Journal of Petroleum & Environmental Biotechnology

Ichor et al., J Pet Environ Biotechnol 2016, 7:4
DOI: 10.4172/2157-7463.1000286

Research Article Open Access

Time Dependent Influence of Aerobic Heterotrophic Bacteria – Cyanobacteria Interaction during Biodegradation of Poly Aromatic Hydrocarbons

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Abstract

Biodegradation of polyaromatic hydrocarbons (PAHs) by aerobic heterotrophic bacteria, cyanobacteria, consortium of cyanobacteria and aerobic heterotrophic bacteria and the control was monitored for a period of 49 days, using GC-MS. The chromatograms showed that after contamination of water with same volume of crude in same quantity of water for all the treatments, PAHs were; 3.68, 23.5, 13.7 and 5.49 mg/L for AHB, CB, AHB + CB and control. The PAHs components were naphthalene, acencephthylene, acenaphthene, fluorence, anthracence, phenanthrene, fluoranthene, pyrene, beiz @ anthracene, chrysene, benzo (h) fluoranthene, benzo (k) fluoranthene, benzo (a) pyrene, dibenzo (a, h) anthracene, indeno (1, 2, 3-c, d) perylene, benzo (g, h, i) perylene. The result showed a decrease in the quantity of PAHs for all the treatment options on the last day of the experiment though some fluctuations in the quantity of PAHs' were observed in all the treatment options throughout the period monitored. The growth of AHB and cyanobacteria however increased steadily with time throughout the sampling period of the experiment in all the treatment options. The degradation of PAH's was statistically significant ($p<0.05$) with time.

Keywords: Polycyclic aromatic hydrocarbon; Cyanobacteria; Consortium; Bodo creek

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds which contain carbon and hydrogen with the carbon arranged in a series of adjoining six member benzene rings and is present in water, crude oil and tars [1]. They can have a range of substituents such as alkyl, nitro, and amino groups in their structure (Nitrogen, sulfur, and oxygen atoms can also be incorporated into their ring system. The precursors for PAHs present in crude oil reportedly include natural products, such as steroids, which have been transformed through chemical conversion to aromatic hydrocarbons over time. They are known to be recalcitrant and are not easily degradable. They are considered hazardous because of their potential trophic biomagnifications and acute toxicity. The behavior of PAHs in the natural environment is of great environmental concern because of their toxic, mutagenic, and carcinogenic characteristics and how they adversely affect both abiotic and biotic processes such as volatilization, photooxidation, chemical oxidation, bioaccumulation and microbial transformation. The role of microorganisms play in the environment through breakdown, mineralization and biodegradation of organic compounds in nature has been reported to be the most effective means of PAH removal from the natural environment [2-6].

Biodegradability of Poly-aromatic hydrocarbons (PAHs) is generally related to the number of fused benzene rings [7]. Half-lives in soil and sediment of the three ring Phenanthrene molecule may range from 16 to 126 days while for the five-ring benzo (a) Pyrene they may range from 299 to more than 1400 days [8]. The persistence of higher molecular weight PAHs is due largely to their low water solubility and resonance energy of their structures [9].

Sediminicola has been reportedly found in the chronically oil and PAH contaminated Liaodong Bay of Bohai Sea, China [10]. Previous studies on PAHs degradation in marine environments implicated *Cycloclasticus* as a major PAH degrader during oil spills [11-16]. *Marinobacter* has also been reported to have degraded alkane and PAH whereas *Flavobacterium* was implicated PAH degradation [17,18]. Newton reported that in one sample set which was collected while oil was visibly washing ashore from Orange Beach in June, OTUs related to *Marinobacter* were reported to have largely increased in the community. Naphthalene, the simplest form of PAHs has been observed previously to be the most readily degraded PAH. Bacteria initially oxidized it by incorporating both atoms of molecular oxygen into the aromatic molecule to form cis-1,2-dihydroxy naphthalene. Salicyalmehyde and pyruvate and produced through ring cleavage of 1,2 – dihydroxy naphthalene. The aldehyde is then oxidized to salicylate that is subsequently converted to catechol which is further oxidized via the ortho or Meta pathway as per described earlier for benzene. In naphthalene degradation by the themophilic *Bacillus thermoleovorans*, mesophilic microorganisms might have a different pathway for Polyaromatic degradation compared to mesophiles. *Pseudomonas* has been reported to degrade naphthalene in experimental systems and is thought to be involved in the co-metabolization of fluorene [19]. Annweiller reported intermediates such as 2,3-dihydroxy naphthalene; 2-carboxynnarmic acid and phthalic and benzoic acid were identified in addition to typical metabolites of naphthalene degradation known from mesophiles in the pathway of *B. thermoleovorans*. *Thalassospira* is also implicated in degradation of fluorene and naphthalene and was found to be associated with oil on water surface during the DWH blowout previously by Liu and Liu [20,21].

Sphingomonas, Mycobacterium, Pseudomonas, and *Burkholderia;* and consortiums of bacteria such as *Pseudomonas* with *Flavobacterium* reportedly degraded benzo[α]pyrene [22]. Within a mixed culture of *Flavobacterium* sp. and *Pseudomonas* sp., the degradation of benzo[α]

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Received May 02, 2016; **Accepted** June 01, 2016; **Published** June 09, 2016

Citation: Ichor T, Gberikon GM, Jerry T (2016) Time Dependent Influence of Aerobic Heterotrophic Bacteria – Cyanobacteria Interaction during Biodegradation of Poly Aromatic Hydrocarbons. J Pet Environ Biotechnol 7: 286. doi: 10.4172/2157-7463.1000286

pyrene was also reported [18]. The utilization of fluoranthane, a HMW PAH, as a sole source of carbon and energy is possible in a seven-member bacteria consortium [23]. Pyrene was also reportedly metabolized by a variety of bacteria through different pathways for the different bacteria [24,25]. Catabolism of a PAH molecule as reported by previous research findings started initially by the oxidation of PAH to a dihydrodiol using a multicomponent enzyme system. The dihydroxylated intermediates produced could then be processed through an ortho cleavage or a meta cleavage pathway, leading to the production of intermediates such as protocacechuates and catechols which can be converted to tricarboxylic acid cycle intermediates.

Marine cyanobacteria have been reported in previous literatures to have oxidized aromatic hydrocarbons under photoautotrophic growth conditions. The genera of Cyanobacterial reported in previous studies to have degraded hydrocarbons under aerobic conditions include; *Aphanocapsa*, *Anabaena*, *Microcoleus*, *Nostoc*, *Oscillatoria* and *Phormidium* [26]. *Microcoleus chthonoplastes* and *Phormidium corium* cultures were observed to have degraded n-alkanes [27]. *Oscillatoria* sp. and *Agmenellum quadruplicatum* had oxidized naphthalene to 1-naphthol [28,29]. *Oscillatoria* sp. strain JCM oxidized biphenyl to 4-hydroxybiphenyl and *A. quadruplicatum* had previously been reported to have metabolized phenanthrene into trans-9, 10-dihydroxy-9, 10dihydro-phenanthrene and 1-methoxy-phenanthrene as reported by Narro [24,30]. Several other strains were reported from earlier research findings to have degraded crude oil and other complex organic compounds such as surfactants and herbicides [31-34].

This research was however prompted by previous research findings on the capability of resident microbial flora to biodegrade petroleum hydrocarbons and the controversy that generated about the specific roles played by each of aerobic heterotrophic bacteria and cyanobacteria in the consortium during petroleum hydrocarbon degradation since microbial mats appear at almost every site of oil pollution and whether cyanobacteria possess the capability for biodegradation of petroleum hydrocarbons. In the present study, we monitored biodegradation of PAHs in brackish surface water of petroleum hydrocarbon contaminated Bodo creek by indigenous aerobic heterotrophic bacteria, cyanobacteria and the consortium as the main aim of the study. The objectives were:

A. Monitor the biodegradation of poly aromatic hydrocarbons by aerobic heterotrophic bacteria, cyanobacteria and the aerobic heterotrophic bacteria - cyanobacteria consortium.

B. Identify key bacterial and cyanobacteria resident populations in Bodo creek involved in poly aromatic hydrocarbon degradation using conventional culturing and molecular methods.

C. Determine the Phylogenetic relationship of the consortium of aerobic heterotrophic bacteria – cyanobacteria.

Materials and Methods

The study area is oil contaminated water body, Bodo creek located in Ogoni land in Gokana, LGA of Rivers State, in Niger Delta region.

Media and incubation conditions

BG-11 medium consisting (g/liter) of $NaNO_3$ (1.5), K_2HPO_4 (0.004), $MgSO_4$. $7H_2O$ (0.0075), $CaCl_2$. $2H_2O$ (0.036), Na_2CO_3 (0.02), citric acid (0.006), ferric ammonium citrate (0.006), disodium magnesium EDTA (0.001) and trace metal solution 1.0 mL/liter comprising of H_3BO_3, $MnCl_2$. $4H_2O$, $Co(NO_3)_2$. $6H_2O$, $ZnSO_4.7H_2O$, $Na_2MO_4.2H_2O$, $CuSO_4.5H_2O$ was prepared for the growth of cyanobacteria and adjusted to a pH of 7.4. The medium was prepared according to the methods of Ichor and Salleh [35,36].

Enumeration of cyanobacteria

Cyanobacteria were enumerated as described by Ichor [35]. Ten brackish water and sediment samples collected from crude oil contaminated Bodo creek in Rivers State were taken to Environmental Microbiology Laboratory of University of Portharcourt and analyzed. The water samples were shaken to suspend the sediment. 1 mL of the raw water sample was then removed and diluted into 99 mL of sterile distilled water which was serially diluted and 1 mL removed from the third dilution and plated on BG-11 medium which had been solidified using agar agar that was treated with ciprofloxacin and nystatin in duplicate plates. 1 g of the soil sediment samples were dissolved in 9 mL of distilled water and filtered using Whatman No. 1 filter paper. The filterate was serially diluted and same procedure followed as described for water. An aliquot of cyanobacteria culture was prepared using different medium- water volume ratio of 90: 10, 80: 20, 70: 30, 60: 40 and 50: 50 mL and vice-versa and incubated in a cotton wool corked Erlenmeyer flask for 14 days and exposed to natural sunlight for 12 hr and darkness for 12 hr under ambient temperature as described by Ichor [35]. The set up were monitored and shaken twice daily to ensure uniform distribution and avoid sedimentation of the nutrients from the BG-11 medium.

Preparation of inoculum and biodegradation experiment

Aerobic heterotrophic bacteria aliquot was prepared by transferring a loopful of 24 hr culture of each isolate into 400 mL of sterile nutrient broth in 500 mL Erlenmeyer flask and incubated for 24 hrs.

A bloom culture of cyanobacterial aliquot using BG II medium formulations was also prepared by transterring 1 mL of each viable culture in to 400 mL of the medium and incubated for 48 hrs under natural sunlight for 12 hr and darkness for 12 hr.

Treatment options of brackish water samples labeled AHB and CB were prepared by asceptically transferring 200 mL each of aerobic heterotrophic bacteria and cyanobacteria aliquot into 500 mL of sterile distilled water in two separate 1000 mL flask respectively. AHB + CB was prepared by transferring 200 mL each of aerobic heterotrophic bacteria and cyanobacteria aliquot into another 500 mL of sterile distilled water in 1000 mL flask. Each option was standardized using 0.5 M Macfarland solution [37]. The inoculum size used for each treatment option was 100 mL of standardized aliquot. The water containers for the experimental set up were each filled with 11 litres of water and labeled AHB, CB, AHB + CB and C for the control and contaminated with 32300 ppm of sterile Bony light crude oil sample obtained from shell petroleum development company. Water samples AHB, CB, AHB + CB were inoculated with aerobic heterotrophic bacteria, cyanobacteria and aerobic heterotrophic bacteria + cyanobacteria respectively while the control was left uninoculated. Sample CB was treated with 0.25 mg/mL of ciprofloxacin and nystatin, AHB was treated with $CuSO_4$ and nystatin to prevent cyanobacteria and fungal activities in the setup, AHB + CB was treated with only 0.25 mg/mL of nystatin while the control was left untreated. The containers for the treatments were washed with detergents and rinsed severally with distilled water prior to the experimental set up [37].

Molecular analysis of bacterial and cyanobacterial isolates

DNA extraction, polymerase chain reaction: Gene sequencing

and analysis for bacterial isolates used was done as reported by Ichor [37]. Universal primers 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 16SrRNA gene (70) were synthesized. A semi nested PCR reaction was carried out with the first reaction using primers CYA 106F and CYA 781R and followed by a reaction with primers CYA 359F and CYA781R. the PCR was carried out in a 25 µL final volume of reaction mixture containing 100 ng of DNA 2.5 µL of 10x PCR buffer with 200 µg bovine serum albumin (nuclease free) and 0.2 u Taq DNA polymerase (Banngalore Genei, India) in a 1 cycler (BioRad, USA). The thermal cycling profile was thus; initial denaturation for 3 mins at 94°C followed by 35 amplification cycles each consisting of 1.5 min denaturation at 94°C, 1 min annealing at 59°C and a 2 min elongation at 72°C with a final 5 min elongation at 72°C [35].

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). Species were identified based on the percentage similarity with known sequences in the data base [35].

Results

Biodegradation of polyaromatic hydrocarbons (PAHs) by aerobic heterotrophic bacteria, cyanobacteria, consortium of cyanobacteria and aerobic heterotrophic bacteria and the control was monitored for a period of 49 days, using GC-MS. The chromatograms showed that after contamination of water with same volume of crude in same quantity of water for all the treatments, PAHs were; 3.68, 23.5, 13.7 and 5.49 mg/L for AHB, CB, AHB + CB and control. The PAHs components were naphthalene, acencephthylene, acenaphthene, fluorence, anthracence, phenanthrene, fluoranthene, pyrene, beiz @ anthracene, chrysene, benzoth) fluoranthene, benzo (k) fluoranthene, benzo (a) pyrene, dibenzo (a, h) anthracene, indeno (1, 2, 3-C, d) perylene, benzo (g, h, i) perylene. The result showed a decrease in the quantity of PAHs for all the treatment options on the last day of the experiment though some fluctuations in the quantity of PAHs' were observed. For instance, on day 14, PAH's reduced to 1.66 mg/L but rose again to 2.79 mg/L on day 42 for AHB, 0.99 mg/L in week 2 (14 days) to 1.26 in week 3 (21 days) force 0.13 mg/L in week 4 (28 days) to 1.79 mg/L in week 6 (42 days) for AHBC + CB and 0.79 mg/L for week 5 (35 days) to 0.85 mg/L in week 6 (42 days) for control. This trend was observed in all the treatment options. Figure 1 shows the degradation of PAH's by the various treatment options introduced with time. Though with some observed fluctuations in the degradation of PAH's the growth of AHB and cyanobacteria steadily increased with time throughout the sampling period of the experiment in all the treatment options (Figures 1-5). The result from the chromatogram revealed complete degradation of naphthalene in week (3) (21 days) for all the treatments though it had the highest concentration during the first day of sampling in all the treatment. Dibenzo (a h) anthracene disappeared on week 4 for treatment AHB + CB; ideno (1, 2, 3-c, d) perylene and Benzo (g, h, i) perylene were completely degraded from week 6 from the same treatment. In treatment CB, Dibenzo (a, h) anthracene, ideno (1, 2, 3, c, d) perylene and Benzo (g, h, i) perylene were completely degraded by week 5. Complete degradation of same PAHs components occurred in week 4 for the control. This trend was not observed in AHB though fluoranthene and pyrene were completely degraded in week 3 and week 5 respectively. Acenaphtylene fluctuated throughout the period monitored in all the treatments but disappeared after week 7 for the control. This pattern of fluctuation was observed in Acenaphthene, fluorene, anthracene, phenanthrene, Benz (a) anthrcene, chrysene, Benz (k) fluoranthene, Benz (a) pyrene, dibenzo (a, h) anthracene and benzo (g, h) perylene for all the treatment including the control. The fluctuation is probably due to novel synthesis where a complex compound could have broken down to release other PAHs components which were previously lost. Biodegradation of components of PAHs was statistically significant ($p<0.05$) with time for sample AHB, CB, C and AHB + CB (Figure 6).

Discussion

Biodegradation of polyanomatic hydrocarbon (PAHs) by the treatment options was also measured using GC-MS. The result

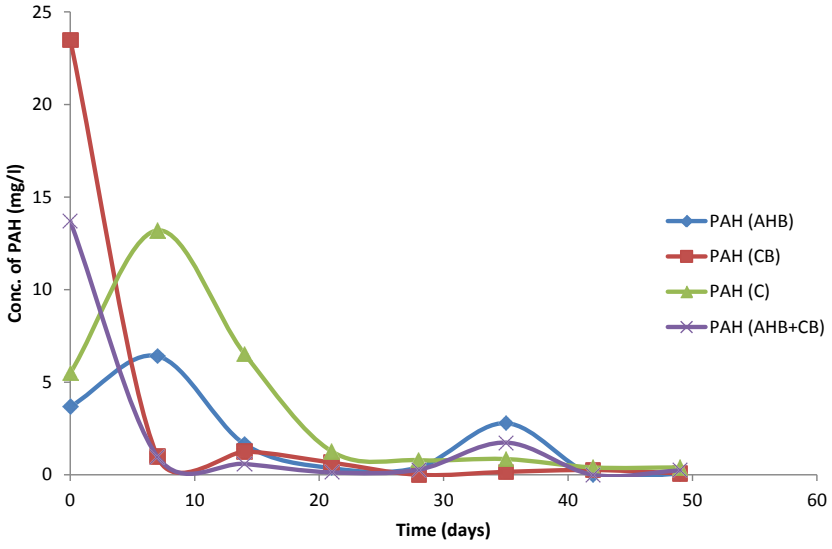

Figure 1: Biodegradation of PAHs by the treatment options.

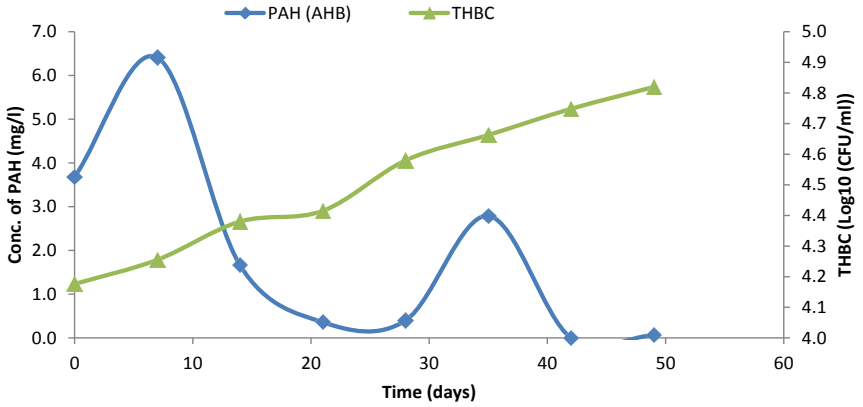

Figure 2: Biodegradation of PAHs by AHB for 49 days.

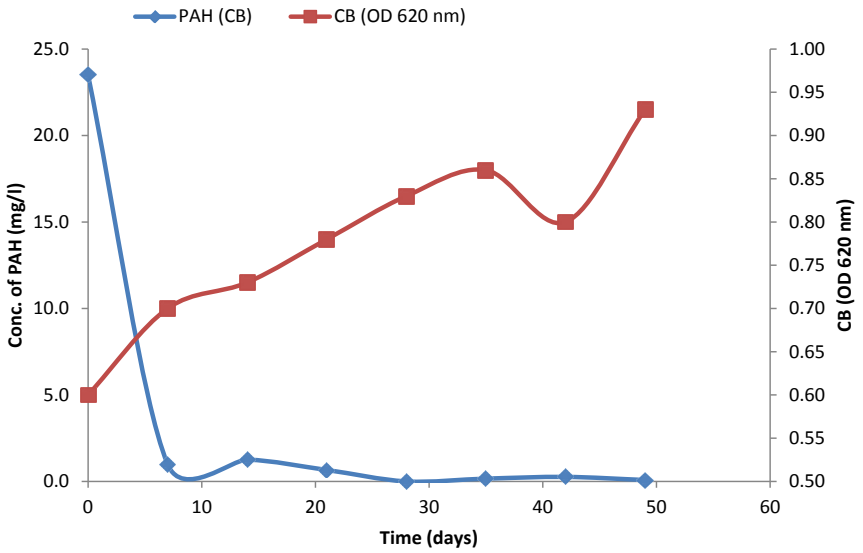

Figure 3: Biodegradation of PAHs by CB for 49 days period.

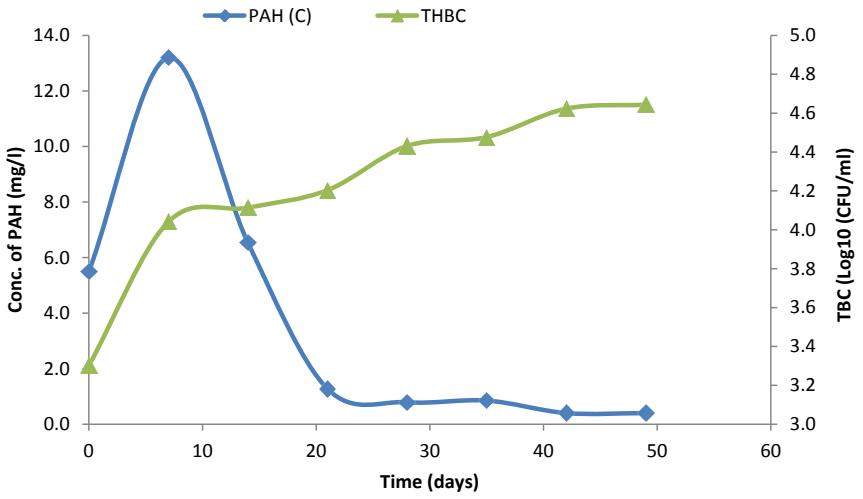

Figure 4: Biodegradation of PAHs in crude oil contaminated control.

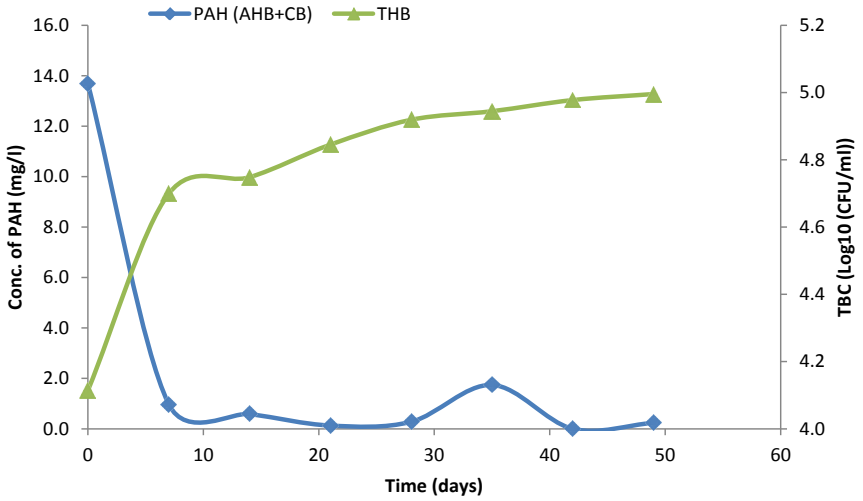

Figure 5: Biodegradation of PAHs by consortium of AHB and CB for 49 days.

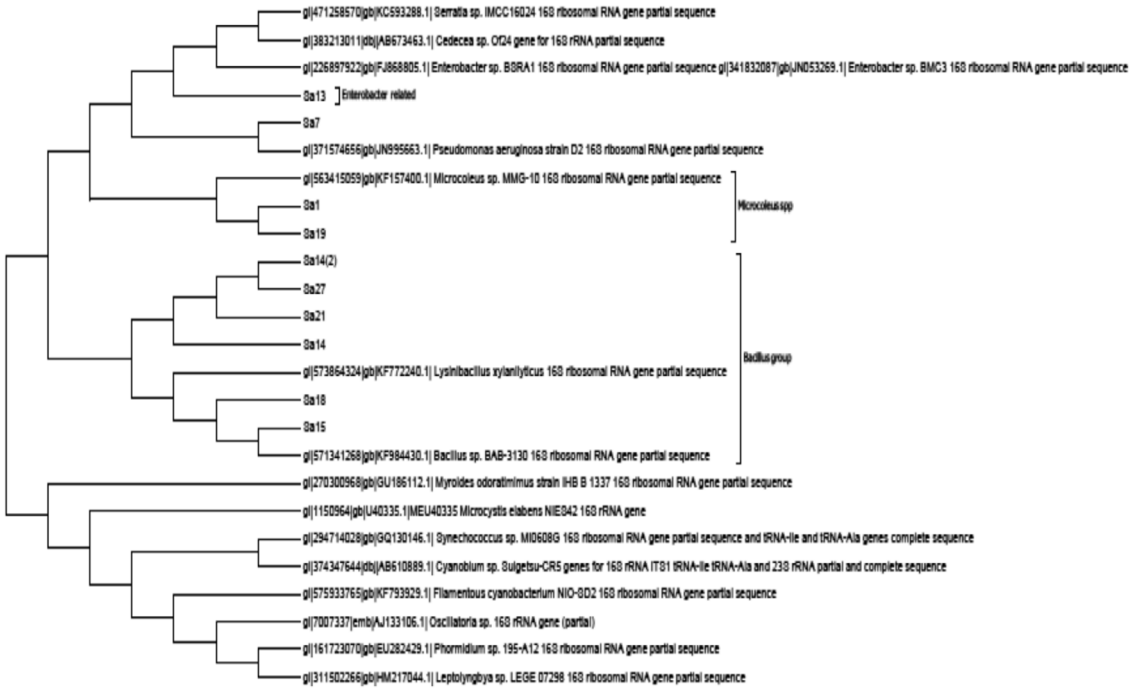

Figure 6: Unrooted phylogenetic tree showing affiliation of bacterial and cyanobacteria consortium based on partial 16S rRNA sequences.

revealed a reduction in the quantity of PAHs in all the treatment options on day 49 though fluctuations in the quantity were observed. For AHB treatment for instance, PAHs rose progressively from 3.68 on day 1 to 6.41 on day 7 and reduced to 1.66 on day 14. This pattern of fluctuation continued in other treatments though not observed negative impact on growth of microorganisms used in the experiment and the control hence microbial growth increased from day 0 till the last day of the experiment. PAHs are hydrophobic and naturally carcinogenic in marine organism and pose serious health concern to heath of both aquatic life and humans through bioaccumulation [38-40]. It is established that they cause cancers, mutagenic and teratogenic conditions in plants, lower animals and this will definitely get into man in the food web of repeated chances of eaten and being eaten.

Earlier reports have shown that microbial activity has the most significant influence in PAH's removal from the environment [4-6]. The metabolic pathways and genetics of degradation of low molecular PAHs such as naphthalene, phenanthrene and anthracene by gram-negative bacteria has been the focus of research in recent times [41]. Arulazhagan reported that a consortain of bacterial utilized 3 mg/L of naphthalene as sole carbon source and nearly 98% of it degraded in four days by bacteria [1]. The result for this study as revealed by GC-MS chromatograms, complete degradation of naphthalene was observed on day 21 from day 1 of the experimental set up in all the treatments including cyanobacteria treatment option. Earlier degradation of naphthalene may be due to its relative solubility in water and high vapour pressure, which biodegradation and volatilization in open

water is known to affect its fate in aquatic ecosystems. Fluorene has been listed as a priority pollutant by EPA. Gomes was as previously reported was utilized by a consortium of bacteria as the sole source of carbon and showed a maximum degradation of 99%, anthracene 95%, phenanthrene and fluorene 97-98% the result showed that the consortium of bacterial degraded PAHs almost completely in (95%) in four days [1,42]. Naphthalene, phenanthrene and authracene which are classified as low molecular mass PAHs were degraded in this present study and the result shows that Dibenzo (a, h) anthracene was completely degraded in 21 days by AHB + CB, Ideno (1, 2, 3-c, d) perylene and Benzo (g, h, i) perylene completely degraded on day 42 by the same treatment.

Cyanobanteria (CB) apart from degrading naphthalene, degraded dibenzo (a, h) anthranaene, ideno (1, 2, 3-c, d) penylene and Benzo (g, h, i) perylene completely by day 28. Similar pattern of degradation occurred in the control day 21. For AHB treatment, however, fluoranthene completely degraded on day 14, pyrene on day 28, phananthrene on day 42, Acenaphthylene, dibenz (a, h) anthrancene, ideno (1, 2, 3-c, d) diperylene, Benzo (g, h, i) perylene and dizenz (a, h,) anthracene completely degraded on day 49. There were disappearance and reappearnce in the course of the experiment for some PAHs on some days monitored. Our result shows biodegradation of PAHs by both acrobic heterotrophic bacteria and cyanobacteria which completely disagrees with the report of Abed and Abed and Koster that cyanobacteria only plays an indirect role in oil biodegration by providing the associated aerobic heterotrophs with oxygen needed for the breakdown of aliphatic and aromatic hydrocarbons, fixed nitrogen and simple organics [43-45]. In this study apart from naphthalene which was degraded by day 14 by all the treatments, all the other components of PAHs were degraded much earlier by AHB + CB, CB and control compared to AHB. This implies that cyanobacteria exerts to a greater extent an influence on biodegradative activities of the consortium in biodegradation and its presence responsible for earlier degradation of the components of PAHs including in the control which was not cured of any specific microorganism. It is apparent that PAHs cannot be oxidized effectively by one single organism as mixed cultures are seen to outperform single species isolated from a consortium [12]. A dominant benzo (a) pyreme degrading bacteria obtained from a marine enrichment which contained three strains *Cochrabactrum*, *Stenotrophomonas* and *Pseudomonas*) degraded PAHs faster than when tested individually [46]. Pelz reported the biodegradation of 4-chlorosalicylate by combination of *Pseudomonas* MT1 [47], *Pseudomonas* MT4 and *Achromobacter* MT 4 using a ^{13}C-labelled substrates. Strain MT 1 degraded 4-chlorosalicylate and provided carbon seletons for the other strains which degraded the toxic metabolites that could have inhibited MT1 if it was allowed to accumulate. One of the toxic intermediates was taken (4-chlorocatechol) up by strain MT3 and further degraded. Mueller reported that cyanobacteria could play important role in the degradation and transformation PAHS since they are widely distributed in aquatic environment [48]. They reported on its ability to oxidize PAHs under photoautotrophic conditions to form hydroxylated intermediates. Naphthalene and phenathrene according to Narro et al. oxidized to form metabolites, *Oscillatoria* sp strain JCM oxidized naphthalene and *Agmellum quadruplicatum* PR6 metabolized phenathrene to trans-9, 10-dehychoxy-9, 10-dihydrophenanthrene and l-methoxy phenanthrene and Benzo (a) pyrene to form a metabolite Isomeric cis-dihydrodiols which is suggestive of dioxygenase catalyzed reaction [49].

Conclusion

The present study showed significant degradation of petroleum hydrocarbons by the cyanobacteira isolates and varied from previous reports by [44,50-54]. Degradation of components of PAHs was observed throughout the 49 days period of the experiment. The degradation of PAHs was significant with time at $p<0.05$. However, the PAH's level was minimal. Polycyclic aromatic hydrocarbons are reported to be of minimal values in an environment without previous history of crude contamination, and very high in polluted environment that has undergone fire burning. The age of contamination/pollution, the higher the PAH's concentration. In this our investigation, the spiking of crude oil in uncontaminated surface water was a recent effort which could have accounted for low level of PAH's in the contaminated water [55-57].

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