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Correlation and Distribution of Xenobiotics Genes and Metabolic Activities with Level of Total Petroleum Hydrocarbon in Soil, Sediment and Estuary Water in the Niger Delta Region of Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Authors UOE and SPA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author UOE managed the analyses of the study. Author UOE managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

In the Niger Delta region of Nigeria, the major anthropogenic source of xenobiotics is petroleum based hydrocarbons. Studies suggest that hydrocarbon degradation genes with potential for bioremediation have a greater abundance at equatorial biomes. Therefore, the primary aim of this study was to establish the distribution of hydrocarbon utilizing genes and metabolic activities in different ecosystems in Eastern Obolo in the Niger Delta and correlate same with total petroleum hydrocarbons contents of these ecosystems. Samples were designated as 1 to 5 (two soil samples: 1 and 2), epipellic and benthic sediment samples (3 and 4), and estuary water (5) samples). Sample collection, determination of total petroleum hydrocarbon (TPH) contents and molecular analyses were all done using standard methods. Extracted DNAs from the various samples were then subjected to next generation sequencing on Miseq Illumina platform.
Gene calling was then performed on the assembled sequence reads using Frag Gene Scan to

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predict open reading frames (ORFs). The ORFS were then functionally annotated to various taxonomic groups using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ghost KOALA databases. A total of 10 xenobiotics metabolisms were detected and these were amino benzoate and steroid degradation, phenylalanine metabolism, synthesis and degradation of ketones, fatty acid degradation, caprolactam, ethylbenezene, chloroalkane and chloroalkene, geraniol, and limonene and pinene degradations. Mean values of TPH (mg/L) ranged from 1.58 to 23.48 in the samples. Furthermore, sample 1 with the least TPH had no xenobiotics metabolism while sample 2 with the highest TPH had the highest number of xenobiotic metabolisms. Dioxygenase, monoxygenase, dehydrogenase and acyltransferase enzymes were the most commonly annotated genes in our samples. Annotated bacteria classes in decreasing order were Alphaproteobacteria > Firmicutes > Gammaproteobacteria > Betaproteobacteria. Correlation analysis between levels of TPH and various annotated metabolisms gave a significant ($p < 0.05$) and strong positive R^2 value (> 0.90). The findings in this study indicate that TPH coupled with metagenomic assessment of xenobiotics metabolic activities is a better way of monitoring biodegradation capacity of an impacted ecosystem.

Keywords: Metagenomics; bioremediation; hydrocarbon degrading genes; xenobiotics; Eastern Obolo; TPH.

1. INTRODUCTION

The 21st century witnessed unprecedented civil and technological advancements as well as intensive usage of xenobiotics [1]. Amongst the xenobiotics that are of environmental significance, petroleum hydrocarbons (PHC) are of greatest significance [2] especially so in crude oil producing communities such as the Niger Delta of Nigeria [3,4]. Crude oil exploration, production and transportation activities in the Niger Delta coupled with its widespread use have resulted in incessant spillages of PHC into the environment affecting various terrestrial and aquatic ecosystems [2,3,5]. PHC are well known for their neurotoxicity effect on humans and animals [6,7]. Furthermore, they have been shown to affect diversity of plants, animals and microorganisms [3,8-10]. Gross estimation of various PHC products is usually done using total petroleum hydrocarbon (TPH) [3,11].

Crude oil spillage is a daily occurrence in the Nigeria Delta [5]. Despite the well established diversity loss and alteration of microbial communities associated with PHC pollution, culturable based techniques are often used in assessing microbial diversity in pristine and impacted environments, and bioremediation potentials of hydrocarbonoclastic micro-organisms [10,12-14]. These techniques are deficient in capturing the unculturable majority in various ecosystems and as a result more sensitive techniques capable of capturing this unculturable majority have emerged [10,14].

Metagenomics stands out amongst the molecular techniques that can be used to capture microbial and functional diversities of an ecosystem [10]. Furthermore, it has been applied in the discovery of novel pathways and genes involved in PHC degradation [15]. Our understanding of microbial community, genes and pathways involved in PHC degradation is still limited [15,16]. Microbial remediation (bioremediation) of crude oil polluted sites remains one of the leading techniques of restoring petroleum hydrocarbon contaminated sites [4]. Bioremediation of PHC and other xenobiotics is a complex process and is made possible by degrading genes that utilizing microbes elaborate [4].

Studies suggest that biodegradation and biosurfactant genes of potential in bioremediation have a unique geographical distribution with the former having greater abundance in equatorial biomes [17]. In the Niger Delta, various studies exist that have assessed levels of total petroleum hydrocarbon in various aquatic and terrestrial ecosystems. However, to the best our knowledge, none exist that have examined the distribution of degradation genes. Therefore, the main aim of this study was therefore, to determine the distribution and correlation of xenobiotics genes and metabolic activities with TPH levels in various ecosystems.

2. MATERIALS AND METHODS

2.1 Sampling Site Description and Sample Collection

Samples were collected from Eastern Obolo Local Government Area of Akwa Ibom State which is located in the Niger Delta Region of Nigeria. The coordinates of the sampled areas

are latitudes 4º 32′ 0″ N & longitude 7º42′ 0″ E. (See map below for more details). Collected samples were designated as samples 1 to 5. Samples 1 and 2 were soil, 3 and 4 were epipellic and benthic sediments and 5 was estuary water sample, respectively. Samples were collected using standard techniques previously described [5,10,13]. All samples were collected in triplicates and then mixed to form composite samples which were then used for further analysis.

2.2 Total Petroleum Hydrocarbon

Determinations of total petroleum hydrocarbon (TPH) content of the five composite samples were carried out as previously described [18-21]. Briefly, this was done using Agilent 6890N Gas Chromatography - Flame Ionization Detector (GC – FID). Estuary water sample (5) was transferred into a 1000 ml separating flask to which 30 µg/ml of 1-chlorooctadecane in 1ml of dichloromethane (DCM) was added. To the mixture, 20 ml of DCM was then added and shaken with release of pressure periodically. This was allowed to stand for some time, filtered and concentrated to 1ml in a fume cupboard. Similar treatments were given to all the soil and sediment samples (1 to 4) but samples 3 and 4 were allowed to air dry to get rid of most of the moisture. Exactly 5g of anhydrous Na_2SO_4 was added to the samples and stirred. To the mixture, 30 mL of extracting solvent DCM was added and shaken for 5 to 6 hours using an electrical mechanical shaker at room temperature. After which they were then filtered as done for sample 5. Pretreatment and detection were done as described previously [21]. TPH in soil and sediments samples were reported in mg/kg while that of water sample was in milligram per litre.

2.3 Molecular Analysis

Genomic DNA extractions on the samples were done using ZYMO soil DNA extraction Kit (Model D 6001, Zymo Research, USA) following manufacturer's instructions and procedures as reported recently [10]. Following genomic DNA

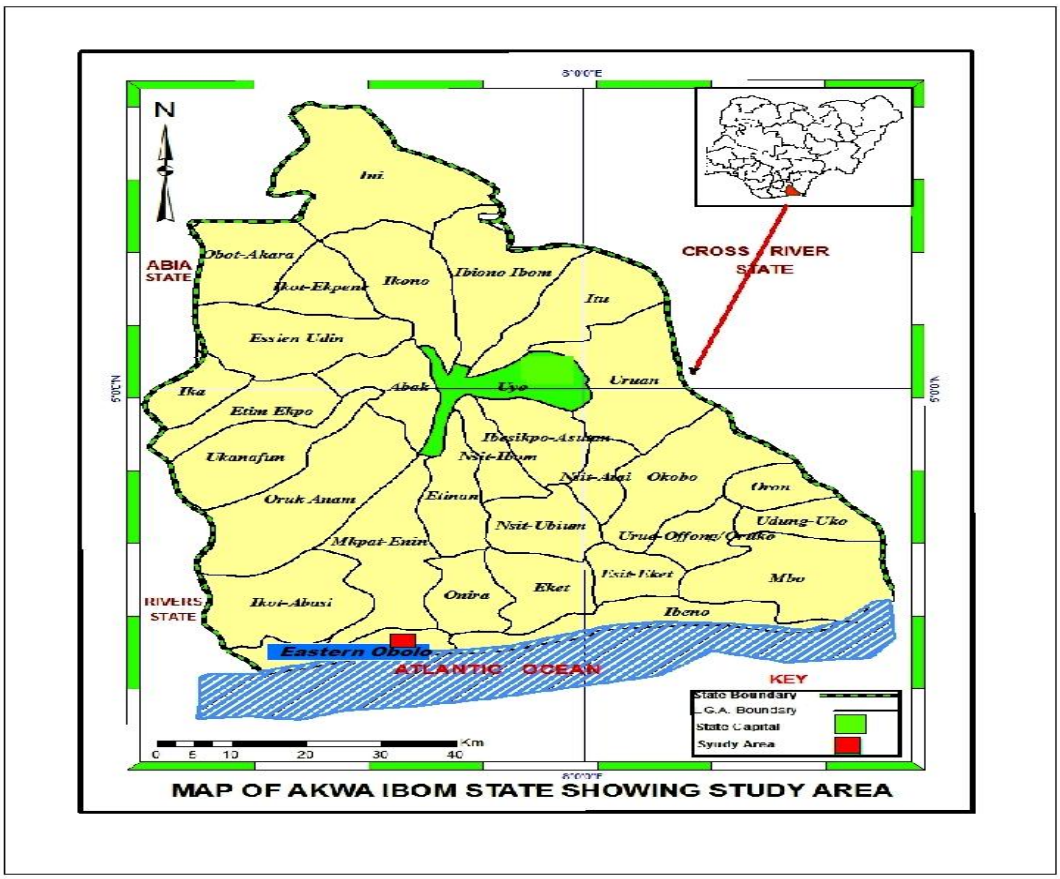

Fig. 1. Map of Akwa Ibom State showing the study area

extractions, gel electrophoresis and amplification were done using the universal primer pair 341 Forward (5′-TCGTCGGCAGCGTCAGATGTG TATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 785 Reverse (5′-GTCTCGTGGGCT CGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC-3′) together with Illumina specific adapters added to the 5′ end of the primers [22]. Next generation sequencing and analysis of reads were all done as described [22,10]. KEGG and Ghost KOALA [23] were employed for functional analyses of the metagenomic reads. Open reading frames were predicted via gene calling performed on the assembled sequenced reads using Frag Gene Scan [24]. The ORFs were then functionally annotated and assigned to the KEGG and GhostKOALA. In GhostKOALA, each query gene is assigned a taxonomic category according to the best-hit gene in the Cd-hit cluster supplemented version of the non-redundant pangenome dataset [25].

2.4 Statistical Analysis

Levels of TPH were analyzed using clustered column bar charts while stacked plots were used to analyze the reads counts of the various phyla and the relative abundance of the xenobiotics metabolisms. These were done as previously described [26]. Correlation analysis was performed by plotting the number of the various xenobiotics metabolisms annotated against the levels of TPH in the various samples using an X-Y plot. Regression analysis was also performed at 95% confidence level.

3. RESULTS

The various xenobiotics metabolic activities and enzymes, annotated phylotypes, and as well as the TPH are presented in the Tables and figures below. As evident from Fig. 2, the various xenobiotics degradation activities as obtained from KEGG were amino benzoate and steroid degradations, phenylalanine metabolism, synthesis and degradation of ketones, fatty acid, caprolactam, ethylbenezene, chloroalkane and chloroalkene, geraniol, and limonene and pinene degradations. No xenobiotic degradation was detected from the KEGG analysis of the sample 1 metagenome. However, in other samples they were detected. The abundance of the xenobiotic degradation obtained in the samples were Sample 2 > Sample 3 > Sample 4 > Sample 5 in that order.

From Fig. 3, Sample 1 had the least quantity of TPH of 1.58 mg/kg while sample 2 had the highest 23.48 mg/kg.

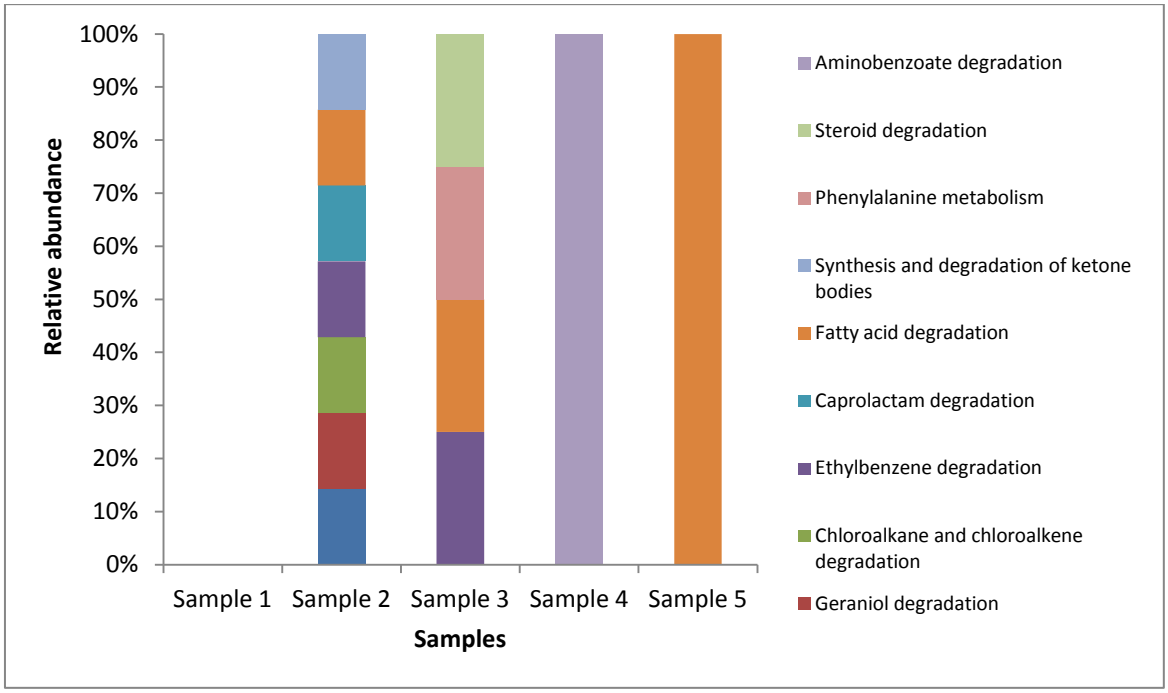

Fig. 2. Relative abundance of the various xenobiotics metabolisms detected in the various samples as obtained via KEGG presented as 100% stacked plots

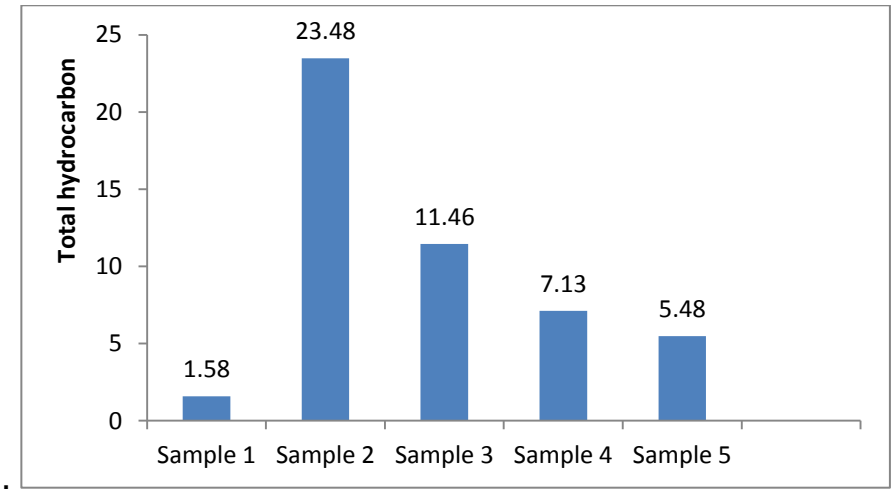

Fig. 3. TPH levels in the various samples. Samples 1 to 4 are expressed in mg/kg while 5 was in mg/l

Fig. 4 shows the relative abundance of the various phyla detected in samples 1 to 5. After the unknown phyla, Proteobacteria, Acidobacteria, Gemmatimonadetes, Planctomycetes, Chloroflexi, Chlamydiae and Verrumicrobia showed higher abundance in samples (2 to 5) than sample. However, Actinobacteria and Firmicutes abundance levels

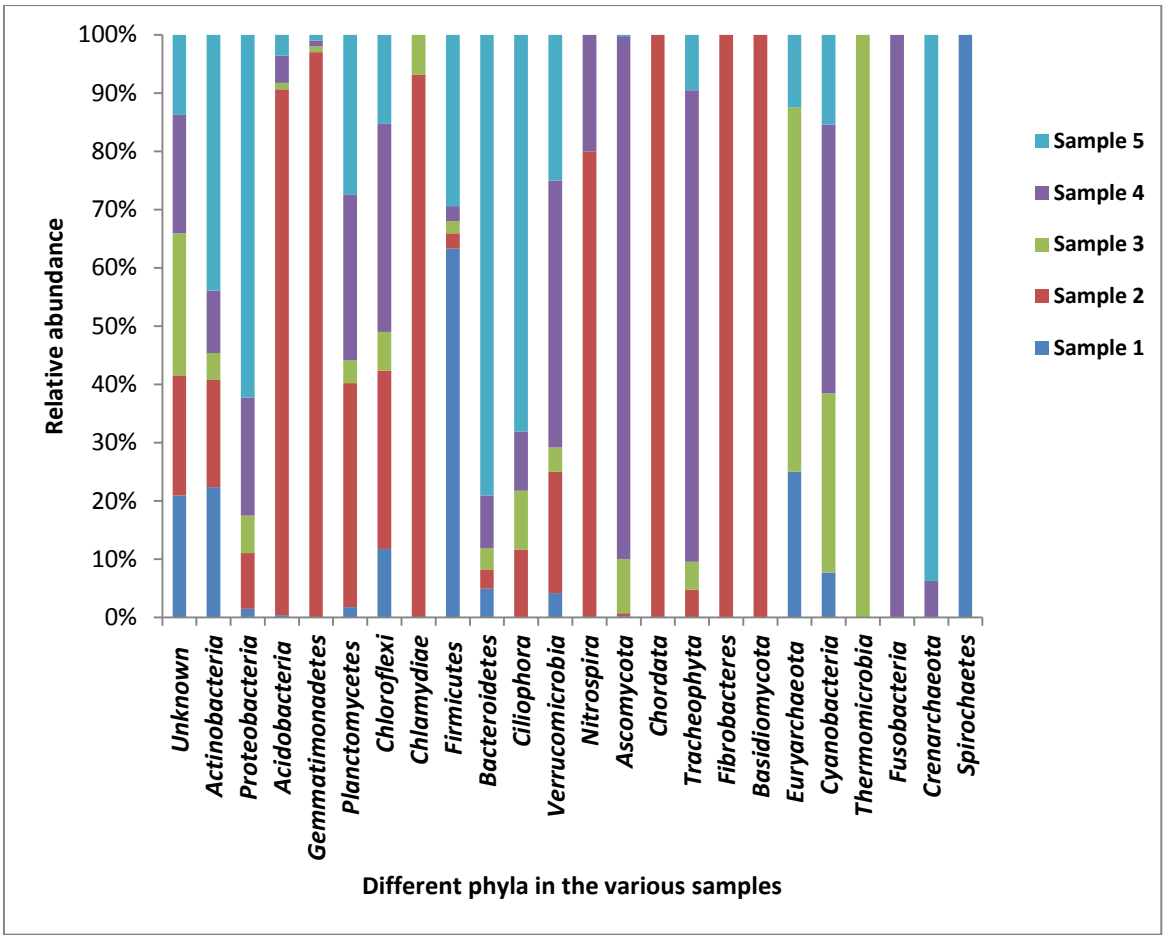

Fig. 4. Relative abundance stacked plot of the different phyla in samples 1 to 5

were in this order sample 5 > sample 1 > sample 2 > sample 4 > sample 3 and Sample 1 > sample 5 > sample 2 = sample 4 > sample 3, respectively.

Tables 1 to 3 show the various xenobiotic metabolic profiles detected in the samples 2 to 5. Sample 1 had no annotated xenobiotics metabolism. In sample 2, the annotated metabolisms were as captured in Table 1. Metabolism of terpenoids and polyketides was detected and the annotated enzyme was aldehyde dehydrogenase elaborated by the betaproteobacteria *Paraburkholderia xenovorans.* Geraniol and benzoate degradation were also detected in *Kyrpidia tusciae* and the enzyme was acetyl-CoA acyltransferase. In addition, the alphaproteobacteria *Parvibaculum lavamentivorans* was also involved in benzoate degradation. Chloroalkane and chloroalkene degradation was detected by the betaproteobacteria *Paraburkholderia xenovorans* and the annotated enzyme was aldehyde dehydrogenase. Ethylbenzene degradation was also annotated and the enzymes was acetyl-CoA acyltransferase and from *Kyrpidia tusciae.* Caprolactam degradation was also detected in *Defluviimonas alba* and the enzymes were alkane 1-monooxygenases (alk B1 and alk M). Fatty acid degradation was one of the most annotated xenobiotic degradation obtained in the study. The phylotypes were *Parvibaculum lavamentivorans, Kyrpidia tusciae, Defluviimonas alba* and *Paraburkholderia xenovorans*. The respective enzymes were acetyl-CoA C-acetyltransferase, acetyl-CoA acyltransferase, alkM alkane-1-monooxygenase and aldehydes dehydrogenase (NAD+). Synthesis and degradation of ketones bodies was also detected in the phylotype *Parvibaculum lavamentivorans* and the enzyme was acetyl-CoA C-acetyltransferase. In addition to geraniol, other living sources of hydrocarbon were also detected and these were limonene and pinene degradations.

Table 1. Distribution of various xenobiotics degrading enzymes amongst various phylotypes in soil sample obtained from KEGG in sample 2

Xenobiotic metabolism	Bacterial class (species)	Enzymes
Metabolism of terpenoids and polyketides	B (*Paraburkholderia xenovorans*)	Aldehydes dehydrogenase (NAD+)
Geraniol degradation	F (*Kyrpidia tusciae*)	Acetyl-CoA acyltransferase (fadl)
Benzoate degradation	F (*Kyrpidia tusciae*)	Acetyl-CoA acyltransferase (fadl)
	A*lavamentivorans*)	Acetyl-CoA C-acetyltransferase
Chloroalkane and chloroalkene degradation	B (*Paraburkholderia xenovorans*)	Aldehydes dehydrogenase (NAD+)
Ethylbenzene degradation	F (*Kyrpidia tusciae*)	Acetyl-CoA acyltransferase (fadl)
	F (*Kyrpidia tusciae*)	Acetyl-CoA acyltransferase (fadI)
Caprolactam degradation	A (*Defluviimonas alba)*	alkane 1-monooxygenase (alkB1, alkM)
Fatty acid degradation	A (*Parvibaculum lavamentivorans*)	Acetyl-CoA C-acetyltransferase
	F (*Kyrpidia tusciae*)	Acetyl-CoA acyltransferase (fadI)
	A (*Defluviimonas alba)*	Alkane 1-monooxygenase (alkM)
	B (*Paraburkholderia xenovorans*)	Aldehydes dehydrogenase (NAD+)
Synthesis and degradation of ketone bodies	A (*Parvibaculum lavamentivorans*)	Acetyl-CoA C-acetyltransferase (atoB)
Limonene and pinene degradation	B (*Paraburkholderia xenovorans*)	Aldehyde dehydrogenase (NAD+)

Key: B = Betaproteobacteria, A = Alphaproteobacteria, F = Firmicutes

Table 2. Distribution of various xenobiotics degrading enzymes amongst various phylotypes in epipellic sediment obtained from KEGG in sample 3

Xenobiotic metabolism	Bacterial class (species)	Enzymes
Fatty acid degradation	A (*Methylobacterium extorguens*)	3-Phenylpropionate/trans-cinnamate dioxygenase ferredoxin
	F (*Heliobacterium modesticaldum*)	Phenylacetate-CoA ligase
	A (*Methylobacterium extorguens)*	3-Phenylpropionate/trans-cinnamate dioxygenase ferredoxin
	G (*Pseudomonas putida)*	Trans-feruloyl-CoA hydratase / vanillin synthase
Caprolactam degradation	A (*Komagataeibacter xylinus*)	Gluconolactonase
Steroid degradation	A (*Methyloceanibacter caenitepidi)*	Cholesterol oxidase

Key: A = Alphaproteobacteria, F = Firmicutes, G = Gammaproteobacteria.

In sample 3 as captured in Table 2, the various xenobiotics metabolisms obtained were fatty acid degradation, caprolactam ad steroid degradation. The enzyme and phylotypes for steroid and caprolactam degradations were cholesterol oxidase and *Methyloceanibacter caenitepidi,* and *Komagataeibacter xylinus* and gluconolactonase, respectively. The annotated enzymes for fatty acid degradation were 3-phenylpropionate/trans-cinnamate dioxygenase ferredoxin, phenylacetate-CoA ligase and trans-feruloyl-CoA hydratase / vanillin synthase while the corresponding phylotypes were *Methylobacterium extorguens, Heliobacterium modesticaldum* and *Pseudomonas putida.*

Table 3 shows the various metabolic activities in the benthic sediment and estuary water samples (sample 4 and 5). In the benthic sediment sample, aminobenzoate degradation was the only xenobiotic degradation detected. The enzyme associated with aminobenzoate degradation were vanillin dehydrogenase, acetate CoA/acetoacetate CoA-transferase alpha subunit, and the enzyme for phenylacetic acid degradation was operon negative regulatory protein, and their corresponding phylotypes were *Immundisolibacter cernigliae, Coprococcus catus, Aneurinibacillus* species*,* respectively*.*

Fig. 5 shows a graph of the number of annotated xenobiotics metabolisms against the levels of TPH in the various samples. Regression analysis gave significant probability ($p < 0.05$) while correlation coefficient (R^2) from the regression analysis and X-Y plots gave a strong positive value (> 0.90).

4. DISCUSSION

The effects and impacts of crude oil activities are well studied in Eastern Obolo communities and ecosystems and as well as other oil producing communities in and around the Niger Delta

Table 3. Distribution of various xenobiotics degrading enzymes amongst various phylotypes in benthic sediment and estuary water samples as obtained from KEGG in samples 4 and 5

Xenobiotic metabolism	Bacterial class (species)	Enzymes
Sample 4		
Aminobenzoate degradation	G (*Immundisolibacter cernigliae*)	Vanillin dehydrogenase
	F *(Coprococcus catus)*	Acetate CoA/acetoacetate CoA-transferase alpha subunit
	F (*Aneurinibacillus* species)	Phenylacetic acid degradation operon negative regulatory protein
Sample 5		
Lipid metabolism	Al (*Aureimonas* species AU20)	Long-chain alkane monooxygenase
	A (*Sinorhizobium meliloti*)	IclR family transcriptional regulator, pca regulon regulatory protein

Key: A = Alphaproteobacteria, F = Firmicutes, G = Gammaproteobacteria

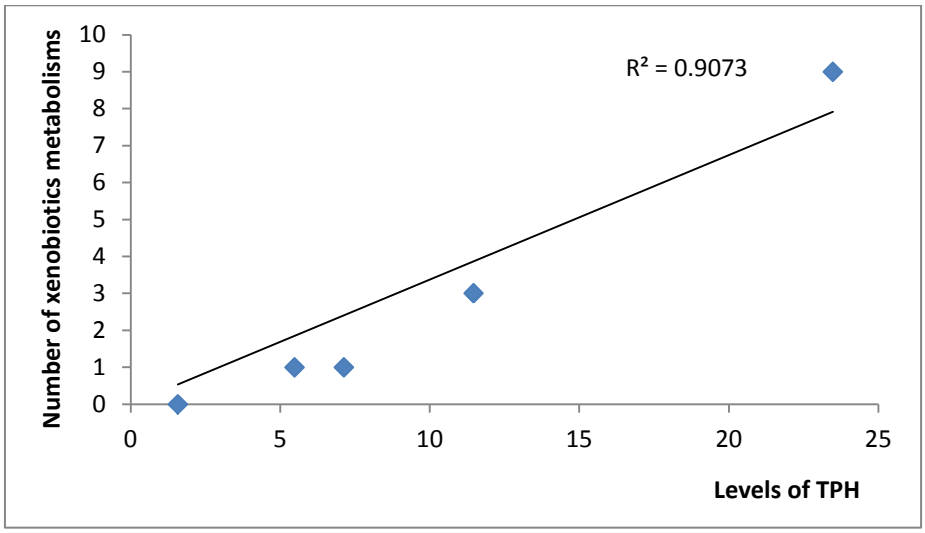

Fig. 5. An X-Y plot of the number of annotated xenobiotics levels of TPH in the various samples

region of Nigeria [5,27,20,18,28-30]. These studies which include our recent studies confirm anthropogenic impact on Eastern Obolo ecosystems.

In surface and groundwater samples collected from the vicinity of Nigerian National Petroleum Corporation (NNPC) in south west Nigeria, TPH valves ranging from 20.34±1.79 to 27.40±5.32 and 2.67±0.80 to 13.03±2.21mg/L respectively were reported. Furthermore, control sample from University of Ibadan gave TPH values that ranged from 13.18±2.41 to 1.58±0.22 mg/l. Elsewhere, TPH values in sediment samples along Bonny creek showed seasonal variations with values ranging from > 0 < 800 μg/g [30]. Furthermore, in various samples from Cross River Estuary, high values of TPH were also recorded for both surface and groundwater that ranged from 20.34±1.79 to 27.40±5.32 and 2.67±0.80 to 13.03±2.21 mg/L respectively, as against the control of 13.18±2.41 and 1.58±0.22 mg/L [29]. Our findings were within ranges of the aforementioned studies. However, our findings were lower than those of Olufemi et al. [19] who reported higher levels of TPH of 73.50 ±4.80 mg/L and 73.50 ±4.80 mg/L, respectively for surface water and sediment samples and those of ground and surface water [18]. TPH have also been reported in various shell fishes from the littoral waters of the Bight of Bonny [28].

Microbes in marine ecosystems have been exposed to crude oil hydrocarbons for millions of years via natural seeps. Consequently, hydrocarbon degradation abilities amongst degraders have been acquired via evolution and they are widespread in nature [31]. Important degraders include member of the Gammaproteobacteria, Alphaproteobacteria and the Actinobacteria [32]. In another study, taxonomic classification of the bacterial groups associated with petroleum hydrocarbon contaminated ecosystems revealed the following phyla: Actinobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Proteobacteria, Verrucomicrobia, Chlorobi, and Firmicutes with Proteobacteria being the most abundant taxa [4]. These phyla were also detected in our samples. However, after unknown phyla, Proteobacteria, Acidobacteria, Gemmatimonadetes, Planctomycetes, Chloroflexi, Chlamydiae and Verrumicrobia were higher in samples (2 to 5) than in sample 1 which had the lowest TPH levels. Furthermore, Actinobacteria and Firmicutes abundance levels were in this order sample 5 > sample 1 > sample 2 > sample 4 > sample 3 and sample 1 > sample 5 > sample 2 = sample 4 > sample 3, respectively. Elsewhere, all the phyla identified in our study were also detected in a microbial consortium of natural crude oil seepage except for Ciliophora, Chordata, Tracheophyta, Fibrobacteres and Basidiomycota [33]. In India, metagenomic analysis revealed complexity in microbial community and functional diversity in a hexachlorocyclohexane (HCH) polluted pond. Functional gene analysis revealed gene implicated in HCH degradation. Significant and abundant phyla were Actinobacteria, Chloroflexi, Firmicutes, Euryarchaeota, Proteobacteria and Verrumicrobia in their study and these were all present in our various samples [34].

A study on geographical distribution of hydrocarbon degradation and biosurfactant producing genes suggested that near equator biomes have higher abundance of both genes with potential for bioremediation [17]. Terrestrial biomes elaborate more cyclic compounds degradation enzymes and less surfactant genes compared to aquatic biomes [17]. Sierra-Garcia et al. [15] reported some xenobiotic degradation metabolisms in an oil reservoir to include geraniol, biphenyl, bisphenol A, 3-Chloroacrylic acid, 1,2 dichloroethane napthalene and anthracene, ethylbenzene, flourene , caprolactam, gamma-hexacholorcyclehexane, trinitrotoluene, 1,1,1-Tricloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation, 1- and 2-methylnaphthalene degradation and benzoate degradation via coA ligation and hydroxylation degradations. These xenobiotics showed variations amongst the various fosmids they employed in their study. Our findings revealed not just petroleum based xenobiotics but also living sources of hydrocarbons such as geraniol and limonene and pinene metabolisms. Furthermore, ethylbenzene, caprolactam and benzoate metabolisms were also detected in our study. The levels of xenobiotics degradation metabolism could be a pointer to the degradation capacity of microorganisms in an impacted ecosystem.

5. CONCLUSION

Our findings of a positive correlation between TPH levels and annotated xenobiotics metabolisms suggest that the level of TPH could be an indicator of the biodegradation capacity of a particular ecosystem given the number of different xenobiotic degradation annotated in our various environmental samples. Thus, future studies aim at examining the pollution status of an ecosystem via TPH assessment should also include assessment of xenobiotics degradation genes/metabolisms.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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