 0.3 mg/l. This could be due to the sample being kept under aerobic condition. The sample had foul rotten egg odour which is associated with the production of hydrogen sulfide (Zhu *et al.*, 2004). It indicates that sulphate may have been used up at this level of oxygen tension. The pH (Fig. 6) is suitable for the growth of sulphate reducing bacteria, which can tolerate a wide range (5.5 to 9.5) of pH values.

The conductivity of various treatment options decreased (Fig. 7) indicating uptake and exchange of ions. However, the conductivity value of flask A remained constant. This could be due to the fact that the treatment option contained sterile sample with killed cells, thus there is little or no electron exchange.

At the end of the study period the nitrate level in flask C was reduced to 0 from 660 mg/l (Fig 10). The results of nutrient utilization measurements revealed that there were limiting nutrient conditions, especially with respect to nitrogen. From the quantity of nutrient applied flask C (NS) which received 2 g of 15:15:15 NPK fertilizer contained 660 mg/l NH_4NO_3 and 460 mg/l PO^{-2}_4 in a (1.4:1) N: P ratio (Abu and Ogiji, 1996). Phosphorous and nitrogen are among the major elemental nutritional requirements of microorganisms (Gottschalk, 1985). This explains the lower degradation in unamended options and the option treated with biocide. The drastic reduction in nitrate concentration indicates that there was uptake of nitrate, in this case by microorganisms, to build up biomass. In this treatment option 57 % of hydrocarbon was degraded; the remaining (43 %) undegraded hydrocarbon could be attributed to either recalcitrant groups or nitrogen and phosphorus limitations.

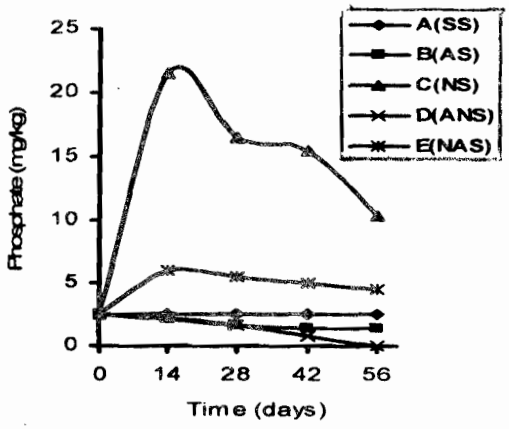

Fig. 8. Time series analysis of phosphate of the treatment options

The utilization of nitrate under anaerobic condition is significant. Nitrate was not detected in the other flasks during the study period. Monitoring of various non-oxygen electron acceptors revealed that nitrate, a bioenergetic analog of sulphate (Abu, 1992) is preferred (Gottschalk , 1985; Engvall, 1986). This is supported by a perfect positive correlation (r = 1) between SRB and nitrate level but perfect negative correlation (r = -1) between SRB and sulphate.

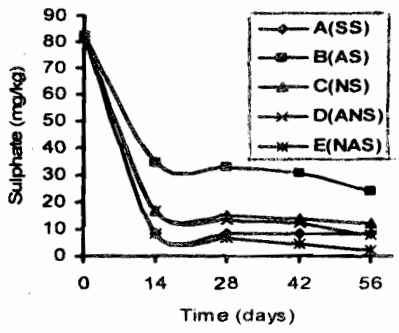

Fig. 9. Time series analysis of sulphate of the treatment options

This means there were non sulphate reducing anaerobes present. They could be denitrifying bacteria. Bioremediation is driven by nutrient limitation following Monod kinetics. One way to enhance pollutant removal in this ecosystem would be by careful manipulation of the nutritional requirements of the microorganisms especially nitrogen.

The decrease in the trend of the sulphate level of flasks A, C, D and E could be attributed to heat applied to the sample during sterilization since some sulphate decompose on heating (Bastos-Gonzalez *et al.*, 1996), but also to uptake by microorganisms. Flask B recorded higher sulphate concentration than other treatment options and this could be as a result of its being kept under aerobic condition, so there is the supply of other terminal electron acceptors such as oxygen. Oxygen is usually a preferred electron acceptor to sulphate and even nitrate (Abu, 1992). Thus, sulphate was not a limiting nutrient nor was it a preferred electron acceptor under the conditions tested. The negative correlation recorded by C (NS) is significant because it shows that increase in the number of organisms, led to decrease in the concentration of sulphate.

The titer of sulphate reducing bacteria recorded a high level at day 0, supporting the high concentration of sulphate (Engvall, 1986). The heterotrophic bacteria comprised Gram negative and Gram-positive rods as well as Gram-positive cocci. The sulphate reducing bacteria were Gram positive rods with central spores. No further identification was carried out. This is in line with results of other workers (Coates *et al.*, 1997). The biocide treated set-up gave results comparable to the other non sterile set-ups, but different from the sterile set-up. This set-up could also serve as a positive control for anaerobiosis. This is because the biocide (sodium azide) used is usually effective against aerobes only.

A few centimeters and often only a few millimeters below the sediment surface, wetland sediments are anaerobic. Therefore, oxygen is likely to be a limiting factor for oil biodegradation in wetlands such as Egbara (Eweta-Egbara stream). The results of this investigation revealed utilization of hydrocarbon by microorganisms under anaerobic condition. The sulphate reducing bacteria which are known to be associated with petroleum degradation, under anaerobic condition were detected and enumerated from the contaminated site. Total hydrocarbon (THC) and total petroleum hydrocarbon (TPH) were degraded under anaerobic condition tested in the laboratory. Non-oxygen terminal electron acceptors such as nitrate and sulphate are essential in anaerobic degradation of hydrocarbon.

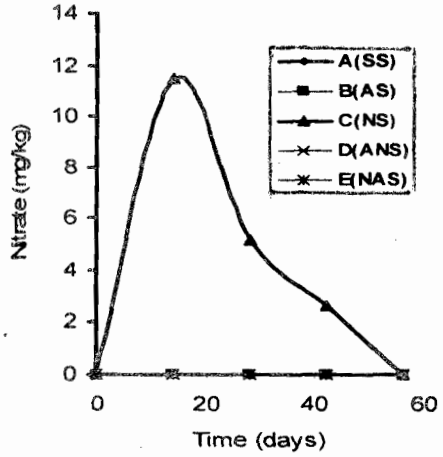

Fig.10. Time series analysis of nitrate of the treatment options

The fact that the metabolism of hydrocarbon can be linked to sulphate reduction is most beneficial because sulphate is one of the most abundant electron acceptors in the environment. Thus, if inputs of hydrocarbon pollution can be controlled, much of the existing hydrocarbon contamination may eventually be eliminated. Furthermore nitrate can also serve as a bioenergetic analog of sulphate (Abu, 1992).

The results suggest that anaerobic degradation of hydrocarbon contaminants may also be a useful strategy for the ex-situ treatment of dredged sediment which must be remediated prior to disposal. This could be done through introduction of nutrients in the form of inorganic fertilizers such as the 15:15:15 NPK . Our results indicate that the nitrate in the fertilizer may not only serve to supply nitrogen but may also act as terminal electron acceptors in coupled reactions for hydrocarbon degradation under anaerobic conditions. We thus opine that there is need to optimize anaerobic bioremediation under field conditions in Nigeria. Hydrocarbon utilizing, sulphate and nitrate reducing bacteria could be used to bioaugment indigenous microorganisms for anaerobic bioremediation. Understanding the bioenergetics of hydrocarbon degradation in our Niger delta environment is thus of considerable significance.

ACKNOWLEDGEMENT

The cooperation of the Ejamah Ebubu community during field sampling is hereby greatly appreciated and acknowledged. Anaerobic chamber services were obtained from Fugro Nigeria Ltd.

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