FATE OF 2- & 3 - RING POLYCYCLIC AROMATIC HYDROCARBONS IN ESTUARINE MUDFLAT FROM IKO RIVER ESTUARY, NIGERIA

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ABSTRACT

The present study was designed to evaluate the PAH status, fate of PAH suites (2- & 3- ring PAHs) and their degradation in the epipellic sediment (mudflat) of Iko River estuary using standard analytical and microbiological techniques. The results of the analysis have shown that the mudflat of Iko Estuary ecosystem contained detectable levels of PAHs (ranging from 8.61 to 9.02mg/kgdw) with 2- and 3- ring PAHs being predominant with a concentration of 4.20 mg/kgdw in the three stations sampled, while 4-ring and 5-ring PAHs ranged from 1.15 to 1.74 and from 2.14 – 2.47 mg/kgdw respectively. The 6-ring PAHs were the least detected with a range of 0.20 – 1.52 mg/kgdw. Enrichment of mudflat samples with indigenous hydrocarbon degraders showed that the 5 bacterial species isolated from the sediment exhibited varying PAH utilizing potentials, while *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Micrococcus luteus* were found to possess strong PAH degrading capabilities. A 100% degradation of Naphthalene was observed from *Pseudomonas* sp, this was followed by *Bacillus subtilis* (95%) and *Micrococcus* sp (93%), and similarly, *Pseudomonas* sp had the highest anthracene degrading potentials. *Pseudomonas* sp reduced the concentration of anthracene from 1.10mg/l (initial concentration) to 0.015mg/l (final concentration) showing a 98.6% degradation, while *B. subtilis* and *Micrococcus* sp reduced the same initial concentration to 0.076mg/l and 0.097 mg/l showing a 93.1% and 91.2% degradation respectively. Findings have shown that the fate of PAH suites including the dominant suite of PAHs (2- and 3-ring PAHs) in estuarine sediment is short-lived due to the presence of potent hydrocarbon degrading bacterial species which can sequester the recalcitrant hydrocarbons over time.

INTRODUCTION

Increasing crude oil exploitation in the Niger Delta has led to widespread contamination of most of its creeks, rivers, swamps soils, underground water and coastal regions. These contaminations result from crude oil spills, improper disposal of drilling muds and cuttings dispersant application and other wastes resulting from crude oil extraction, transportation and refining processes (Okpokwasili and Nnubia, 1995; Udotong *et al.*, 2008; Essien *et al.*, 2015; Abraham and Essien, 2016). Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous environmental pollutants and some of them are known to be mutagenic or carcinogenic (Ravindra *et al.*, 2008). PAHs are released to the environment through natural (biogenic and geochemical) and anthropogenic activities such as the production and combustion of fossil fuels, and biomass (Omar *et al.*, 2002) and enter surface waters through different pathways including atmospheric fallout, urban runoff and municipal/industrial effluents (Zhu *et al.*, 2004). The wide distribution of environmental PAHs sources coupled with its global transport phenomena has resulted in their ubiquitous distribution. This has led to an increased environmental concern due to their toxic, mutagenic, and carcinogenic properties.

PAHs can be absorbed to organic-rich soils and sediments, accumulate in fish and other aquatic organisms, and may be transferred to humans through seafood consumption (Abraham and Essien, 2016; Essien *et al.*, 2015). The transport and fate of pollutants in our environment are

governed by different biotic or abiotic processes and depend on several factors. These factors include soil/water properties, chemical compounds properties, biota activity, sequestration and environmental factors (Essien *et al.*, 2011, Cachada *et al* 2018). Soil is the ultimate sink for many pollutants especially the most persistent such as PAHs, PTEs and POPs, where they may persist for many years. Once in soils pollutants, contaminants can be incorporated into more stable solid phases over time, for instance they can be retained in the organic phase of a soil, and this process which is known as aging (or sequestration) can be virtually irreversible (Cachada *et al* 2018) but for scavenging effects of microbes. Studies have shown that because of the near anoxic (reduced) status and high repository capacity of the epipellic (mudflats) or intertidal sediment, low microbial activities of epipsammic and benthic sediments of aquatic bodies, they readily serve as haven for pollutants in aquatic systems (Essien, 2020). In this study we evaluate the role of oil degrading bacteria on the PAH status, fate of PAH suites (2-, 3-, 4-,5- and 6 –ring PAHs) and their degradation in the epipellic sediment (mudflat) of Iko River estuary, Nigeria

MATERIALS AND METHOD

Study Area

The study area is a mangrove ecosystem located in Iko LGA (Fig. 1) within the petroleum belt of the Niger Delta, Nigeria (7^0 30' N and 7^0 45'N, and longitude $7^0$30' E and 7^0 40'E). Iko River estuary has semi-diurnal tides and a shallow depth ranging from 1 to 7 m at flood and ebb tides.

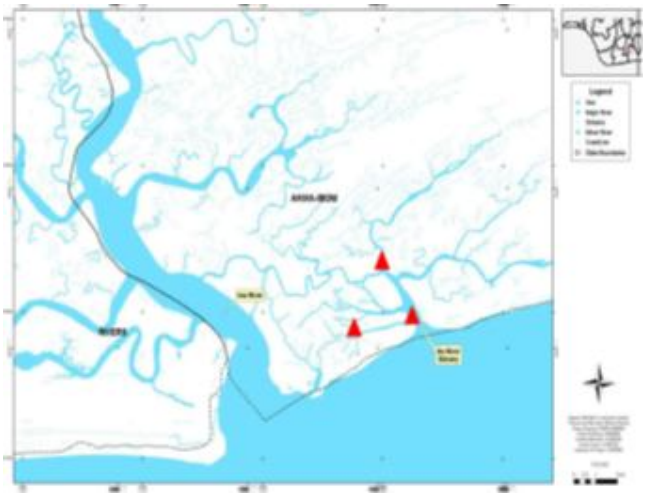

Figure 1: Iko River Estuary showing the sampling locations (*solid triangles*)

Soft dark mudflats (Fig. 2) are usually exposed during low tide while the shoreline is characterized by mangrove swamps, shoals and sandbars. The estuary is more than 20 km long with an average width of 5 m. Iko River takes its course from Qua Iboe River catchments and drains directly into the Atlantic Ocean at the Bight of Bonny (Ekpe *et al.*,1995; Benson and Etesin, 2007). It has many adjoining tributaries and part drains into Imo River estuary, which opens into the Atlantic Ocean. The river is characterized by a humid tropical climate with rainfall reaching about 3,000mm per annum.

Sample Collection

With the aid of Shipek grab sampler, the epipellic sediment samples were collected from three sample stations (IB_1, IB_2 and IB_3) selected at random within the course of the estuary. The samples were contained in amber bottles, stored in ice–packed chest cooler to preserve its quality and transported to the laboratory for analysis.

Figure 2: Mudflat or epipellic sediment at Iko estuarine coast

Determination of PAHs Content of Sediment

The epipellic sediment samples were extracted and fractionated as described (Olajire *et al.*, 2005). Dried and sieved sediment samples (50g) were weighed and spiked with pre-deuterated PAH cocktail as internal standard and extracted with dichloromethane (DCM) using temperature programmed Soxhlet extractor at 65 ^{0}C for 24 h. The extracts were reduced to dryness, re-dissolved in n-hexane then fractionated on a glass column packed with 30g of alumina deactivated with 4.5% water. Aliphatic and polycyclic aromatic hydrocarbons were eluted with 50 ml of hexane: DCM (95/5%, w/v) and the polar fractions were eluted with DCM. The PAH fractions were then concentrated by rotary evaporation. Before quantification using GC/MS analysis, fractions were dried under nitrogen and re-dissolved in DCM.

A gas chromatograph (GC, Hewlett-Packard HP 6890 series) coupled to a mass spectrometer (MS, Model 5971, Hewlett-Packard) was used to quantify extractable organic PAHs and the PAHs determined in selective ion-monitoring mode (SIM mode) with ionization energy of 70 eV. The m/z peak corresponding to the molecular masses of the individual PAH was used for identification and quantification. Concentrations of PAH were calculated relative to the pre-deuterated internal standard.

Enrichment and Isolation of Indigenous Species of Hydrocarbon and PAH Utilizing Bacteria

To assess the PAH utilizing bacterial community, precisely 10 g of the sample was inoculated into two sets conical flask containing 100 ml of sterile minimal salt medium (MSM) [KH_2PO4 1 g/L, Na_2HPO_4 1.3 g/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4$ 0.2 g/L, $FeSO_4{\cdot}7H2O$ 0.05 g/L, $CaCl_2$ 0.02 g/L, $ZnSO_4{\cdot}7H_2O$ 5 mg/L, $MnCl_2{\cdot}4H_2O$ 5 mg/L, $NaMoO_4{\cdot}2H_2O$ 1 mg/L, $CuCl_2$ 0.5 mg/L] (Abraham and Essien, 2016). The first flask was enriched with 0.5g/l Naphthalene while the second flask was enriched with 0.5g/l Anthracene as carbon sources and incubated at 28 °C in shaker incubator (100 rpm) for 5 days. After 5 days of incubation, 5ml of the aliquot was transferred into a freshly prepared MSM containing the same quantity of naphthalene and anthracene respectively. This repeated enrichment was carried out for four consecutive times after which, the culture suspension was serially diluted and plated on a MSM supplemented with 0.5g/l naphthalene and 0.5g/l anthracene respectively and 15g/l agar-agar as solidifying agent and incubated for 5 days at 28^{o}C. Discrete colonies with clear zones on the plate were isolated, purified and stored in agar slants.

Identification of the Hydrocarbon Degrading Isolates

The bacterial isolates obtained from the assay were characterized based on their cultural and biochemical properties using the methods described by Collins *et al.*, 2004; Chessbrough (2006). The Gram stain, oxidase, catalase, nitrate reduction, urease, citrate utilization, motility, H_2S production, indole production, esculin hydrolysis, arginine hydrolysis, mehyl red- Voges Proskauer (MR-VP) and sugar utilization tests as described by Collins *et al.*, (2004) and Chessbrough (2006) was adopted for the characterization of the bacteria. Tentative identification

of the bacterial strains was based on their biochemical and cultural attributes as described by Holt *et al*., (1994) and Collins *et al.,* (2004).

Napthalene (2-ring PAH) and Anthracene (3-ring PAH) Degradation by Mangrove Sediment Bacteria

To determine the potentials of PAH utilizing bacterial isolates to degrade naphthalene and anthracene, the test isolates (1.4×10^4 cells per ml) were cultured in 100ml of MSM, (Abraham and Essien, 2016; John *et al.,* 2012), containing low levels 4% of naphthalene and anthracene respectively for 24 days at 28^{o}C. Additional experiment, under the same conditions excluding the test isolates was carried out to serve as un-inoculated controls. The controls were poisoned with 1% $HgCl_2$ to avoid microbial activity (Diaz *et al.,* 2002). The degree of degradation of PAHs by the isolates was measured first by estimation of the bacterial biomass and changes in pH in a 3-days interval for 24 days. Secondly, by the subtracting the concentration of the residual PAH in the medium after 24 days from the initial concentration and comparing it with that of the control. In this case, the concentrations of PAHs were calculated relative to the squalane standard. Squalane and bromotetradecane were added as internal standards and the amount of individual aromatic compounds was determined by GC-MS, relative to standard. The degree of biodegradation was determined as percentage values of the amount of PAHs lost during the degradation course.

RESULTS

PAH Profile of the Sediment Sample

The PAH concentration in the epipellic sediments of the sample station from Iko River estuary are presented in Table 1. The results have shown that the 2 -3 rings PAHs are the dominant suite of PAHs in mudflats from Iko River Estuary with concentration of 4.20 mg/kgdw in the three stations sampled, while 4-ring and 5-ring PAHs ranged from 1.15 to 1.74 and from 2.14 – 2.47 mg/kgdw respectively. The 6-ring PAHs were the least detected with a range of 0.20 – 1.52 mg/kgdw. Figure 3 shows the group profiles of PAHs normalized by the total sum of all the PAHs in each sample.

Table 1: Total PAHs concentration (mg/kgdw) in sediments from Iko River Estuary

PAH Weight (Ring)	Analyte	Sediment Samples IB_1	IB_2	IB_3
2-3ring PAHs	Naphthalene	1.00	1.00	1.00
	2methylnaphthalene	0.20	0.20	0.20
	Acenaphthylene	1.00	1.00	1.00
	Acenaphthalene	0.70	0.70	0.70
	Flourene	0.70	0.70	0.70
	Phenanthrene	0.20	0.20	0.20
	Anthracene	0.40	0.40	0.40
∑2-3ring PAHs		4.20	4.20	4.20
4ring PAHs	Flouranthene	0.20	0.20	0.20
	Pyrene	0.25	0.22	0.23
	Benzo(a)anthracene	0.53	0.33	0.56
	Chrysene	0.40	0.40	0.75
∑4ring PAHs		1.38	1.15	1.74
5ring PAHs	Benzo(b)flourenthene	0.58	0.74	0.91
	Benzo(k)fluranthene	0.47	0.25	0.46
	Benzo(a)pyrene	0.39	0.20	0.31
	Dibenzo(a,h)anthracene	0.20	0.47	0.59
	Benzo(g,h,i)perylene	0.60	0.48	0.20
∑5ring PAHs		2.24	2.14	2.47
6ring PAHs	Indeno(1,2,3,cd)pyrene	1.04	1.53	0.20
TOTAL PAHs		**8.86**	**9.02**	**8.61**

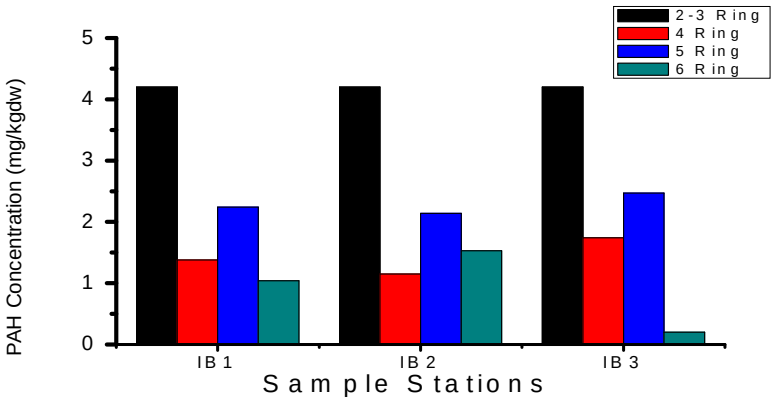

Figure 3: Group profile of sedimentary PAHs normalized by total PAHs in the sediments of Iko River estuary

Bacteriological properties of the estuarine sediment samples

Table 2 shows the distribution and Percentage prevalence of the PAH utilizing bacterial isolates obtained from the various sediment samples. Five bacterial genera were obtained from the continuous enrichment isolation procedure. These include *Pseudomonas aeruginosa, Acetobacter, Bacillus subtilus, Micrococcus leteus* and *Alcaligenes*. Of these, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Micrococcus luteus*, proved to be the best utilizers as there were able to utilize the PAH samples (Naphthalene and Anthracene) and were associated with the three sample stations.

Table 2: Distribution and Percentage prevalence of the bacterial isolates

	IB1		IB2		IB3		
Isolates	Naph	Anth	Naph	Anth	Naph	Anth	% Prevalence
Pseudomonas aeruginosa	√	√	√	√	√	√	100
Acetobacter sp	√	√	-	-	√	√	66.7
Bacillus subtilis	√	√	√	√	√	√	100
Micrococcus leteus	√	√	√	√	√	√	100
Alcaligenes sp	√	-	-	-	√	√	50

Key: √ = Present; - = absent

PAH degradation

Growth profile of the selected PAH degraders on Naphthalene and Anthracene:

The growth profile of the selected best PAH utilizers (*Pseudomonas aeruginosa*, *Bacillus subtilis* and *Micrococcus luteus*) on the 4% concentrations of naphthalene and anthracene respectively is as shown in Figure 4.

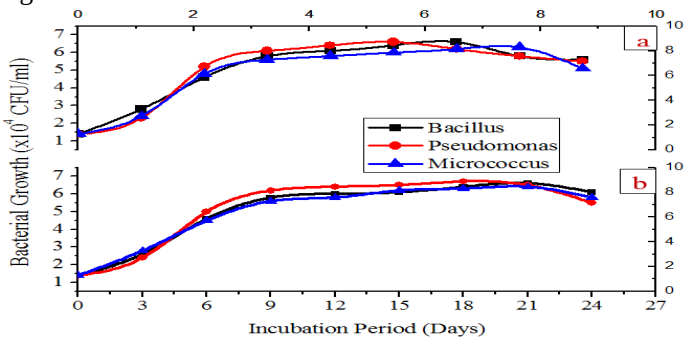

Figure 4: Growth Profile the PAH degraders when cultured on Naphthalene (a) and anthracene (b) supplemented medium

The test isolates had a longer stationary phase when cultured on anthracene supplemented medium than when cultured with naphthalene. Of all, *Pseudomonas* has a shorter stationary phase when cultured on naphthalene, this may be attributed to a faster rate of utilization of the substrate. On the other hand, a slight change in the pH was observed in the various growth medium (Figure 3) within the 24-days degradation course

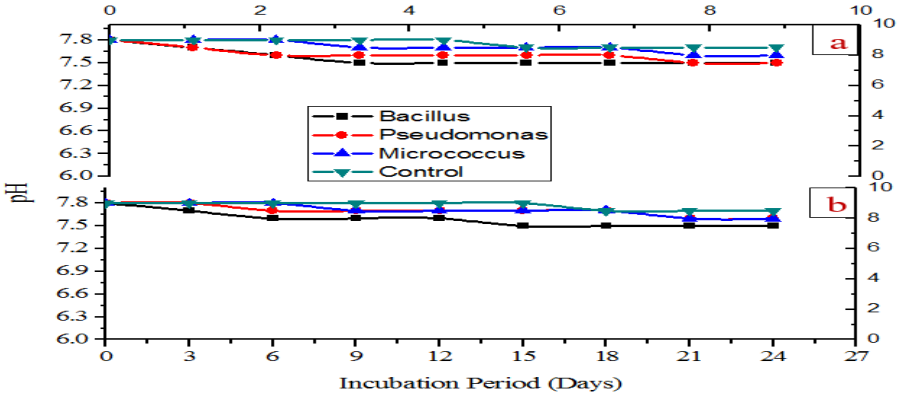

Figure 5: changes in the pH of the bacterial growth medium supplemented with Naphthalene (a) and anthracene (b).

PAH degradability of the Test Isolates:

The various PAH degrading potentials of the test bacterial isolates is as shown on Table 3 and 4. This was determined by comparing the concentration of the residual PAHs in the growth medium with the concentration at the initial and control set up as revealed by the analysis

Table 3: Naphthalene degrading Potentials of the Isolates (mg/l)

Isolate	Initial Concentration	Residual Concentration	Amount degraded (%)
Pseudomonas sp.	1.300	0.000	1.300 (100)
Bacillus subtilis	1.300	0.066	1.234 (95)
Micrococcus sp.	1.300	0.087	1.213 (93)
Control	1.300	1.289	0.011 (0.85)

Table 4: Anthracene degrading Potentials of the Isolates (mg/l)

Isolate	Initial Concentration	Residual Concentration	Amount degraded (%)
Pseudomonas sp.	1.10	0.015	1.085 (98.6)
Bacillus subtilis	1.10	0.076	1.024 (93.1)
Micrococcus sp.	1.10	0.097	1.003 (91.2)
Control	1.10	1.098	0.002 (0.2)

DISCUSSION

Mangrove sediment provides a unique ecological environment for a wide range of microbial ecology. Microorganisms form a good percentage of niches and are of necessity to the survival of their habitats. They are particularly important in controlling the chemical environment of the mangrove ecosystem. The epipellic sediment samples of the Iko River estuary contained detectable level of PAHs. From the compositional study, the 17 target PAHs detected from the sediment samples were divided into groups according to their number of aromatic ring (2–3, 4-, 5- and 6-ring) as presented in Table 2. Based on the grouping, the 2-3 ring PAHs tend to be the most abundant PAHs groups in the sediment. This was followed by the 5-ringed PAHs. The total PAH loads in the three samples stations showed a close similarity and ranged between 8.61 to 9.02 with station two (IB2) having the highest PAH load followed by IB1 and IB3 had the least. The distribution of the PAH groups in the ecosystem is as shown on Figure

3. This finding agrees with Udotong *et al.*, (2008) who in their study suggested that these PAHs are introduced into the estuary by oil spills, sabotage to well heads, disposal of industrial waste and other human activities in addition to persistent gas flaring from oil facilities with horizontal nozzle pointed at the sediment and vegetation.

Studies have shown that lower molecular weight (LMW) PAHs (composed of 2 or 3 fused benzene ring) are easily degraded by bacteria whereas the high molecular weight (HMW) PAHs (consisting of 4 or more benzene rings) are recalcitrant to biodegradation and persist in the environment (John *et al.*, 2012). The present study has revealed the present of five bacterial species (*Pseudomonas* sp, *Acetobacter* sp, *Bacillus subtilis*, *Micrococcus* sp and *Alcaligens* sp) with varying potentials of utilizing PAHs (Table 2). The bacterial strains were identified as 2- and 3- ring PAH utilizers based on the ability to grow and form clear zones on MSM supplemented with Naphthalene and Anthrancene as carbon and energy sources. Of these, *Pseudomonas* sp., *Bacillus subtilis* and *Micrococcus* sp were the most prevalent and were selected for the degradation study.

The potential of the selected bacterial species to degrade 2- and 3- ring (Naphthalene and anthracene respectively) PAH was evident by the increase in their biomass within the 24-day study and well as the significant difference in the amount of residual PAH after the study. The growth indices (biomass and pH) of the test isolates in PAH-supplemented minimal salt medium shown in Figure 4 and 5 respectively revealed that *Pseudomonas aeruginosa* had the highest growth in the sterilized MSM supplemented with the test PAHs. This was followed by *Bacillus subtilis* and *Micrococcus* sp. The high growth rate exhibited by *P. aeruginosa* was not surprising, not because it was isolated from PAH-polluted environment but also because it is known to possess a more competent and active hydrocarbon degrading enzyme than other bio-degraders (Abraham and Essien, 2016; Ekpo and Udofia, 2008). It is established to be fast growing and capable of degrading a wide variety of organic compounds (Ijah and Okang, 1993).

Profiles of the naphthalene and anthracene degrading activities of the test organisms presented in Figure 3. pH of the PAH- supplemented medium was affected by the biodegradation process. Utilization of organic substrate such as naphthalene and anthracene by microorganisms resulted not only to growth but also to concomitant production of acidic metabolic products. The acidic metabolites are responsible for the decrease in the pH of the growth media of the test isolates. In this study, pH reductions from 7.8 to 7.5, 7.6 and 7.8 to 7.7; and 7.6, 7.6 and 7.7 were observed for *B. subtilis*, *Pseudomonas* and *Micrococcus* sp grown on naphthalene and anthracene supplemented medium respectively. Similar results have earlier been reported by Itah and Essien (2005) on the biodegradation of tarballs by hydrcarbonoclastic bacteria isolated from the Bight of Bonny in Nigeria.

Substantial decrease in concentration of the PAHs in the setup discovered after 24 days of exposure (Table 3 and 4) also points to the PAH degrading potentials of the test isolates. A 100% degradation of Naphthalene was observed from *Pseudomonas* sp, this was followed by *Bacillus subtilis* (95%) and *Micrococcus* sp (93%), and similarly, *Pseudomonas* sp had the highest anthracene degrading potentials. *Pseudomonas* sp reduced the concentration of anthracene from 1.10mg/l (initial concentration) to 0.015mg/l (final concentration) showing a 98.6% degradation, while *B. subtilis* and *Micrococcus* sp reduced the same initial concentration to 0.076mg/l and 0.097 mg/l showing a 93.1% and 91.2% degradation respectively. The result of this study agrees with Bamforth and Singleton (2005) who in their review indicated that PAH-degrading microorganisms are ubiquitously distributed in the natural environment, such as in soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi). Many PAH contaminated soils and sediments host active populations of PAH-degrading bacteria. The principal mechanism for the aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form cis-dihydrodiols. These dihydrodiols are dehydrogenated to form dihydroxylated

intermediates, which can then be further metabolised via catechols to carbon dioxide and water.

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. Figure 6 shows the main principle of aerobic degradation of hydrocarbons (Olajire and Essien, 2014) and 94. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen which is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate and pyruvate. Sugars required for various biosyntheses and growth are synthesized by gluconeogenesis (Olajire and Essien, 2014).

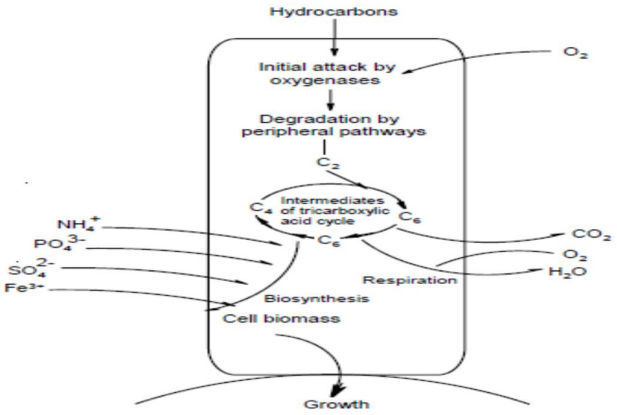

Figure 6: Main principle of aerobic degradation of hydrocarbons by microorganisms
Source: Olajire and Essien (2014) and Essien (2020)

CONCLUSION AND RECOMMENDATION

The high level of LMW PAHs coupled with the isolation of bacteria with PAH utilizing potentials from the mudflat is an indication that tropical mangrove ecosystems have strong capability to recover from hydrocarbon pollution impacts. The recovery of the PAH-contaminated ecosystem could also partly be attributed to transformation, sequestration in sediment, and volatilization of low molecular weight PAHs and could be stimulated by high nutrient availability (bioattenuation) or sediment transport, which may wash out the sediment to the sea. This study has shown that the intertidal sediment of Iko river estuary, despite is contamination with high PAH concentration also harbors hydrocarbon degrading strains of *B. subtilis, Pseudomonas* and *Micrococcus* that are capable to breakdown the pollutants significantly. These bacteria species are recommended for enhanced remediation of crude oil contaminated wetlands in the Niger Delta of Nigeria.

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