African Journal of Microbiology Research. Vol.(2) pp. 217-223 August, 2008
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2008 Academic Journals

Full Length Research Paper

Biodegradation of hydrocarbons in untreated produce water using pure fungal cultures

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Accepted 8 August, 2008

Biodegradation studies of hydrocarbons in untreated produce water from an oil production facility in Nigeria were undertaken over a period of time using pure fungal cultures *(Penicillium* sp. and *Aspergillus niger).* The rate of reduction in some petroleum hydrocarbon fractions, such as *n*-alkanes, aromatics, nitrogen, sulfur and oxygen (NSO)-containing compounds and polycyclic aromatic hydrocarbons (PAHs), were monitored by means of gas chromatography and mass spectrometery, using mechanically treated produce water as a reference. Gas chromatographic analysis showed that untreated produce water with an oil and grease content of 1407 mg/l contained various petroleum hydrocarbon fractions, including *n*-alkanes (608 mg/l), aromatics (13.88 mg/l), NSO compounds (12.68 mg/l) and PAHs (0.833 mg/l). Upon mechanical treatment, the oil and grease content of the produce water was reduced to 44 mg/l, while *n*-alkanes, aromatics, NSO compounds and PAHs were reduced to 38.4, 2.65, 1.78 and 0.0655 mg/l, respectively.A pure culture of *Penicillium* sp. reduced the oil and grease content to 72.3 mg/l, comprising of *n*-alkanes (65.50 mg/l), aromatics (0.98 mg/l), NSO compounds (1.64 mg/l) and PAHs (0.0021 mg/l) after 120 days of exposure. However, *A. niger* reduced the oil and grease content to 59.1 mg/l, comprising of *n*-alkanes (56.50 mg/l), aromatics (0.65 mg/l), NSO compounds (0.96 mg/l) and PAHs (0.008 mg/l) after 120 days of exposure. The results indicate that produce water is readily biodegradable and that fungal cultures have the capability to degrade the recalcitrant PAH component of the petroleum hydrocarbon mixture in produce water. Biodegradation rates were, however, slightly more enhanced by using *A. niger* than *Penicillium* sp.

Key words: Biodegradation, fungal cultures, petroleum hydrocarbons,produce water.

INTRODUCTION

The water produced along with the oil and gas during crude oil production is called produce water. It is also called formation water or oil field brine effluent (Read, 1978). Produce water is usually treated before disposal, because it constitutes a source of chronic pollution since they are continuously discharged into the open sea over a long period of time.

Produce water are also known to be toxic to fishes and other marine animals (Middledich, 1984). A major cause for concern in recent times has been the presence of polycyclic aromatic hydrocarbons (PAHs), such as anthracene, phenanthrene, benzo(a) pyrene and benzo(a)anthracene, in produce water. Some of these compounds are recalcitrant and potential carcinogens, and they have the capability to bioaccumulate in food chains since they are not easily biodegradable (Neff, 1985). This is a problem, because the produce water treatment systems currently in use by most oil production companies is primarily designed to remove particulate or dissolved oil and therefore has little effect on the concentrations of dissolved petroleum hydrocarbons and other organics in produce water (Lysyj, 1982).

Both mixed and pure fungal cultures have been used successfully in the degradation of PAHs (Andrea et al., 2001). The degradation of hydrocarbons by yeasts and filamentous fungi has been investigated previously and it was concluded that most fungal species are excellent hydrocarbon degraders (Sutherland, 2004; Gadd, 2001). However, these studies did not investigate the degradation of produce water hydrocarbons by fungal isolates.
An investigation carried out on the biodegradation of produce water hydrocarbons by Okoro and Amund (2002) showed that produce water hydrocarbons are readily degradable by the indigenous microbial flora. Since fungal species form part of the produce water microflora, an attempt was made to establish the role of

fungi in the biodegradation of produce water hydrocarbons by exposing the hydrocarbons to pure fungal cultures. In this study, the ability of two fungal isolates, *Penicillium* sp. and *A. niger*, to degrade petroleum hydrocarbon components of the produce water, especially the PAHs, was demonstrated. It is expected that a significant degradation of PAH in produce water by the indigenous fungal species will help to reduce the problem of bioaccumulation of these organic compounds in the marine animals, and also the resultant risks of potential health hazards associated with the consumption of the contaminated sea foods.

MATERIALS AND METHODS

Sample collection

Untreated produce water samples were collected in sterile 1000 ml Wheaton glass bottles at a point before the final process stream of the Wemco treatment plant at Chevron's Escravos tank farm. Treated produce water samples were collected at a point after the final process stream where it is being discharged to the receiving water.

Enumeration of hydrocarbon-utilizing fungi

Hydrocarbon-utilizing fungi in produce water were obtained by Plating out low dilutions (10^{-1} to 10^{-2}) of samples onto mineral salt medium (Mills et al., 1978). The medium has the following composition [in (g/l)]: NaCl (10), $MgSO_4.7H_2O$ (0.42), KCl (0.29), KH_2PO_4 (0.83), Na_2HPO_4 (1.25), $NaNO_3$ (0.42), Agar (15), distilled water (1000 ml), and pH (7.2). The medium was autoclaved at 1.1 kg/cm^2 for 15 min. The inoculated mineral agar plates were then inverted over sterile membrane filters, moistened with crude oil (Escravos light) and held in the lid of the petridishes. The petridishes were wrapped with masking tape so as to increase the vapor pressure within the dishes during incubation of the Petridishes at 29^{o}C for 7 days. After incubation, the fungal cultures were stained with methylene blue and observed under a high-power resolution microscope (x40). Each fungal culture was identified based on its morphological characteristics.

Separation of aliphatic and aromatic components of hydrocarbons in produce water

A measured volume of the oil sample (10 ml) was introduced into the bond elute filter to separate the hydrocarbons from the nitrogen, sulfur and oxygen (NSO)-containing compounds. The filtrate (2 ml), containing both the aliphatic and the aromatic components of the petroleum mixture, was subjected to high performance liquid chromatography (HPLC) (Waters 486, Waters Inc., USA). The aliphatic component eluted after 18 min, while the aromatic component eluted after 45 min. Each fraction (0.2 µl) was subsequently analyzed by means of gas chromatography attached to a mass selective detector.

Analysis of total hydrocarbons, *n*-alkanes and polyaromatic hydrocarbons

The analysis was performed, as previously described by Neff et al. (1985). The hydrocarbon extract was concentrated in a Kuderna-Danish flask, on a 70^{o}C water bath, to approximately 1.0 ml and the concentrated extract was then transferred to a 1-dram vial with a disposable pipette. The flask was rinsed twice with 1 ml of methylene chloride. The rinse solutions were added to the vial and the volume of the extract was reduced to about 1 ml with a gentle stream of purified nitrogen gas.

Total *n*-alkanes and aromatic concentrations were determined by GC-MS analysis of the F1 and F2 fractions, respectively. Both resolved and unresolved hydrocarbons were quantified. The resolved concentrations were determined by summing the total resolved area with valley integration, and then using an average *n*-alkane or PAH response factor to calculate an amount relative to the internal standard. The unresolved concentrations were calculated by integrating the total area of the chromatogram (comprising both resolved and unresolved complex mixture) and then subtracting the resolved area and determining the amount relative to the internal standard.

Gas chromatography of residual oils

Fresh and degraded oil were analyzed by gas chromatography using a Hewlett Packard 5890 series 11 gas chromatograph, equipped with a single flame ionization detector (FID) and fitted with a Perkin Elmer Nelson analog digital converter (900 series) and a Compaq computer. A DB-1 capillary column of 15 cm length and an internal diameter of 0.32 mm wide bore of 1micron film thickness were used (J and W Scientific). A temperature program of 50 - 305^{o}C, increasing at 3.5^{o}C per minute for 27.15 min was employed. Hydrogen with a flow rate of 2 ml/min was used as a carrier gas, while the flow rate of air was 400 ml/min. The detector temperature was 325^{o}C, while the injection port temperature was 305^{o}C. The oil extracts of culture supernatants were dissolved in methylene chloride, while a sample volume of 0.2 µl was injected. The nC_{17}/Pristane and nC_{18}/Phytane ratios were subsequently calculated from the height of various chromatograms.

Biodegradation and growth studies

Growth and degradation studies over a time course were carried out using untreated produce water from the Escravos tank farm as the sole carbon and energy source. The untreated produce water used for the study had an initial oil and grease content of 1407 mg/l. Starter cultures were originally prepared using the minimal salts formulations of Mills et al. (1978) and the produce water as the sole carbon and energy source. Ten (10) ml each of the pure fungal cultures *(A. niger* and *Penicillium* sp.*)* were introduced into 500 ml of produce water in a 1000 ml capacity Wheaton glass bottle. The bottle was covered with non-absorbent cotton wool and placed in a slanted position to allow air passage through the pores of the cotton wool. The bottles were shaken manually at regular intervals to allow adequate mixing and homogeneity of the contents. The experimental setup was monitored for a period of 120 days. At intervals of 30 days, culture samples were collected and analyzed for microbial load, while the residual hydrocarbon was extracted with methylene chloride and analyzed by gas chromatography.

RESULTS

Untreated and mechanically treated produce water

The untreated produce water used for the experiment had an oil and grease content of 1407 mg/l, comprising of *n*-alkanes (608 mg/l), aromatics (13.88 mg/l), NSO compounds (12.68 mg/l) and PAHs (0.833 mg/l), and initial nC_{17}/Pristane and nC_{18}/Phytane ratios of 1.41 and 2.93, respectively. The mechanically treated produce water used as a reference had an initial oil and grease

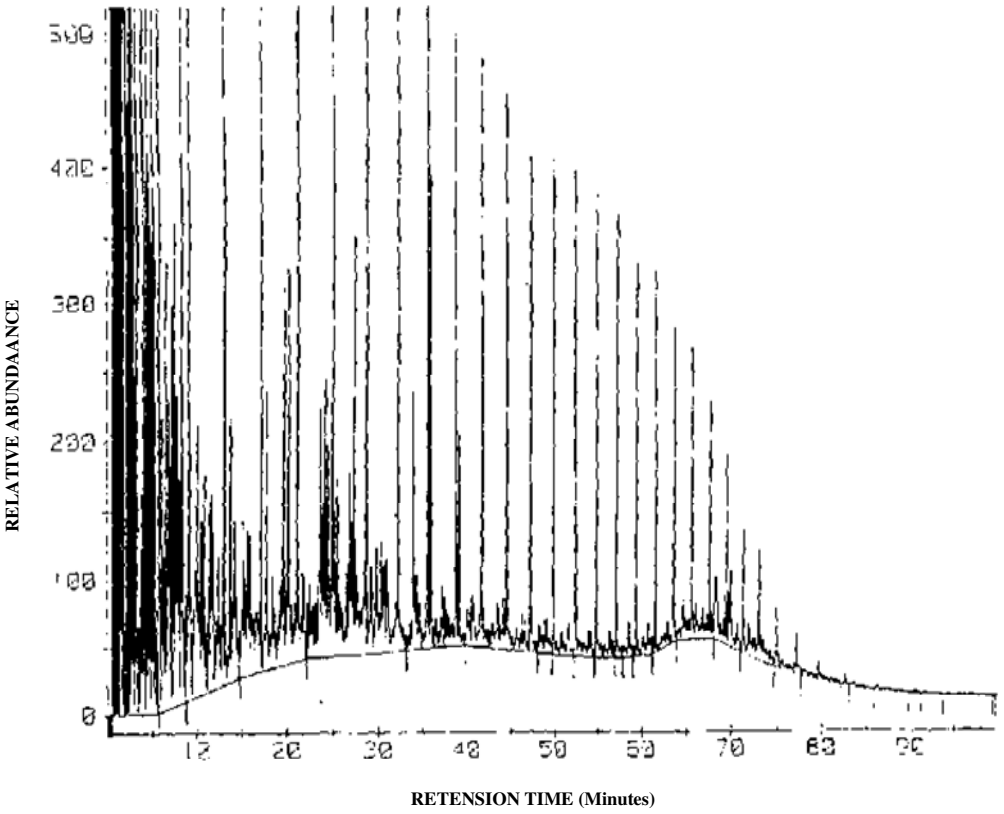

Figure 1. GC Chromatogram of untreated produce water from the Escravos tank farm (nC_{17}/Pristane, Ratio = 1.41; nC_{18}/Phytane, Ratio = 2.93).

content of 44 mg/l, comprising of *n*-alkanes (38.4 mg/l), aromatics (2.65 mg/l), NSO compounds (1.78 mg/l) and PAHs (0.0655 mg/l), and initial nC_{17}/Pristane and nC_{18}/Phytane ratios of 1.24 and 3.0, respectively. The GC chromatograms of both the untreated and the mechanically treated produce water are shown in Figures 1 and 2, respectively.

Biodegradation of produce water hydrocarbons with a pure culture of *Penicillium* sp.

The GC chromatograms of untreated produce water after a 120 days treatment with a pure culture of *Penicillium* sp. are shown in Figure 3. The corresponding nC_{17}/Pristane and nC_{18}/Phytane ratios are shown in Figure 4. With regards to the degradation of individual petroleum hydrocarbon components, it was observed that significant concentrations of aromatic components of the petroleum hydrocarbons were removed by the fungal culture after 120 days of exposure. The percentage reduction in the concentrations of individual petroleum hydrocarbon components after 120 days of exposure with *Penicillium* sp. were as follows: *n*-alkanes (89.3%), aromatics (93%), NSO compounds (87.1%) and PAHs (99.7%).

Biodegradation of produce water hydrocarbons with a pure culture of *Aspergillus niger*

The GC chromatograms of untreated produce water after a 120 days treatment with a pure *A. niger* culture are shown in Figure 5, and the corresponding nC_{17}/Pristane and nC_{18}/Phytane ratios are shown in Figure 6. With regards to the degradation of individual petroleum hydrocarbon components, it was observed that significant concentrations of aromatic components of the petroleum hydrocarbons were removed by the fungal culture after 120 days of exposure. The percentage reduction in the concentrations of individual petroleum hydrocarbon components after 120 days of exposure with *A. niger* were as follows: *n*-alkanes (90.7%), aromatics (95.32%), NSO compounds (92.43%), and PAHs (90.4%).

DISCUSSION

The two fungal isolates used in this study, *A. niger* and *Penicillium* sp., were the predominant fungal isolates found in produce water effluent from Chevron's Escravos tank farm (Okoro, 1999). They form part of the microflora of the produce water effluent and therefore can tolerate the slightly alkaline pH (7.9), low biological oxygen demand (BOD) and high salinity of the produce water. It

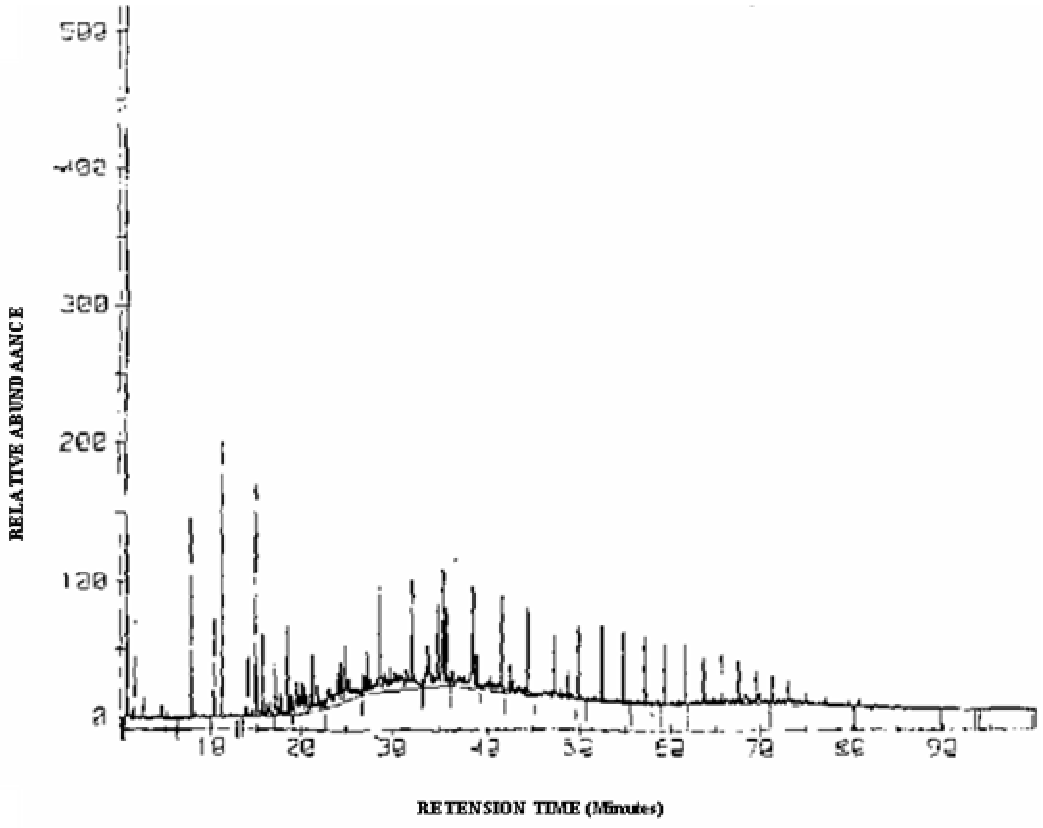

Figure 2. GC Chromatogram of mechanically treated produce water from the Escravos tank farm (nC_{17}/Pristane, Ratio = 1.24; nC_{18}/Phytane, Ratio = 3.00).

has been reported in the literature that a significant number of soil fungi utilize petroleum hydrocarbons very efficiently, though slowly (Canniglia et al., 1980).
Moreover, when degradation is carried out by mixed cultures of bacteria and fungi, fungal cultures are usually outgrown by their bacterial counterparts. Since the bacteria are usually fast degraders, the degradation potential of fungi are not usually observable until the time that the population density of the bacterial species has dropped significantly (Okoro and Amund, 2002).

However, comparing the degradation efficiency of Bacteria and Fungi on Polycyclic Aromatic Hydrocarbons (PAH), it is evident from this study that Fungi have a greater capacity and enzymatic capability to degrade the recalcitrant PAH than Bacteria. The pure cultures of *Penicillium sp.* and *A. niger* used in the study showed greater capability in the degradation of PAHs than the pure cultures of bacteria used by Okoro and Amund (2002). Other researchers, e.g. Andrea et al. (2001), Cerniglia (1992), Gadd (2001) and Sutherland (2004), have also reported that fungi are good PAH degraders. It is apparent from this study that both *A. niger* and *Penicillium* sp. were able to degrade the original level of petroleum hydrocarbon fractions, including PAHs present in the produce water, to an insignificant concentration after 120 days of exposure.

The index used to monitor the progress of biodegradation is the rate of decrease in the ratios of nC_{17}/Pristane and nC_{18}/Phytane. Pritchard and Coaster (1991) used the decreasing rate in the ratios of nC_{17}/Pristane and nC_{18}/Phytane to monitor the progress of biodegradation during the EPA Alaska oil spill bioremediation project. The application of this concept was based on the principle that during biodegradation, decreases of total oil residues could occur because of other non-biological processes. Thus, changes in hydrocarbon composition that are indicative of biodegradation must be measured accurately and this is done historically by examining the weight ratio between hydrocarbons known to be readily biodegradable, such as the C_{17} and C_{18} alkanes, and those that biodegrade slowly, such as the branched alkanes (Pristane and Phytane), but with very close chromatographic behaviour. The two fungal isolates showed a similar trend in the decreasing ratios of nC_{17}/Pristane and nC_{18}/Phytane up till the stage that the recalcitrant Pristane and Phytane were degraded.

In conclusion, *A. niger* and *Penicillium* sp., which form part of the microflora of the produce water effluent, showed an appreciable level of degradation of petroleum hydrocarbons after the 120 days of exposure. The two isolates also showed significant levels of degradation of the recalcitrant polycyclic aromatic hydrocarbon (PAH)

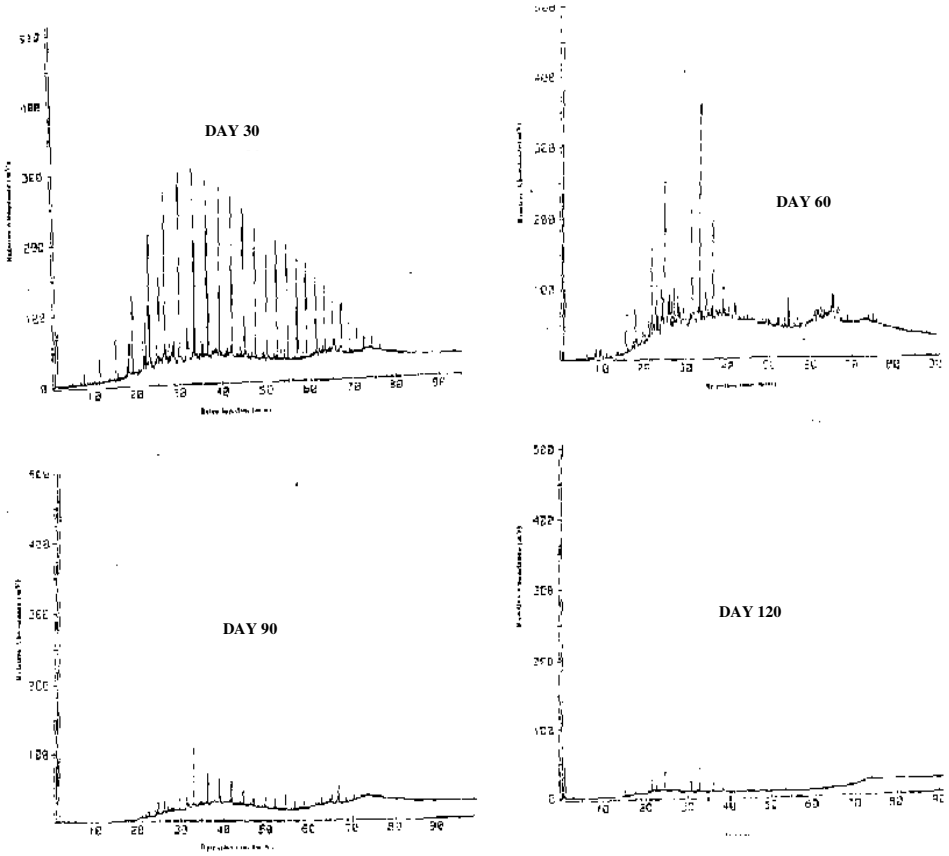

Figure 3. GC Chromatograms of untreated produce water after a 120 days exposure to a pure *Penicillium* sp. culture, without nutrient supplementation.

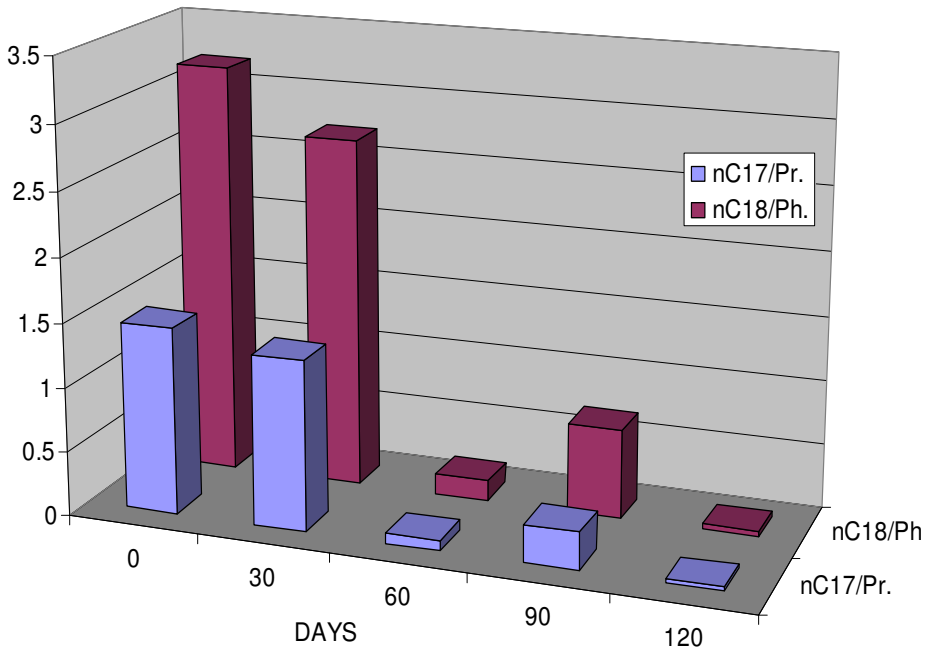

Figure 4. Biodegradation of untreated produced water, without nutrient supplementation, and treated with a pure *Penicillium* sp. culture. The result is evaluated as decrease in nC_{17}/Pristane and nC_{18}/Phytane ratios.

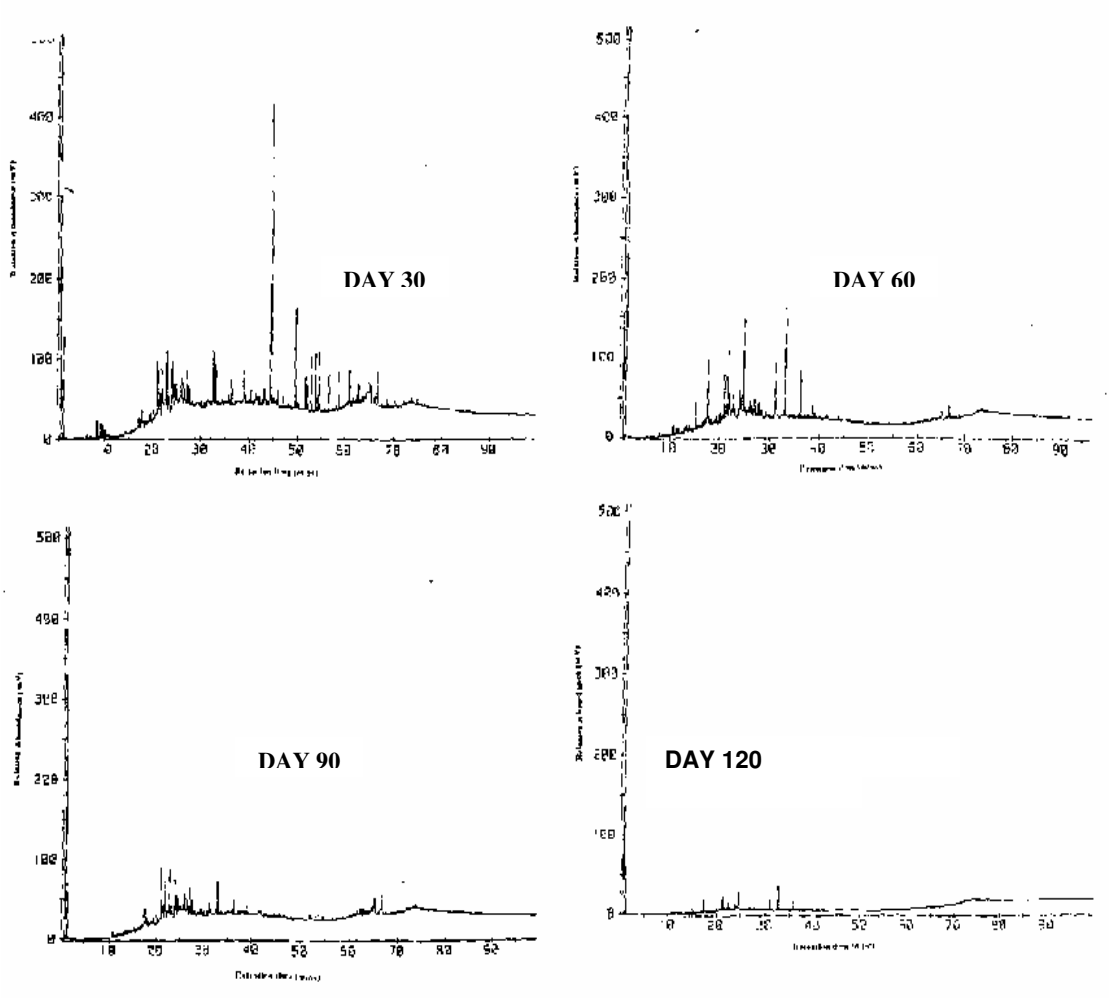

Figure 5. GC Chromatograms of untreated produce water after a 120-day exposure to a pure *Aspergillus niger* culture, without nutrient supplementation.

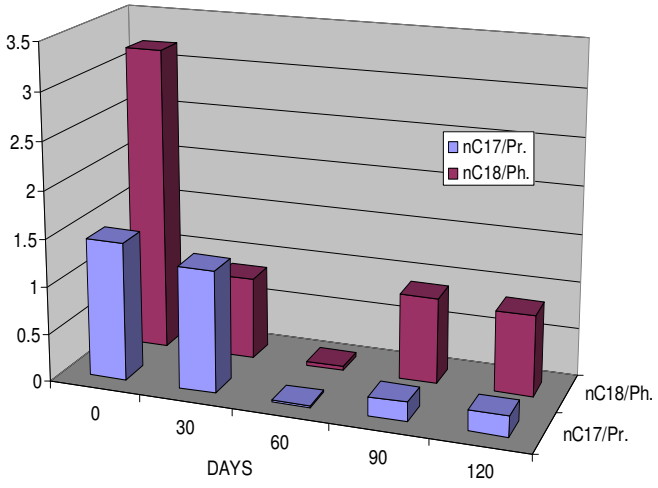

Figure 6. Biodegradation of untreated produced water, without nutrient supplementation, and treated with a pure *Aspergillus niger* culture. The result is evaluated as decrease in nC_{17}/Pristane and nC_{18}/Phytane ratios.

component of the hydrocarbon mixture present in the produce water in an experiment carried out under natural conditions.

ACKNOWLEDGEMENTS

Chevron Nigeria Limited is gratefully acknowledged for providing facilities and logistics for this research.

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