Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (5): 415-425, 2007
ISSN 1816-4935

Quantitative and Qualitative Assessment of Hydrocarbon-Degrading Bacteria and Fungi in Qua Iboe Estuary, Nigeria

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Abstract: The number (quantity) and nature (quality) of hydrocarbon-degrading bacteria and fungi in Qua Iboe estuary, Nigeria and their biodegradation potentials were assessed in this study to ascertain the estuary's inherent capacity for natural attenuation following an oil spill. Water and sediment samples collected 9 months after a minor oil spill revealed, upon analysis that the fraction of total heterotrophic microorganisms that could utilize crude oil as sole source of carbon and energy was up to 64% in sediments and 15% in near shore waters. There was significant correlation ($r = 0.78$; $p<0.05$) between total hydrocarbon content (THC) and percentage abundance of hydrocarbon utilizers in the samples. Different taxonomic and physiological groups of bacteria, yeast and mold were identified. Their carbon dioxide evolution potentials as groups, in terms of rate and extent of evolution, were only marginally better than those from their constituent pure culture studies. A pH-dependent periodic succession of these groups and their species was observed during the study. From the foregoing therefore, Qua Iboe estuary has a great capacity to self-purify in the event of an oil spill and such natural attenuation might even be more environment-friendly than other remediation protocols particularly bioaugmentation.

Key words: Number, nature, hydrocarbon degradation, bacteria, fungi, Qua Iboe Estuary

INTRODUCTION

According to industry estimates, almost 80% of the world's trade is moved by ships, making commercial maritime shipping one of the cornerstones of modern world economy. In Nigeria, the high rate of petroleum-related activities in the Niger Delta region causes frequent oil spills with attendant ecological problems associated with their coastal waters (Antai, 2005). The ecological damage of such spilled oil is influenced by the relative rate and importance of three basic processes namely; emulsification, biodegradation and mineralization; which form a logical sequence in the recycling of individual hydrocarbon molecules (Bartha and Atlas, 1977).

The microbial ecology of hydrocarbon degradation, emphasizing both environmental and biological factors that are involved in determining the rate at which and extent to which hydrocarbons are removed from the environment by biodegradation was reviewed by Atlas (1981, 1991) and Leahy and Colwell (1990). Biodegradation of petroleum and other hydrocarbons in the environment is a complex process whose quantitative and qualitative aspects depend on the nature and amount of oil or hydrocarbon present, the ambient and seasonal environmental conditions and the composition of the autochthonous microbial community.

The relative importance of natural biodegradation by the autochthonous microbial community and seeding of oil-impacted areas (bioaugmentation) with specially prepared bio-formulated microbial cultures has been a subject of controversy over time. Seeding of petroleum-contaminated aquatic

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environments has been attempted, with mixed results. Tagger *et al*. (1983) observed no increase in petroleum degradation in seawater inoculated with a mixed culture of hydrocarbon-degrading bacteria whereas Atlas and Busdosh (1976) reported increased degradation of oil in a saline Arctic pond after inoculation with an oil-degrading *Pseudomonas* sp., but no improvement in a fresh water pond.

In the present study, the quantitative and qualitative composition of the natural hydrocarbon-biodegrading bacterial and fungal populations in the Qua Iboe river estuary and their biodegradation potentials was assessed in order to ascertain the capacity of the estuary for natural attenuation in the event of an oil spill.

MATERIALS AND METHODS

Site Description

Qua Iboe estuary is a mesotidal estuary of the Qua Iboe river located within the Niger Delta region of Nigeria on Lat. 4°30' - 5° 30' N, Long. 7° 30' - 8° 20' W (Fig. 1). Ekwere *et al*. (1992) and King and Nkanta (1991) described the estuary as having fine sandy beaches fringed with tidal mud flats and mangrove swamps. The estuary has been under constant influence of petroleum exploration and exploitation activities.

Sample Collection

Sediment samples comprising inter-tidal (epipellic) and sub-tidal (benthic) sediments were collected from three locations on the estuary namely (A) Iwookpom, (B) Mkpanak and (C) Upenekang

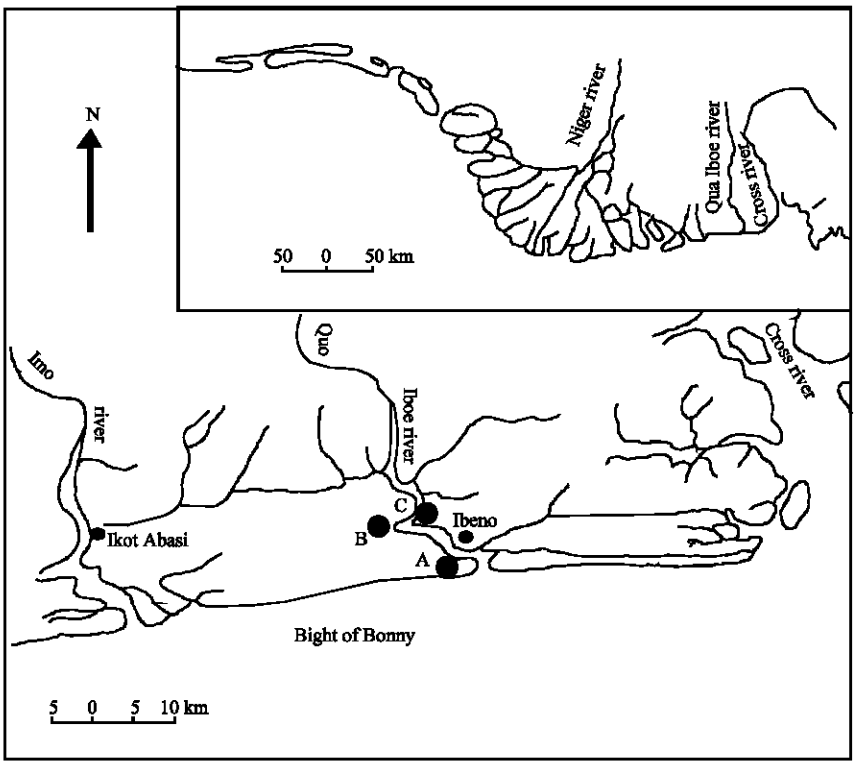

Fig. 1: Map of the Niger delta region of Nigeria showing A: Iwookpom, B: Mkpanak and C: Upenekang on the Qua Iboe estuary where samples were collected

(Fig. 1), each location separated from the other in that order by a distance of 500 m. Three samples were collected from each location from epipellic and benthic sediments with overlying near shore surface water samples. Epipellic sediment samples were collected with a hand scoop while benthic sediment samples were collected with Shipek grab sampler. The top 2 to 3 cm of sediments was collected, by placing the bottom of a sterile petri plate over the loose sediments and slicing the material (Hood *et al*., 1975), into plastic bags. Two replicates of all sample types were taken, properly labeled and preserved in ice-packed coolers and transported to relevant laboratories for processing and analysis.

Physicochemical Analysis

Replicate samples labeled for physicochemical analysis were transported to Aluminium Smelter Company of Nigeria (ALSCON) laboratory, Ikot Abasi, Nigeria for analysis. Determinations of pH, Salinity, Chloride content, Nitrate, Phosphate, Carbonates, Biochemical Oxygen Demand (BOD), Total Hydrocarbon Content (THC) (APHA, 1998), Total Organic Carbon (TOC) (Jakobsen, 1992) and exchangeable cations (nutritive salts) (Black *et al*., 1965; Radojevic and Bashkin, 1999) were made within 24 h of sampling.

Microbiological Analysis

Serial dilutions of water samples and supernatants of epipellic and benthic sediment samples from the three locations were made with sterilized aged seawater. Appropriate dilutions were plated by means of the surface spreading technique onto Nutrient Agar (NA) and Sabourund Dextrose Agar (SDA) for enumeration of total aerobic heterotrophic bacteria and total fungi respectively. Three replicas of the preparations were set up. Colony forming units were enumerated from the maximal colony development after 7 days of incubation at room temperature (28±2°C).

A basal medium made up of 750 mL seawater, 250 mL distilled water, 0.5 g NH_4Cl, 0.5 g K_2HPO_4 and 0.5 g Na_2HPO_4 (Hood *et al*., 1975) and solidified with agar (Oxoid), was used for the enumeration of oil-degrading bacteria, yeast and mold. The portion of medium meant for isolation of oil-degrading bacteria was supplemented with 50 μg mL^{-1} of nystatin to inhibit interfering yeast and mold and pH adjusted to 7.6. The portion meant for enumeration of oil degrading yeasts was supplemented with 50 μg mL^{-1} of chloramphenicol and the pH adjusted to 4.5, while that for mold isolation was supplemented with 50 μg mL^{-1} of each of penicillin G and streptomycin to inhibit interfering bacteria and pH adjusted to 5.8. The vapour phase transfer method of Thijsee and van der Linden (1961) was used with Nigerian light crude oil as carbon and energy source supplied from the lid of the plates. Test preparations as well as the controls (without crude oil) were made in triplicates and incubations made at room temperature (28±2°C) for 7 days.

Colony Forming Units (CFUs) were enumerated and the number of hydrocarbon-utilizing bacteria, yeast and mold were calculated by subtracting the number of CFUs in control from those in test cultures (Antai and Mgbomo, 1989). The ratio of hydrocarbon-utilizing bacteria and fungi to their total heterotrophic counts were determined and expressed in percent. In addition, each developed colony that exhibited distinctly different colony morphology was noted and enumerated.

Each distinct bacterial, yeast and mold colony on oil-degrading enumeration plates was purified by repeated sub-culturing onto Nutrient Agar (NA), Yeast-Peptone-Dextrose (YPD) agar (containing 2% glucose, 1% yeast extract [Difco], 2% peptone [Difco] (Urano *et al*., 2001) and Sabouraud Dextrose Agar (SDA), respectively. The isolates were also respectively characterized and identified based on the schemes of MacFaddin (1980), Barnett and Pankhurst (1974) and Barnett and Hunter (1987) and Efiuvwevwere (2000). Pure cultures of isolates were maintained on slants containing media used for their purification and preserved at 4°C in a refrigerator until required.

All hydrocarbon-utilizing isolates were screened for oil-utilization capabilities in Mineral Salts broth Medium of Okpokwasili and Okorie (1988), under stationary and agitated conditions of incubation. Filamentous fungi (mold) were also screened by the agar method of Thijsee and van der Linden (1961).

Biodegradation Potentials of Isolates

Oil biodegradation potentials of isolates were assessed by the carbon dioxide evolution method of Cornfield described by Ekpenyong and Antai (2006a). Residual hydrocarbon in mineralization study flasks were determined by the gravimetric method of Odu (1972). The potentials of eight pure cultures of hydrocarbonoclastic isolates were assessed singly and as different assemblages of bacteria, yeast and mold. Determinations of pH and viable counts of bacteria, yeasts and molds from the mixed consortial study flasks (comprising 4 bacteria, 2 yeasts and 2 mold isolates) were made at 2 day intervals over 16 days of incubation.

Statistical Analysis

Analysis of variance of appropriate data and correlation analysis were used to establish, at 95% confidence limit, significant relationships or otherwise of studied parameters.

RESULTS

Total hydrocarbon content (THC) of 425.63 mg kg^{-1} in the epipellic sample was observed, indicating pollution of the estuary at the time of sampling. There was considerable estuarine health as evidenced by the amounts of nutritive salts (calcium, potassium, sodium and magnesium) in the estuary, with a salinity value of 4.7% (Table 1). There was significant ($p<0.05$) difference in the number of hydrocarbon utilizers among the sampling locations, with a high degree of site-specific variability within sampled regions as evidenced by the large standard deviations obtained (Table 2). There was significant site-specific correlation ($r = 0.78$; $p<0.05$) between THC and number of hydrocarbon-utilizing microorganisms. There were also significant ($p<0.05$) variations in the number of different colonies among sample sites and locations (Table 3). A total of twenty-eight (28) hydrocarbon-utilizing microorganisms (comprising 12 bacteria, 8 yeasts and 8 molds) were isolated in the study by selective enrichment and characterized as species of *Nocardia*, *Bacillus*, *Pseudomonas*, *Micrococcus*, *Proteus* and *Vibrio* (Bacteria); *Geotrichum*, *Rhodotorula*, *Candida, Saccharomyces* and *Sporobolomyces* (yeasts); *Aspergillus*, *Penicillium* and *Rhizopus* (Molds) (Table 3).

Table 1: Physicochemical analysis of water sample, epipellic and benthic sediment samples from Iwookpom (outfall location)

Parameters	Water	Epipellic sediment	Benthic sediment
Chloride (mg L^{-1})	485.7	ND	ND
Salinity (%)	4.7	ND	ND
Carbonates (mg L^{-1})	57.0	ND	ND
BOD (mg L^{-1})	0.6	ND	ND
pH	7.56	3.85	7.18
Total Nitrogen	1.4[a]	0.112[b]	0.084[b]
Nitrate	0.136[a]	1.673[c]	2.092[c]
Total organic carbon (%)	ND	3.904	2.372
Total hydrocarbon content	100.2[a]	425.6[c]	137.5[c]
Phosphate	0.002[a]	232.63[c]	432.0[c]
Calcium	40.42[a]	1.124[d]	1.924[d]
Potassium	65.99[a]	2.467[d]	2.517[d]
Sodium	12.22[a]	11.17[d]	9.284[d]
Magnesium	6.402[a]	13.93[d]	15.25[d]

Key: ND = Not Determined [a] (mg L^{-1}); [b](%); [c](mg kg^{-1}); [d](meq/100 g)

Table 2: Microbiological analysis of water, benthic sediment and epipellic sediment samples

Treatments	Water	Epipellic sediment	Benthic sediment
THBC	2.27 (±1.42) $\times 10^7$	2.52 (±2.04) $\times 10^8$	1.46 (±0.92) $\times 10^8$
HFC	1.08 (±1.67) $\times 10^7$	1.84 (±1.18) $\times 10^8$	1.26 (±0.92) $\times 10^8$
HUBC	2.05 (±0.80) $\times 10^6$	1.48 (±0.54) $\times 10^8$	0.41 (±0.87) $\times 10^8$
HUFC	1.66 (±2.05) $\times 10^6$	1.18 (±2.25) $\times 10^8$	0.49 (±1.41) $\times 10^8$
%HUB	9.03	58.73	28.08
%HUF	15.37	64.13	38.89

THBC = Total Heterotrophic Bacterial Count; HFC = Heterotrophic Fungal Count; HUBC = Hydrocarbon-Utilizing Bacterial Count; HUFC = Hydrocarbon-Utilizing Fungal Count; %HUB = fraction of THBC that utilized crude oil × 100 %HUF = fraction of HFC that utilized crude oil × 100 values presented in the tables are mean counts; ±standard deviations from the mean of triplicate determinations

Table 3: Occurrence of hydrocarbonoclastic bacteria, yeast and mold genera in study samples and locations

	Water			Epipellic sediment			Benthic sediment		
Microbial genera	A	B	C	A	B	C	A	B	C
Bacteria									
Nocardia (2)[a]	-	-	-	+	+	+	+	+	-
Bacillus (4)	+	+	+	+	+	+	+	+	+
Pseudomonas (2)	+	+	+	+	+	+	+	+	+
Micrococcus (1)	+	+	+	+	-	+	-	+	+
Proteus (2)	+	+	-	+	+	-	-	-	-
Vibrio (1)	+	+	-	-	-	-	-	-	-
Yeasts									
Geotrichum (1)	-	-	-	+	+	+	-	-	-
Rhodotorula (1)	-	-	-	+	+	-	-	-	-
Candida (4)	+	+	+	+	+	+	+	+	+
Saccharomyces (1)	-	-	-	+	+	+	+	+	-
Sporobolomyces (1)	+	+	-	+	+	+	+	+	-
Molds									
Aspergillus (4)	+	+	+	+	+	+	+	+	+
Penicillium (3)	+	+	+	+	+	+	+	+	+
Rhizopus (1)	+	-	-	+	+	+	-	-	-

a = Number of distinct colonies (species), + = Present, - = Absent, A = Iwokpom, B = Mkpanak and C = Upenekang sampling locations,

There was no significant ($p>0.05$) difference between stationary and agitated screen test conditions among the yeasts; however bacteria performed better in agitated systems while the molds preferred stationary conditions of incubation (Data not shown).

Biodegradation potentials of isolates obtained from the results of the screen tests, as pure cultures and as consortia (bacterial, yeasts, mold and mixed microbial consortium) are shown in Fig. 2. Highest amount of CO_2 evolved after 16 days incubation was observed in pure culture studies of *P. frequentans* ESM-02 (an epipellic sediment mold). *Candida marina* SY-06 (both epipellic and benthic sediment yeast) and *Pseudomonas* sp. WB-04 (a water bacterial isolate) evolved 64.6 and 61.4 mg of CO_2, respectively. The yeast consortium produced the highest amount of CO_2 during the study, amassing 74.8 mg of CO_2 in 16 days. Significant correlation between amounts of carbon dioxide evolved and percent weight loss of crude oil during biodegradation was observed in all pure culture studies but one (*Bacillus* sp. ESB-12). Such correlation was not observed in culture studies involving any of the microbial consortia.

Determination of viable counts of bacteria, yeast and mold in the mixed microbial consortium revealed that bacterial counts increased from 4.8×10^6 (day 0) to a maximum of 28.1×10^6 CFU mL^{-1} (day 10). Mold counts could not be taken on day 10 as plates were overcrowded (colony counts > 300) with mold colonies. The crowdedness however reduced by day 12 till the end of the sampling period. Yeast counts increased gradually throughout the duration of the study until plate counts could no longer be determined on days 14 and 16 (Table 4).

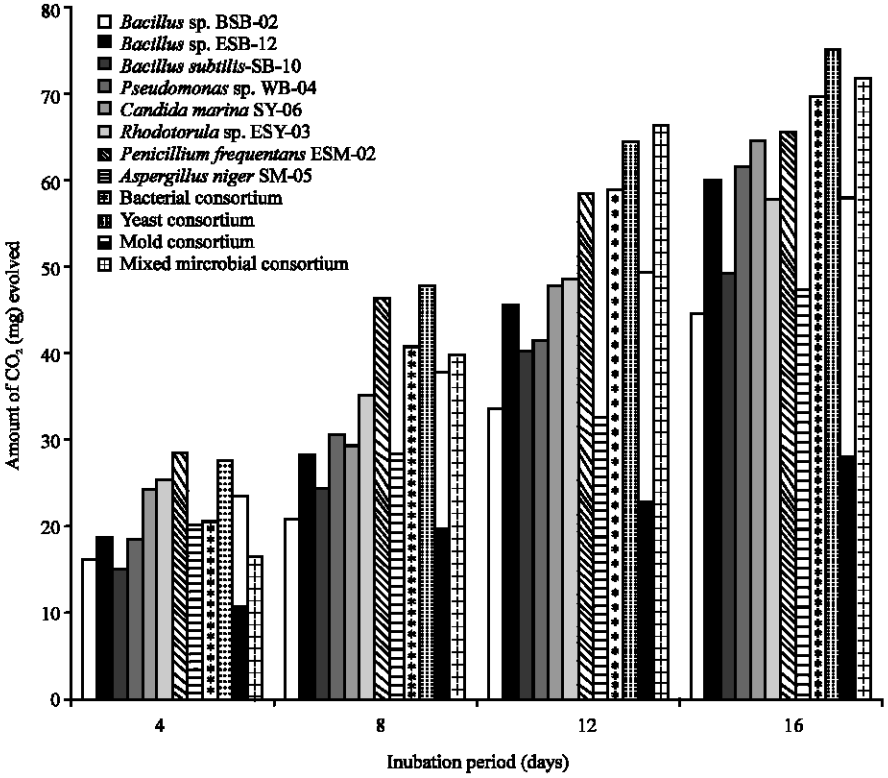

Fig. 2: Oil biodegradation potentials of microbial isolates and consortia

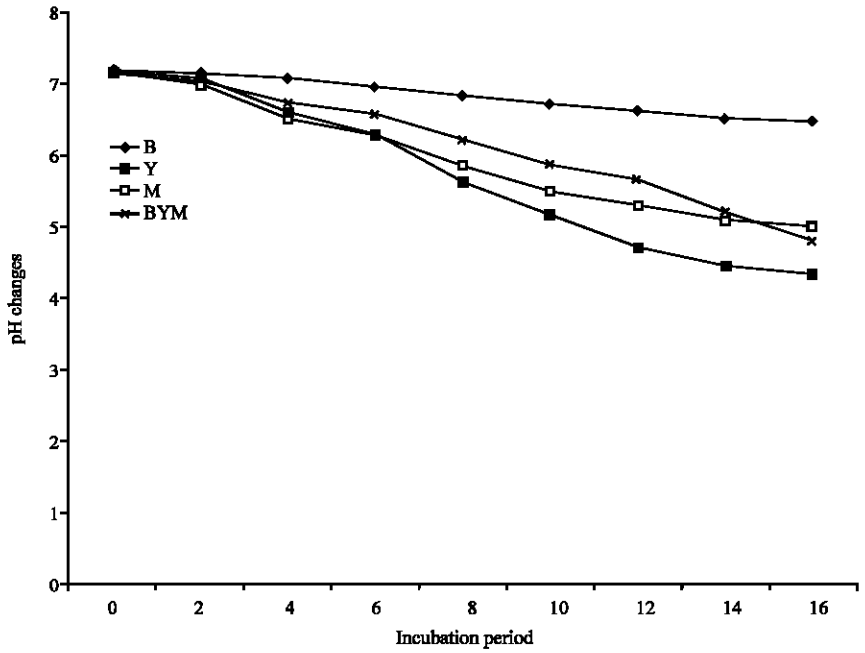

Fig. 3: pH changes during oil biodegradation by microbial consortia

Table 4: Viable count changes of taxonomic groups during oil biodegradation by mixed microbial consortium

	Viable counts ($\times 10^6$ CFU mL^{-1})		
Incubation period (days)	Bacteria	Yeast	Mold
0	4.8	3.20	3.8
2	5.3	13.9	4.2
4	10.8	14.9	6.7
6	17.9	15.3	7.3
8	22.7	25.9	10.2
10	28.1	27.3	> 300 colonies
12	21.6	28.7	26.8
14	18.4	>300 colonies	22.8
16	12.2	> 300 colonies	23.1

Figure 3 shows the gradual pH changes in the study flasks of four different microbial consortia. The drop in pH as a result of oil degradation was most pronounced in yeast consortium (Y) and least in bacterial consortium (B). Intermediate final pH values of 5.23 and 5.04 were observed for mold (M) and mixed microbial consortia (BYM) biodegradation studies, respectively.

DISCUSSION

It was assumed, in this study, that the temperature, pH, dissolved oxygen, NO_3^- and HPO_4^{2-} concentrations in the immediate 1000 m radius of the outfall location (Iwookpom) in the Qua Iboe estuary were relatively constant, but that salinity fluctuations would be a function of tidal movements (Amund and Igiri, 1990). It was also assumed that owing to the hydrodynamics of the receiving water, the petroleum pollutant was unevenly distributed in the estuary (Grahl-Nielsen, 1987) and that the area of high contamination is often localized to the vicinity of the outfall and decreases with distance (Wake, 2005). Physicochemical analysis was therefore fully conducted on samples obtained from Iwookpom (the outfall sampling location A), with an assumed highest total hydrocarbon content (THC). Preliminary results of physicochemical analysis of samples from other distant locations (data not shown) firmly ratified the afore-stated assumptions.

Although there was no significant ($p>0.05$) difference in the counts of aerobic heterotrophs among the 3 sampling locations, there was significant ($p<0.05$) difference in the proportion of hydrocarbon utilizers among the different locations, with a high degree of site-specific variation in hydrocarbon-utilizing microbial counts. Both hydrocarbon-utilizing bacterial (HUB) and hydrocarbon-utilizing fungal (HUF) counts were up to 10^8 CFU mL^{-1} in the sediments and 10^6 CFU mL^{-1} in overlying waters, a finding which was earlier reported in the review of Bartha and Atlas (1977) and a later study by Antai and Mgbomo (1989). Whereas HUB counts were generally higher than those of HUF, the results revealed that the fraction of heterotrophic fungi that could utilize petroleum hydrocarbons as sole source of carbon and energy was higher than that of bacteria. However, the relative importance of bacteria versus fungi in the self-purification of petroleum-impacted environments (especially coastal areas) has been a matter of controversy over time. Both absolute counts and proportions of hydrocarbon-utilizing microbial counts were highest in the epipellic (intertidal) sediments (Roubal and Atlas, 1978). The significant correlation ($r = 0.78$; $p<0.05$) between THC and the percentage abundance of hydrocarbon utilizers in the epipellic sediment suggests that by reason of ocean waves, currents and tidal movements, the bulk of hydrocarbons, even re-suspended ones from the submerged (benthic) sediments would accumulate, given time, in the near shore emergent (epipellic) sediments. This is further strengthened by the fact that the pH of this sediment sample was fairly acidic (3.85) suggesting high organic acid levels resulting from active organic matter biodegradation (Essien and Ubom, 2003). Such high numbers of oil-degrading microorganisms suggest either chronic low-level pollution of the estuary (Crow *et al*., 1975), a previous history of oil contamination (Atlas, 1991;

Delille and Delille, 2000), or a recent pollution, as occurred in the estuary nine months before sampling. The last suggestion is consistent with that of Pinholt *et al*. (1979), who observed that, eight months after contamination of soil by crude oil, hydrocarbon-utilizing bacteria increased to almost 50% of the total heterotrophic bacterial count.

It has been demonstrated that microbial communities can affect chemical pollutants, but the presence of toxicants can also affect microbial community structure (Delille and Siron, 1993; Long *et al*., 1995). These chemicals can alter the community structure through selection of pollutant degraders thus determining the qualitative factors of pollutant biodegradation. In this study, a total of twenty-eight hydrocarbon utilizers, comprising twelve bacteria and eight each of yeast and mold, were identified. The organisms were representatives of six bacterial, five yeasts and three mold genera. The filamentous group of bacteria (actinomycetes) was represented by *Nocardia*; the Gram-negative group by *Vibrio*, *Proteus* and *Pseudomonas* and the Gram-positive group by *Bacillus*, *Micrococcus* and *Nocardia*. Majority of the hydrocarbon-utilizing bacterial genera were rod-shaped bacteria, but the supremacy of Gram-negatives over Gram-positives was not established in this study. The spore-forming group which would survive adverse environmental conditions and reactivate to continue the biodegradation process at the turn of favourable conditions, was represented by *Bacillus* and *Nocardia*. Aerobic and facultatively anaerobic groups of bacteria were observable. The salt-tolerant bacterial group was represented by *Vibrio*. Acidophillic and acid-tolerant groups of yeasts and fungi were even more expressive. The fermentative yeast *Saccharomyces* and other well-documented hydrocarbon-utilizing yeasts like *Candida* and *Rhodotorula* were present. *Aspergillus* and *Penicillium*, which contain species that could utilize crude oil in quiescent and turbulent waters, were isolated. The amylolytic, lipolytic and nitrate-reducing species of *Bacillus*, *Pseudomonas* and *Aspergillus* were widely distributed in study samples. Qua Iboe estuary is therefore qualitatively stable in terms of the great diversity of physiological groups of hydrocarbon-utilizing bacteria (Sanchez *et al*., 2006), harboured by its waters, near shore and submerged open sea sediments.

Biodegradation potentials of the organisms were potentials for complete biodegradation to CO_2 and H_2O (mineralisation). Results of correlation analysis between amounts of CO_2 evolved and percentage weight loss of crude oil showed that such correlation did not always exist as particularly observed in pure culture studies of *Bacillus* sp. ESB-12 (an epipellic sediment bacterium), as well as bacterial, yeast and mixed microbial consortial studies. The observation in pure culture studies was earlier reported by Ekpenyong and Antai (2006a), where they suggested that enzyme systems for initial oil biodegradation were quite distinct from those for CO_2 production. Haughton and King (1961) reported that decarboxylase enzyme systems are mostly adaptive and as such oil-degrading microorganisms will first have to generate from their initial attack on the hydrocarbon structure, intermediates that are capable of inducing the expression of a non-existent, or activating an already existing but inactive decarboxylase enzyme systems, to be qualitatively useful in bioremediation exercises.

The pH changes in consortial oil biodegradation studies revealed that yeasts were responsible for much of the pH depressions in test systems, probably because of their utilization of fermentative catabolic pathways of hydrocarbon biodegradation. Fermentative metabolism of organic substrates has been reported to produce more acidic end products than oxidative metabolism (Hugh and Leifson, 1953; Ekpenyong and Antai, 2006b). In studies involving mixed microbial consortium, the pH depression was not as much as was observed in the yeast or mold consortial studies, suggesting possible neutralizing effect by basic intermediate products mostly from organisms that utilize oxidative biodegradation pathways. Changes in viable counts, reported to be more reliable than optical density measurements in toxicity studies (Ekpenyong and Antai, 2006b) revealed periodic succession of microbial groups during oil biodegradation. There was a strong positive correlation ($r = 0.92$) between pH changes and the nature of microbial group dominating biodegradation at any given time. Apart from

the direct environmental pH effect in selecting dominant microbial groups or species, differential participatory roles of selected microbial groups during oil biodegradation in the natural environment may not be unconnected with the differential toxicities of some components of crude oil to microbial groups and species, influenced indirectly by pH changes. Sulphides, for instance, are known to be removed by bacteria (Concawe, 1979) but have the opposite relationship with pH, that is, toxicity increases with decreasing pH.

The findings from this study revealed that Qua Iboe estuary has a great capacity to self-purify in the event of an oil-spill owing to the large number (quantity) of oil-degrading microorganisms, the nature of the physiological groups of bacteria and fungi and their inherent biodegradation potentials (quality) which gets better with time owing to adaptation by acquisition of transferable plasmids, on which are encoded genes for hydrocarbon-biodegradation pathways. The use of native or non-native microbial consortium with hydrocarbon-utilizing capabilities as seed (bio-augmentation) in this chronic oil-impacted environment therefore might not amount to much, as bio-stimulation by nutrient addition might prove a more workable remediation technique (as is being demonstrated in preliminary studies in our laboratory) especially in near shore areas where active hydrocarbon biodegradation activities are observable, as was the case in the Exxon Valdez disaster.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Etesin of Aluminum Smelter Company of Nigeria (ALSCON) laboratory, Ikot Abasi, Nigeria for conducting the physicochemical analysis of the samples. They also appreciate the efforts of Mr. Sammy Onoyom of the Cross River State Water Board and Mr. Godwin Iwatt for assisting in the microbiological and statistical analysis of the study, respectively.

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