Biodegradation Potential of Polycyclic Aromatic Hydrocarbons (PAHS) of Effluents from Forcados Terminal in Delta State by Some Bacteria Isolates

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ABSTRACT
The biodegration potential of polyclic aromatic hydrocarbons (PAHS) of effluents from Forcados Terminal in Delta State by some bacterial isolates was carried out by incubating the isolates in a mineral salt broth amended with PAHs. Gas chromatographic method was used to determine the levels of PAHs left after 21 days incubation period. The bacteria isolated from effluents of Forcados Terminal included Pseudomonas sp, Klebsiella pneumoniae, Escherichia coli, Micrococcus sp and Staphylococcus sp. Pseudomonas sp is the most commonly occurring. Result of the 21-day biodegradation test by some bacterial isolates showed that there was a reduction in the original concentration of PAHs used for the test. Test results showed decrease in concentration of PAHs with increase in exposure time. Analysis after 21 days showed complete absence of PAHs. Both pure and mixed cultures of bacteria are found to be potential agents of bioremediation of environment impacted by PAHs. The pure cultures of the bacteria Pseudomonas sp or Klebsiella sp used in this study showed greater capabilities in the degradation of PAHs than the mixed culture of bacteria (Pseudomonas sp and Klebsiella sp) and pseudomonas was found to be an excellent degrader and agent of remediation of environments impacted by PAHs under controlled conditions.

KEYWORDS: *Biodegradation, bacterial cultures, polycyclic aromatic hydrocarbons, effluent*

INTRODUCTION

Petroleum pollution poses the most significant problems to humans and aquatic organisms in areas of oil and gas extraction, shipping and processing. Petroleum refers to a group of naturally occurring hydrocarbons. Even a small petroleum spill into the ocean, terrestrial environment or flaring of gases into the atmosphere may be toxic and cause great material and ecological losses. The discharge of chemicals to different compartments of the ecosphere affects the aquatic environment (Hyland *et al.*,1999). The difficulty in determining petroleum pollution in an environmental sample is based on the petroleum's composition. Nature has an amazing ability to cope with small amounts of water wastes and pollution, but it would be dangerous or detrimental if the billions of gallons of wastewater and sewage produced everyday are not treated before

releasing them back to the environment. Increase crude oil pollution often leads to lots of degradation of plants and animals in the environment (Onwurah et al., 2007). In most developing countries like Nigeria, most industries dispose their effluents without treatment. These industrial effluents have a hazard effect on water quality, habitat quality, and complex effects on flowing water (Ethan *et al*., 2003). Industrial wastes and emission contain toxic and hazardous substances, most of which are detrimental to human health (Jimena *et al*., 2008). Polycyclic aromatic hydrocarbons (PAHs) are molecules made of two, three or more fused aromatic rings in various structural configurations, e.g. pyrene, fluoranthene, phenanthrene, etc (Karthikeyan and Bhandani, 2001). They are a class of compounds found throughout the environment in the air, in the soil and in the water. They are found naturally in crude oil, creosote, coal tar, and coal. They can also be made by incomplete combustion of hydrocarbons in coal, oil, gas, tobacco and during forest fires (Vila *et al*., 2001). Examples of some known PAHs include: naphthalene, 2-methylnaphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzanthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno (1,2,3-cd) pyrene. Concern about PAHs initially focused on their ability to cause cancer, but more recently, concern has turned to their interference with hormone systems and their potential effects on reproduction as well as their ability to depress immune function (Chaloupka, 1993). The objectives of this study therefore, was to isolate and identify bacteria in the effluents of Forcados Terminal, to ascertain the level of pollution by enumerating the number of bacteria found in the effluents and to determine the biodegradation of PAHs by some associated Bacteria.

MATERIALS AND METHODS

Effluent samples for bacteria analysis were collected using the method of Adesemoye *et al* (2006), also used by Nwachukwu (2010) . Sterile 1 litre sample bottles were used to asceptically draw part of the effluent water. The effluent samples were collected from four different locations: Barge Jetty A, Barge Jetty B, Saver Pit 1 and Saver Pit 2. The Saver Pits 1 and 2 effluent samples were collected from the storage tank of the Tank Farm situated at the Core Zone while the Barge effluents A and B were collected from the Main Jetty situated at the Secondary Zone where there are lots of ship and vessel settlers moving in and out of the Terminal. Control surface water samples were collected 500m away from the sampling points, far away from the Terminal, to avoid control sampling areas that may be contaminated by the effluent discharge. After collection, the samples were placed in a cooler containing ice blocks and transported immediately to the laboratory for analysis. 10ml of each effluent samples was added to 90ml of sterile distilled water to get an aliquot. Subsequent serial dilutions were made by adding 1.0ml of the last dilution to 9ml of fresh diluents. Finally, 0.1ml of appropriate dilutions (10^{-2} and 10^{-3}) were inoculated unto sterile solidified agar in petridish and evenly spread out with a sterile glass spreader. Cultures were prepared in duplicates on dry media, and incubated at room temperature. Total Heterotrophic Bacterial Counts of the effluents was determined. The spread plate technique as described by Prescott *et al*. (2005) was adopted. The plates were incubated at room temperature for about 3-5 days after which the colonies were counted and the mean of the counts recorded accordingly. Different bacteria colonies were sub-cultured on sterile Nutrient Agar to get pure cultures. Pure cultures of bacteria were subcultered on Nutrient Agar slants which were then stored in the refrigerator for further use. Polycyclic Aromatic Hydrocarbon contents of the samples were determined by gas chromatographic (GC) analysis. A Schimadzu GC-17A gas chromatograph equipped with flame ionization detector (FID) was used.

Isolation of Hydrocarbon Utilizing Bacteria from Forcados Terminal Effluents

The population of hydrocarbon utilizing bacteria was determined by inoculating 0.1ml aliquot of the 10^{-1} diluted samples onto mineral salt agar media using the spread plate technique as described by Odokuma (2003). The vapour phase transfer method phase method was adopted. It employed the use of sterile filter paper discs soaked in filter sterilized crude oil which served as the only carbon source in the mineral salt agar. The sterile crude oil-soaked filter papers were then aseptically transferred to the inside cover of the inoculated petri dishes and incubated for 5days at room temperature. After the incubation period, mean of the colonies for the triplicate plates were calculated and recorded accordingly

Adaptation of Polycyclic Aromatic Hydrocarbon Degrading Isolates

Bacteria isolates were adapted for polycyclic aromatic hydrocarbon utilization and degradation using mineral salt broth with polycyclic aromatic hydrocarbon as the sole carbon source. Incubation was at 30°C and aerated at 100 strokes per minute (Wang, 1984) for 30 minutes each day for 10 days. A loopful of the adapted culture medium was transferred onto mineral salt agar plates as described by Odokuma (2003). The media contains polycyclic aromatic hydrocarbon as the only carbon source. The plates were incubated at 30°C for 5 days after which discrete colonies that developed were transferred onto Nutrient Agar plates and then incubated at 30°C for 24 hours after which they were stored in the refrigerator for further use.

Preparation of PAHs Standard Solution

An ampoule of polycyclic aromatic hydrocarbon (Sigma, USA) containing 1mg each of naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, 1,2 – benzanthracene, chrysene, benzo(b)fluoranthene, benzo(k) fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene was asceptically mixed with 99ml of sterile normal saline (diluent) making 100ml of stock solution containing 10mg/l of individual polycyclic aromatic hydrocarbon constituent. From the stock solution 0.3ml each of this stock solution (containing 0.03mg/l of each PAH) was added to each experimental flask that contained 99.7ml mineral salt broth and 0.1ml of the microorganisms in the biodegradation experiments.

Biodegradation Experiment

The method used in this study is applicable for the determination of biodegradation rates of organic compounds in an activated sludge process. The test method is designed to evaluate the ability of an aerobic biological reaction system to degrade or destroy specific components such as PAHs in waste streams. This method was also used by Okoro (2008) and Nwachukwu (2010) Preparation of inoculum. Two bacterial isolates, *Pseudomonas sp.* and *Klebsiella sp.*, were subcultured separately on sterile nutrient agar medium followed by incubation at 37°C for 24 hours. A loopful of each was then inoculated into a sterile nutrient broth medium separately for pure cultures and combined for mixed cultures, followed by incubation at 37°C for 24 hours.

The cells were then harvested by centrifuging at 2000 rpm for 30 minutes, after which the cells were individually re-suspended in sterile physiological normal saline and further washed by centrifuging at 2000 rpm for another 30 minutes to obtain neat cells which were suspended in sterile physiological normal saline and further diluted with sterile physiological normal saline to a low density cell suspension of 0.2 absorbance containing 1×10^3 cfu/ml of bacteria. 0.1ml

from this dilution which served as inocula was added to the 3 sets of experimental flasks as shown below.

Composition of PAHs Biodegradation Experiment Flasks

99.7ml mineral salt broth + 0. 3ml (0.03mg/l) solution of PAH + 0.1ml culture of *Pseudomonas sp.,*

99.7ml mineral salt broth +0.3ml (0.03mg/l) solution of PAH + 0.1ml culture of *Klebsiella sp.,*

99.7ml mineral salt broth + 0.3ml (0.03mg/l) solution of PAH + 0.1ml mixed culture Bacteria *(Pseudomonas sp.,* and *Klebsiella sp.,)*

99.7ml mineral salt broth + 0.3ml (0.03mg/l) solution of PAH + 0.1ml sterile Nutrient broth.

Statistical Analysis

The software package SPSS version 17.0 was used to analyze bacteria properties. Means were calculated and compared. The one-way analysis of variance was used to find the levels of significance of the considered parameters. At $P \leq 0.05$, there was significant difference in total heterotrophic bacterial count across the sample locations (see Apendix). At $P \leq 0.05$, there was no significant difference in the quantity of PAHs remaining after 21days exposure to pure and mixed cultures of bacteria.

RESULTS/DISCUSSIONS

Total Heterotrophic Bacterial Counts.

The total heterotrophic bacterial (THB) counts ranged between 2.1×10^3- 9.5×10^3 cfu/ml with a mean value of 4.5×10^3 cfu/ml. The highest bacterial count of 9.5×10^3 cfu/ml was recorded in July at Saver Pit 1 while the lowest count of 2.1×10^3 cfu/ml was recorded in January for Barge Jetty B. Comparison of mean spatial variations in total bacteria counts at the sampling locations and their controls showed that minimum and maximum mean values 3.7×10^3 cfu/ml and 4.6×10^3 cfu/ml occurred at Saver Pit 1 control and Saver Pit 2 respectively.

The microbial load of all the effluent samples were low. Microorganisms are said to be ubiquitous and are known for essential functions which include decomposition of organic materials, bioaccumulation of chemicals and biogeochemical cycling of elements. Their presence, abundance and growth in the environment are greatly influenced by factors such as pH, temperature, Inocula size, availability of nutrients and salinity. In this study, it was observed that effluent samples from Saver Pits 1 had the highest total bacterial count. The result revealed that effluents from Saver Pit 1 had higher total heterotrophic bacterial count than Saver Pit 2, Barge Jetty A and Barge Jetty B. The increased bacterial load may be due to the activities of the sewage compartment of the Forcados Terminal that can contaminate the Saver Pits.

The Barge Jetties A and B had lower bacterial counts. This could be a result of less sewage contamination around the jetties. The boats only anchor there before and after conveying people to the terminal.

Generally, bacterial counts from effluents of Barge Jetties and Saver Pits of Forcados Terminals were higher than those of the control which signifies partial contamination of the test samples by the activities of the oil industries that discharge into Forcados Terminal. However, bacterial counts in test and control effluent samples were not significantly raised. This is as a result of Forcados Terminal being capable of treating the effluents before discharge into the storage tank that settles in the Saver Pits and Barge Jetties.

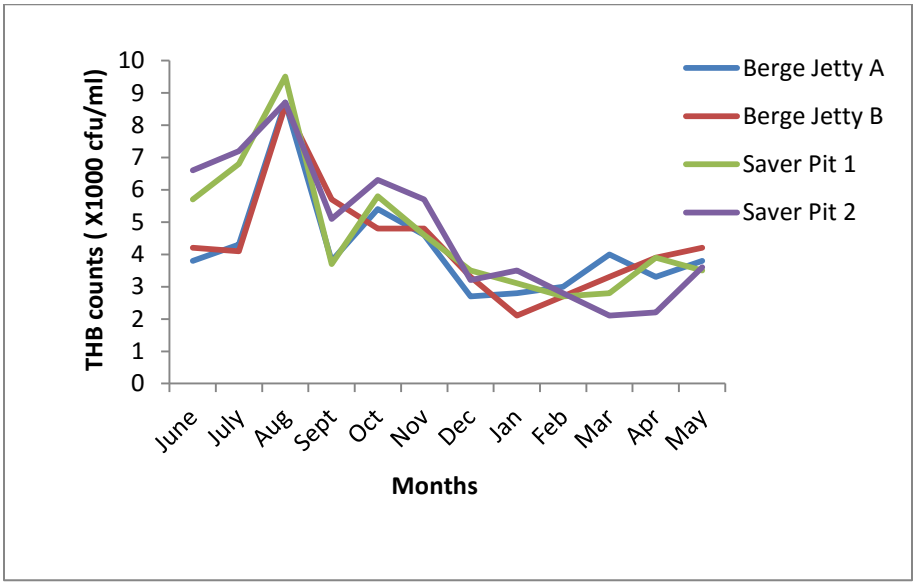

Fig.1: Monthly Variation in Bacterial Counts of Sampling Locations.

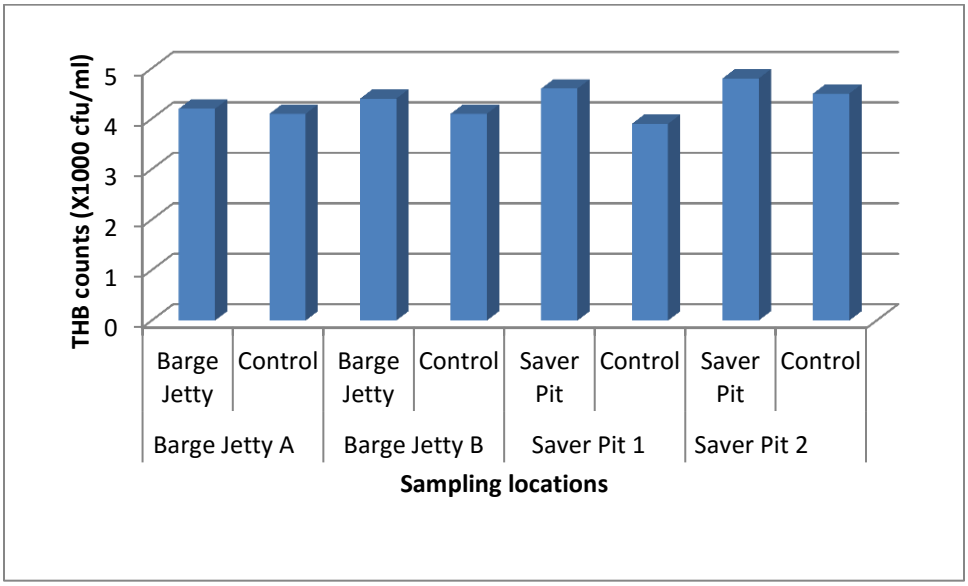

Fig.2: Comparison of mean spatial variations in total bacteria counts with controls

Biodegradation of Individual Polycyclic Aromatic Hydrocarbon by a Pure Culture of Pseudomonas sp.

Figure 3 shows the graphical presentation of the result of the biodegradation of individual PAHs by a pure culture of Pseudomonas sp. after 7, 14, and 21 days. The results after 7 days showed the absence of naphthalene, 2-methylnaphthalene, acenaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd) pyrene and benzo(k)fluoranthene, while fluoranthene had a value of 0.0025mg/L. On the 14th day, fluoranthene had a value of 0.0012mg/L. Analysis after 21 days showed complete absence of all the PAHs.

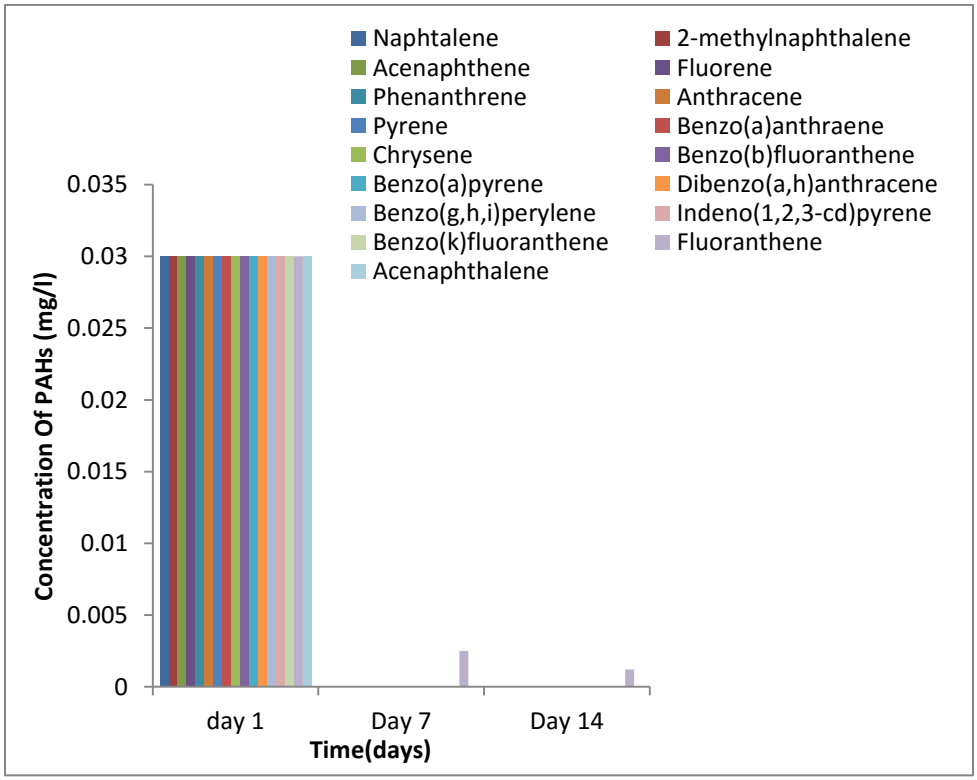

Fig. 3: Biodegradation of individual PAHs by a pure culture of *Pseudomonas* sp.

Biodegradation of Individual Polycyclic Aromatic Hydrocarbon by a Pure Culture of Klebsiella sp.

Figure 4 shows the graphical presentation of the result of biodegradation of individual PAHs by a pure culture of *Klebsiella* sp. after 7, 14, and 21 days. The results after 7 days showed the absence of naphthalene, 2-methylnaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (a) pyrene, dibenzo (a,h) anthracene, benzo (g,h,i)perylene, indeno(1,2,3-cd)pyrene and benzo(k)fluoranthene, while fluoranthene had a value of 0.0025mg/l and acenaphthalene had a value of 0.0043mg/l. On the 14th day, fluoranthene had a value of 0.0015mg/l, while

phenanthrene had a value of 0.0087mg/l. Analysis after 21 days showed complete absence of all the PAHs.

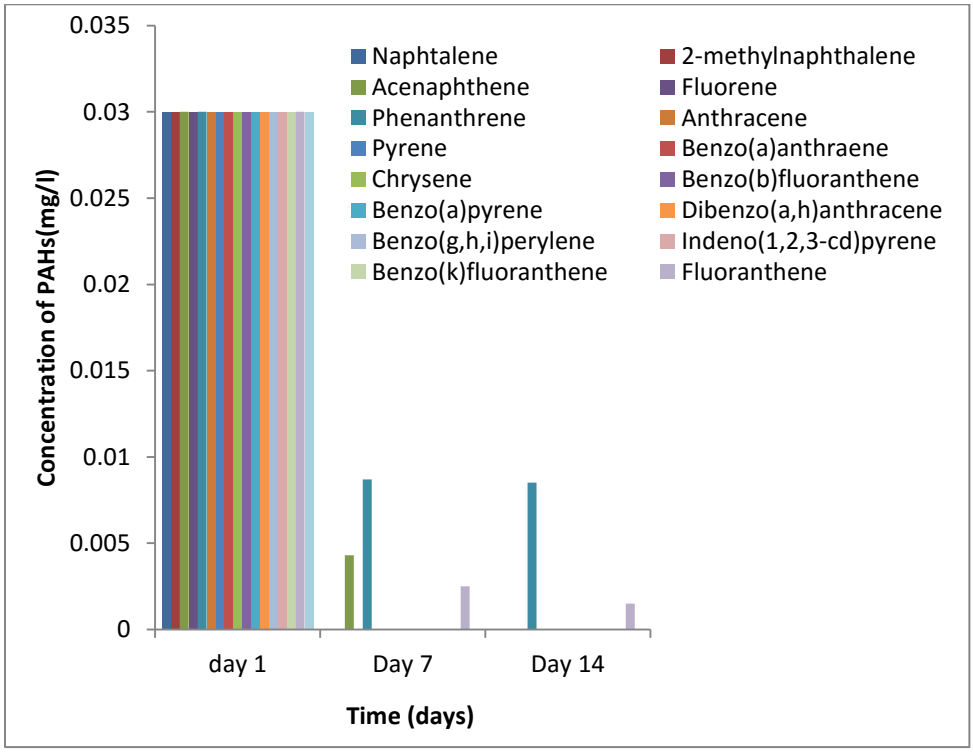

Fig. 4: Biodegradation of Individual PAHs by a Pure Culture of *Klebsiella* sp.

Biodegradation of Individual Polycyclic Aromatic Hydrocarbon by a Mixed Culture of Bacteria (*Pseudomonas sp*. and *Klebsiella sp.)*

Figure 5 shows the graphical presentation of the result of biodegradation of the individual PAHs by a mixed culture of *Pseudomonas sp*. and *Klebsiella sp*., after 7, 14 and 21. The results after 7 days showed the absence of naphthalene, 2-methylnaphthalene, acenaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, chrysene, benzo(b)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd) and benzo(k)fluoranthene, while fluoranthene had a value of 0.0030mg/l and benzo(a)anthracene had a value of 0.0016mg/L. On the 14th day, fluoranthene had a value of 0.0015mg/l. Analysis after 21 days showed complete absence of all the PAHs.

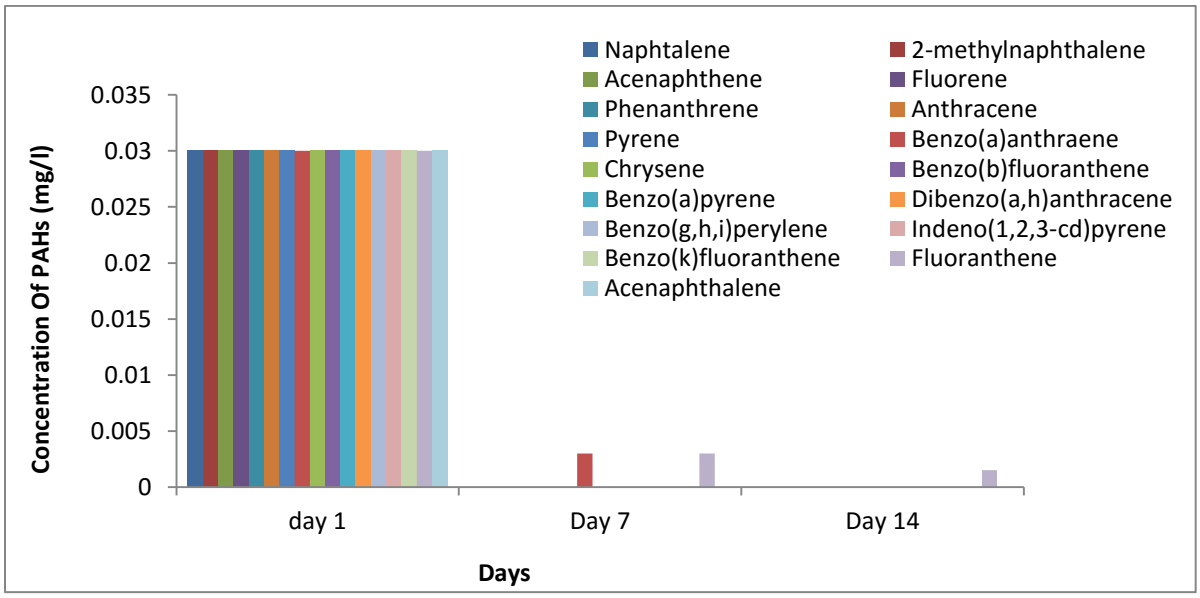

Fig. 5: Biodegradation of Individual PAHs by a Mixed Culture of Bacteria.

Throughout the biodegradation tests, the concentrations of the PAHs reduced between 7 days and 14 days and disappeared completely after 21 days in the experimental and controls. This may be due to abiotic factors such as pH, temperature, salinity, inoculum size and possibly the volume of the effluent. The two bacteria isolates *Pseudomonas* sp and *Klebsiella* sp used in this study were predominant bacteria isolates found in effluents of Forcados Terminal. This agrees with Okoro (1999) who observed that the two fungal isolates used in his study, *Aspergillus* sp and *Penicillium* sp were the predominant fungal isolates found in produced water effluent from Chevron's Escravos tank farm.

The two bacteria isolates *Pseudomonas* sp and *Klebsiella* sp used in this study form part of the microflora of the effluents of Forcados Terminal and therefore can tolerate the pH range (4.86-8.09), slightly low biochemical oxygen demand (BOD_5) and high salinity of the effluent water. According to Okoro and Amund (2002), when degradation is carried out by mixed cultures of bacteria and fungi, fungal cultures are usually outgrown by their bacteria 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 bacteria species has dropped significantly. However, comparing the degradation efficiency of bacteria from the effluents of Forcados Terminal on the PAHs, it is evident that pure cultures of bacteria have a greater capacity and enzymatic capability to degrade the recalcitrant PAHs than mixed cultures of bacteria. The pure cultures of *Pseudomonas* sp used in this study showed greater capacity in the degradation of PAHs than the mixed cultures of bacteria *Pseudomonas* sp and *Klebsiella* sp proving pure cultures of bacteria *Pseudomonas* sp to be an excellent degrader and potential agent of bioremediation of environments impacted by PAHs.

LIMITATION

Throughout the biodegradation tests, the concentrations of the PAHs reduced between 7 days and 14 days and disappeared completely after 21 days in the experimental and controls. This may be due to abiotic factors such as pH, temperature, salinity, inoculum size and possibly the volume of the effluent. Measures should be taken while carrying out this kind of experiment next time to

eliminate the effect of the abiotic factors. The spread plate culture technique used in this study requires series of dilutions.

Methods of other researchers should be subjected to further experiment to eliminate experimental errors before adopting a standard method.

CONCLUSION AND RECOMMENDATION

This research was intended to isolate and identify bacteria in the effluents of Forcados Terminal, to ascertain the level of pollution by enumerating the number of bacteria in the effluents and to determine the biodegradation of PAHs by associated bacteria. The conclusions from this research are as follows.

- The levels of Total heterotrophic bacterial counts were within the recommended limits which indicates that Forcados Terminal has facilities capable of treating effluent samples to be less harmful to the environment, right from the primary treatment.
- The two bacterial isolates *Pseudomonas* sp and *Klebsiella* sp used in this study which form part of the microflora of the effluents of Forcados Terminal showed an appreciable level of degradation of the recalcitrant polycyclic aromatic hydrocarbons after 21days of exposure.
- The pure cultures of the bacteria *Pseudomonas* sp used in this study showed greater capabilities in the degradation of PAHs than the mixed culture the of bacteria (*Pseudomonas* sp and *Klebsiella* sp).
- Periodic monitoring should be given to the Forcados Treatment plant in Delta State in order to maintain their efficiency.
- Replacement of old biological plants with young and fresh ones when due will go a long way in ensuring improved effluent quality.
- Active research into the waste and pollution minimization strategies, waste avoidance technologies, cleaner production processes and zero PAHs emission concepts in Delta State should be encouraged.

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