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Aerobic Degradation of Fluoranthene, Benzo(b)Fluoranthene and Benzo(k) Fluoranthene by Aerobic Heterotrophic Bacteria- Cyanobacteria Interaction in Brackish Water of Bodo Creek

Ichor Tersagh*, Ebah EE and Azua ET

Department of Biological Sciences, University of Agriculture, Makurdi, Nigeria

Abstract

The study aimed at ascertaining the biodegradability of polyaromatic hydrocarbons in the fate of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene by aerobic heterotrophic bacteria - cyanobacteria interaction in crude oil contaminated brackish water of Bodo creek. Samples of brackish water were spiked with known volume of Bonny Light crude oil and inoculated with aerobic heterotrophic bacteria and cyanobacteria isolated from crude oil contaminated waters of Bodo creek and monitored for 56 days. The PAHs investigated were quantified using GC-MS where as the bacteria and cyanobacteria isolates were identified on the basis of 16S rRNA gene sequence analysis. The initial quantity of fluoranthene on day 0 for the treatments of Aerobic heterotrophic bacteria (A) 0.43, Cyanobacteria (B) 0.061, and a consortium of A+B 0.24, and the Control, (C) 0.26; Benzo(b)fluoranthene had 0.53, 0.31, 0.65 and 0.66 whereas benzo(k)fluoranthene had 0.62, 0.31, 0.56 and 0.69 mg/l respectively. There was an observed degradation of the HMW-PAHs which decreased and increased progressively from the treatments with exception of fluoranthene which remained at 0 from week 2 in all the treatment options. Biodegradation did not vary significantly with time in all the treatment options and the control.

Keywords: Petroleum hydrocarbons; Brackish water; Biodegradation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are reportedly toxic, ubiquitous, and organic compounds that can persist in the environment [1,2] and are largely released into the environment due to incomplete combustion of fossil fuels [3]. Yu et al. [4] posited that fluoranthene is a major component of petroleum sludge; it is also reported to be a non-alternant high-molecular-weight PAH that has a five-member ring which has the same structure when compared with other compounds such as dibenzodioxin, dibenzofuran, acenaphthylene, carbazole, fluorene, and dibenzothiophene [2,5]. According to Mishra et al. [3] it is mutagenic, carcinogenic, teratogenic and hydrophobic in nature and binds strongly to soil particles with a restricted bioavailability. However, high-molecular-weight PAHs (HMW-PAHs), such as pyrene, benzo(a)pyrene and benzo(b)fluoranthene, are generally said to be recalcitrant and resistant to microbial attack due to their low solubility and bioavailability hence, highly persist in the environment and can bioaccumulate in organisms [6]. It is widely reported that, HMW PAHs are not easily soluble in water, electrochemically stable and can be acutely toxic, genotoxic and immunotoxic [7,8] or are responsible for disruption hormones [9] based on circumstances and the mode of exposure. They are also implicated in bioconcentration, bioaccumulation and sometimes biomagnification through trophic transfers in terrestrial and marine food webs [10].

Fluoranthene has undergone degradation by bacteria previously as reported by researchers in the genera of *Alcaligenes* [11], *Burkholderia* [12], *Mycobacterium* [13], *Pseudomonas* [14], and *Rhodococcus* [15] *Sphingomonas* [16]. However, a few fluoranthene degraders were isolated from the marine environment and reported to belong to the genera; *Ochrobactrum* [17], *Novosphingobium* [18], *Cycloclasticus* [19] and *Celeribacter* [20]. Zhisong et al. that the degradation of both fluoranthene and pyrene was achieved faster by a consortium of *Cycloclasticus* sp. PY97M and *Marinobacter nanhaiticus* D15-8WT compared to individual strains. Dean-Ross et al. [21] reported on the capability of degradation of fluoranthene by *Mycobacterium flavescens* and *Rhodococcus* spp. with subsequent formation of 9-fluorenone-1-carboxylic acid as a metabolite. Two Marinobacters, *M. sedimentalis* and *M. falvimaris were* isolated from hypersaline sabkhas due to their capability to grow on crude oil and were observed to have utilized Tween 80 and other individual aliphatic hydrocarbons ranging from C_9-C_{40} as carbon sources in the presence of 6% NaCl [22]. Dastgheib et al. [23] isolated a mixed culture (Qphe-SubIV) of *Halomonas* sp. and *Marinobacter* sp. from saline soils contaminated with hydrocarbon from five regions in Iran. The bacteria reportedly degraded several other PAHs as well as fluoranthene as the sole sources of carbon in the presence of 1-15% NaCl.

Benzo(b)fluoranthene and Benzo(k)fluoranthene are polycyclic aromatic hydrocarbons that have the chemical formula $C_{20}H_{12}$. They are colourless solids with poor solubility in most solvents. Impure samples can appear as off white. Benzo(b)fluoranthene is closely related to other isomeric compounds which include benzo(a)fluoranthene, benzo(e) fluoranthene, benzo(j)fluoranthene, and benzo(k)fluoranthene [24]. Benzo(*k*)fluoranthene as a member of the five-ring HMW PAH class was observed to resist biotransformation due to its molecular stability, hydrophobic nature and low water solubility, which is approximately 1 ug L^{-1} or less [25-27]. In the environment, it is present in polluted soils in parts per million range showing different persistence levels; hence

*Corresponding author: Ichor Tersagh, Department of Biological Sciences, University of Agriculture, Makurdi, Nigeria, Tel: +234(0)7039256061; E-mail: smartichor@uam.edu.ng

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it is included as one of the 16 pollutants on the U.S. Environmental Protection Agency's (EPA) Priority Pollutant List [28-31]. In animals, there are existing proves of carcinogenicity caused by benzo(*k*) fluoranthene, and has also been classified as a group 2B carcinogen by the 'International Agency for Research on Cancer' in humans [32].

Zheng, Obbard and Cerniglia [33-35] reported removal of Benzo(b)fluoranthene and Benzo(k)fluoranthene among other HMW-PAHs by some filamentous fungi, white rot fungi, basidiomycetes and deuteromycetes fungi more efficiently than bacteria. *Sphingobium* species strain KK22 biologically transformed benzo(*k*)fluoranthene via the 8,9-carbon position which was the initial position of attack on the parent molecule. This type of attack is structurally analogous to initial 7,8-carbon position attack that was described for fluoranthene by other gram-negative and gram-positive species. The identification of the downstream products 9-hydroxy-fluoranthene-8-carboxylic acid and 1,8-naphthalic anhydride for instance, which may have occurred through 8,9-carbon position initial attack on the benzo(*k*)fluoranthene molecule provides a clear evidence in support of biotransformation mechanism by microorganisms. The first ring fission event of the benzo(*k*)fluoranthene molecule occurred by *ortho*-ring cleavage and resulted in 8-carboxyfluoranthenyl-9-propenic acid, a product that corresponded to $[M-H]^- = 315$, and this is the first report of this product from PAH biodegradation [24]. Ichor et al. [1,36] reported on effective biodegradation of total petroleum hydrocarbons and mineralization of phenanthrene a LMW PAHs by the consortium of aerobic heterotrophic bacteria and cyanobacteria from Bodo creek respectively.

The present study aimed at ascertaining degradation of these HMW-PAHs; fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene from brackish water contaminated with petroleum hydrocarbons by the resident aerobic heterotrophic bacteria and cyanobacteria.

Materials and Methods

Study area

The study was carried out in crude oil contaminated brackish water body; Bodo creek which is located in Ogoni land in Gokana, LGA of Rivers State, in Niger Delta region.

Media and incubation conditions

The method for BG-11 medium preparation described by Ichor et al. [36] was adopted.

Enumeration and molecular characterization of cyanobacteria and aerobic heterotrophic bacteria

Samples of brackish water and sediment obtained from crude oil contaminated Bodo creek in Rivers State, Nigeria. BG-11 aqueous medium was prepared and inoculated with water and filterates from sediment samples using different medium- water volume ratio as described in Ichor et al. [1]. The set up was supplemented with ciprofloxacin and nystatin in order to avoid bacteria and fungi growth. Incubation was done by using a cotton wool to cork the Erlenmeyer flask and exposed to natural sunlight for 12 hr and darkness for 12 hr for 14 days under ambient temperature as described by Ichor et al. [1]. This was shaken twice every day to prevent sedimentation of the nutrients in BG-11 medium. Polymerase chain reaction was performed on the DNA extracted directly from the samples using Universal primers for cyanobacteria CYA 106F (CGC ACG GGT GAG TAA CGC GTG A and CYA 359F(GGG GAA TYT TCC GCA ATG GG) with a 40 nucleotide GC clamp (5^1 CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC G 3^1) on the 5^1 end forward primer and CYA 781R (equimolar mixture of CYA781Ra (GAC TAC TGG GGT ATC TAA TCC CAT T) and CYA 781Rb (GAC TAC AGG GGT ATC TAA TCC CTT T) reverse primers for amplification of a segment of cyanobacterial 16S rRNA gene were synthesized.

The DNA of aerobic heterotrophic bacteria were extracted directly from water samples and filterates from sediment samples and polymerase chain reaction was performed using universal primers used for bacteria; Eub 27F (51-31. AGA GTT TGA TCC TGG CTC AG) forward primer and Eub 1492R (51-31. ACG GCT ACC TTG TTA CGA CTT) for reverse primers. Purification of the PCR sequence products was done using 2 M sodium acetate wash technique before sequencing. Sequences obtained were compared with known sequences in the Gen Bank using the basic local alignment search tool (BLAST) of the National Centre for Biotechnology Information (NCBI) [36]. Phylogenetic tree was constructed based on almost complete 16S rRNA gene sequences (>1300 bp) using MEGA 6.5 software by applying the various methods integrated in it.

Preparation of inoculum and biodegradation experiment

Aerobic heterotrophic bacteria aliquot was prepared via transfer of a loopful of 24 hr old culture of each isolate into 400 ml of sterilized nutrient broth contained in 500 ml Erlenmeyer flask and incubated for 24 hrs. This was supplemented with $CuSO_4$ and nystatin to prevent the growth of cyanobacteria and fungi [1].

The different treatment options of brackish water and sediment filterate samples labelled AHB and CB were prepared asceptically and 200 ml each of aerobic heterotrophic bacteria and cyanobacteria aliquot was transferred to 500 ml of sterile distilled water in two separate 1000 ml flask, AHB+CB consortium was prepared and standardized with 0.5 M Macfarland solution as reported by [36]. The water containers for the experimental set up were filled with 11 litres of water and labeled AHB, CB, AHB+CB and C for the control and spiked with 32300 ppm of sterile Bony light crude oil sample obtained from shell petroleum development company. Treatment of the set up with ciprofloxacin, nystatin and $CuSO_4$ respectively was done as reported in Ichor et al. [35].

Results

Fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene degradation by aerobic heterotrophic bacteria, cyanobacteria and their consortium was monitored using Agilent 7890 GC-MS model. Fluoranthene disappeared after the second week of monitoring in all treatments. Benzo(k)fluoranthene reduced to 0.04 for A, 0.01 (B); 0.02 (A+B) and 0.01 mg/L for the control in week 8. Benzo(k)fluoranthene degraded to 0.04 (A); 0.02 (A+B) and 0.01 mg/L for B and control respectively. The result showed a decrease in the quantity of the PAHs studied for all the treatment options on the last day of the experiment though with observed fluctuations for benzo(b) fluoranthene and benzo(k)fluoranthene throughout the period monitored (Figures 1-4). There was no observed significant difference in the biodegradation of the HMW-PAHs monitored with time ($p>0.05$). Figure 5 is the unrooted phylogenetic tree which shows the relatedness of aerobic heterotrophic bacteria and cyanobacteria present during the biodegradation experiment. The most dominant bacteria were the *Bacillus* species.

Discussion

Biodegradation of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene monitored from Petroleum hydrocarbon contaminated brackish waters of Bodo creek was monitored using

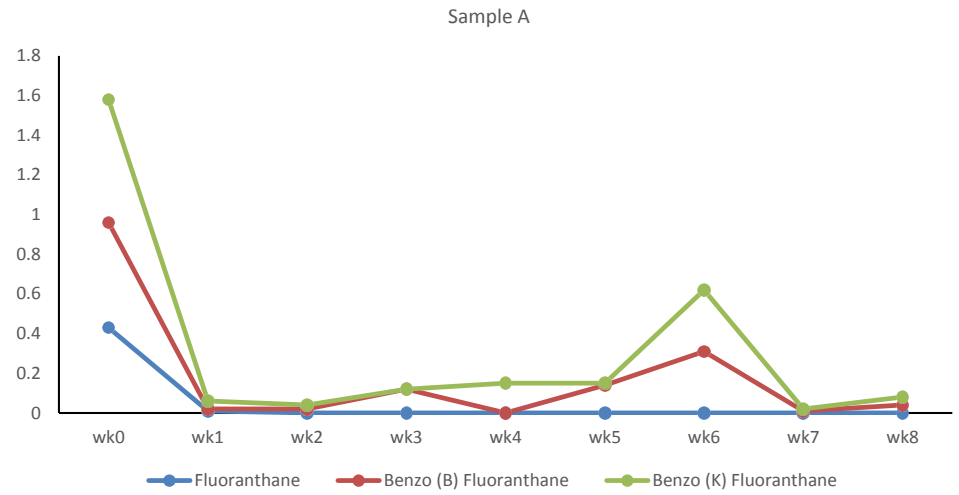

Figure 1: Degradation of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene by aerobic heterotrophic bacteria.

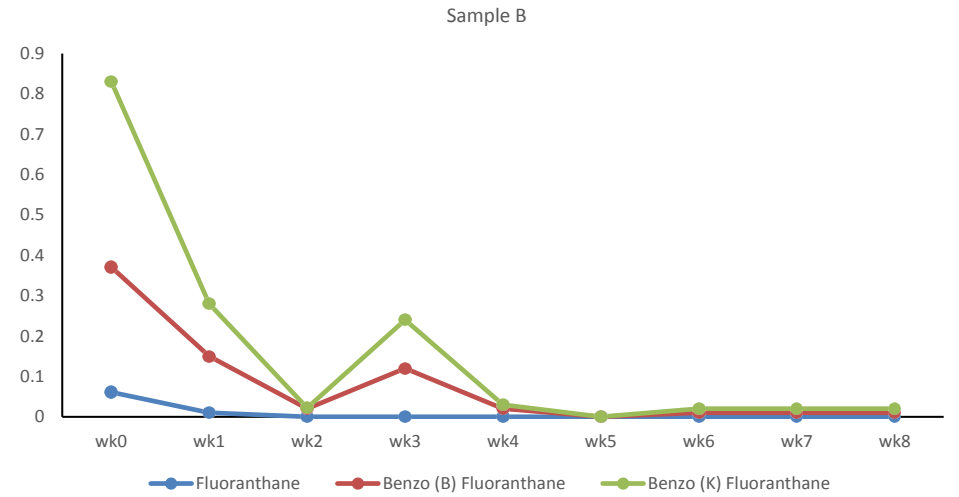

Figure 2: Degradation of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene by cyanobacteria.

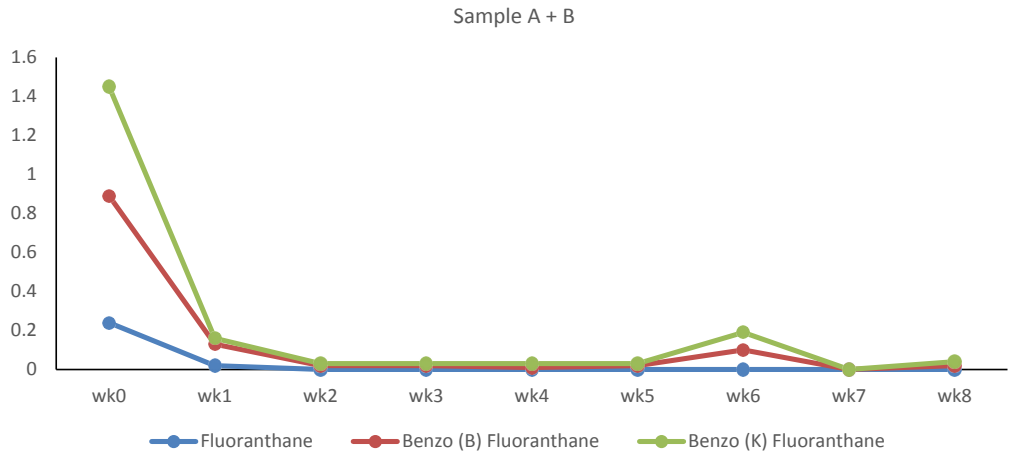

Figure 3: Degradation of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene by consortium of aerobic heterotrophic bacteria and cyanobacteria.

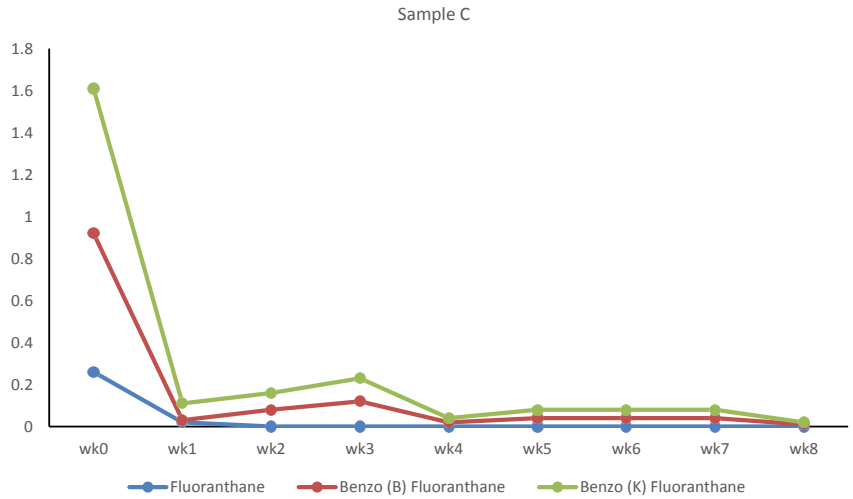

Figure 4: Degradation of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene by control.

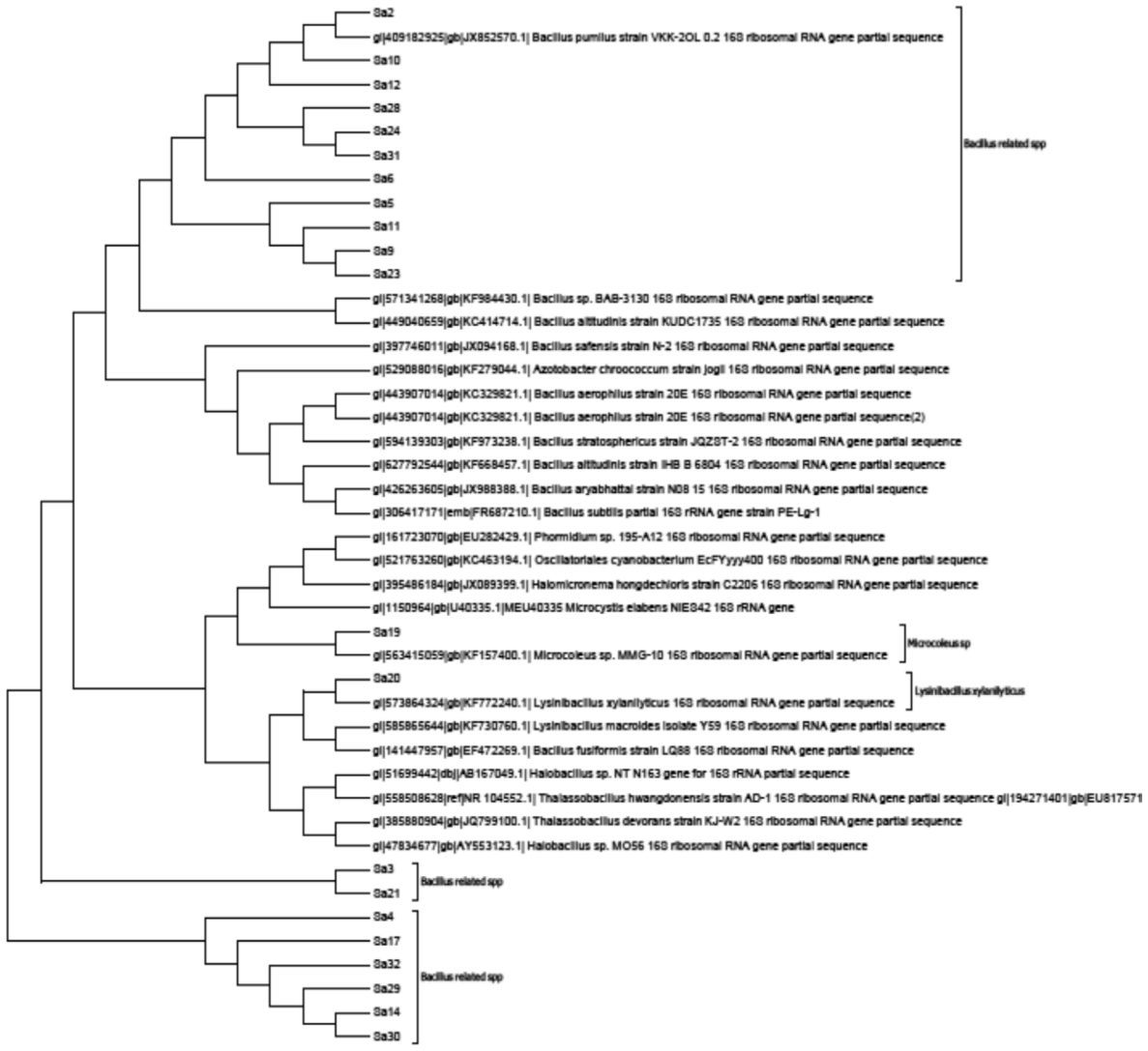

Figure 5: Unrooted phylogenetic tree showing affiliation of bacterial and cyanobacteria consortium based on partial 165 rRNA sequences.

GC-MS. The resident bacteria, cyanobacteria and the consortium mineralized the PAHs studied with observed fluctuations in quantity for benzo(b)fluoranthene and benzo(k)fluoranthene. The observed fluctuation could be as a result of novel synthesis where more complex components of PAHs in the petroleum hydrocarbons may have been metabolized to simpler molecules during biodegradation. Formation of metabolites of benzo(b)fluoranthene and benzo(k)fluoranthene can also account for the fluctuation. Ichor et al. [1] reported similar a trend of fluctuation during phenanthrene degradation without nutritional amendment by aerobic heterotrophic bacteria, cyanobacteria and a consortium of aerobic heterotrophic bacteria and cyanobacteria in petroleum hydrocarbon contaminated Bodo creek throughout the period monitored.

Reports from previous studies had shown the capability of microorganisms to effectively degrade components of poly aromatic hydrocarbon in this study. Forinstance, Maeda et al. [24] reported on transformation of Benzo(k)fluoranthene into four, three and two aromatic ring products after exposure to *Sphingobium* sp strain KK22. Degradation of Benzo(k)fluoranthene achieved 10% biotransformation in the first five days and between this to 10 days biodegradation continued with approximately 73% of Benzo(k)fluoranthene recovered from culture media on the tenth day though it did not support the growth of strain KK22 as a sole source of carbon and energy. Concentration of Benzo(k)fluoranthene reduced by 70% over an experiment that spanned for 20 days. The genus of *Bacillus* species; *Bacillus firmus* remarkably degraded Benzo(b)fluoranthene completely [37] *Bacillus subtilis* completely degraded Benzo(b)fluoranthene [9]; *Psudomonas alcaligenes* degraded Benzo(b)fluoranthene among other HMW-PAHs [38].

Ogbonna et al. [39] reported on biodegradation of benzo(b) fluoranthene, benzo(k)fluoranthene and other individual polycyclic aromatic hydrocarbons by a mixed culture of *Pseudomonas* sp., *Bacillus* sp. and *Klebsiella* sp. Microbial degradation contributes significantly to the ultimate removal of organic molecules including oil from soil, freshwater, brackish water and marine environments [40].

Fluoranthene however disappeared after the first week of sampling and was not detected for the rest of the period sampled. Fluoranthene reportedly served as a growth substrate for *Alcaligenes denitrificans* at the rate of 0.033/h in previous findings of Weissenfeils et al. [11]. Exponential growth of *Mycobacterium* strain BB1 grew slower on fluoranthene though it has higher solubility when compared to pyrene but stopped before fluoranthene was completely degraded. Kumar [41] reported *invitro* degradation of fluoranthene by four bacterial strains which growth invariably corresponded to the degradation potential of fluoranthene. After 168 h of incubation, 61%, 48%, 42% and 41% of fluoranthene was reportedly degraded by PSM 11, PSM 10, PSM 6, and PSM 7 respectively. Nwinyi et al. [42] reported loss of fluoranthene by 7-19% during aerobic degradation by *Enterobacter* and *Pseudomonas* strains. Cao et al. [43] isolated *Celeribacter indicus* P73T; which is reportedly the first fluoranthene degrading bacterium within the family of *Rhodobacteriaceae.*

The observed degradation of fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene as reported in this study is sufficient evidence that aerobic heterotrophic bacteria, cyanobacteria and a consortium of aerobic heterotrophic bacteria and cyanobacteria resident in crude oil contaminated and the uncontaminated Bodo creek used as control are inherently capable of biodegrading petroleum hydrocarbons. The observed degradation was carried out without nutrient amendment or biostimulation which is suggested for further field application [44-47].

Conclusion

The resident aerobic heterotrophic bacteria, cyanobacteria, the consortium and control were capable of degrading fluoranthene, benzo(b)fluoranthene and benzo(k)fluoranthene in brackish waters of Bodo creek. The study proved that the resident flora have inherent abilities for PAHs degradation under moderate salt conditions without nutrient amendment, thus providing evidence of the presence of PAHs degrading halo-tolerant bacteria and cyanobacteria under moderate salt conditions.

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