Assessment of Biological Decomposition of Organic Pollutants with References to Degradation of Petroleum products by Endophytes of Aquatic Plants

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Abstract

Endophytes are microorganisms in plants with much biotechnological prospects. Some physicochemical characteristics and the ability of endophytes of aquatic plants to degrade petroleum hydrocarbons were studied in plants from six-month old contaminated soil at Oloibiri Oil Field (Ogbia LGA) and twelve-month old contaminated soil at Ikarama (Yenagoa LGA) in Bayelsa State, Niger Delta region, Nigeria. Petroleum degradative abilities of endophytes were performed in Minimal Salt Medium containing 1% crude oil, 1% kerosene and 1% diesel. These were incubated for a period of 7 days at room temperature, the residual total petroleum hydrocarbon extracted and analyzed using gas chromatography equipped with single flame ionization (GC/FID).The results of the physicochemical parameters of the two soils were: pH 5.79±0.12 and 5.52±0.11; conductivity 28.42±2.3 µS/cm and 130.08±7.66 µS/cm; nitrate 2.30±0.09 mg/kg and 0.22±0.02 mg/kg, organic carbon (1.11±0.04 % and 2.02±0.07 %), moisture content 6.34±0.13 % and 34.32±1.22 %), and Total petroleum hydrocarbon (741.08±18.47 mg/kg and 343.25±8.38 mg/kg) respectively. The results of the petroleum degradative abilities of endophytes; *Pseudomonas aeruginosa*, *C. indologenes*, *Pseudomonas putida*, *Bacillus* spp., *Proteus* sp., *Providencia rettgeri* and *Sphaerotilus natans* showed crude oil was degraded better by endophytes of the six-month old site with a significant difference ($p<0.05$) occurring between the petroleum and non-petroleum contaminated sites. Significant differences were not recorded in the degradation by individual endophytes ($p>0.05$). No significant differences ($p>0.05$) occurred after degradation by endophytes of the petroleum contaminated sites. The presence of these petroleum degrading endophytes in aquatic plants could be useful in enhancing microbe-assisted phytoremediation in wetland soils on further investigation

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INTRODUCTION

Oil spills have posed and caused extensive damage to the aquatic environments, both marine and freshwater. Freshwater spills are more deleterious to the environment and in terms of public health even though smaller volumes are involved as compared to marine spills. This is because they often occur within populated areas and may directly contaminate surface water and groundwater supplies (Zhu, 2001).

Remediating wetlands still remains an issue as conventional land reclamation means such as landfarming (ex-situ), bioventing and composting are difficult due to the vast and impenetrable nature of the ecosystem as well as its marshy soils (Murygina *et al*., 2000). Fresh water environments (predominant in Bayelsa State, Nigeria) have low levels of dissolved oxygen, and petroleum biodegradation is faster in the presence of oxygen. This anoxic nature of wetlands renders soil cleanup operations in a freshwater wetland environment to be difficult and complex (Purandare, 1999)

Wetlands support hydrophytes which could be vital in oil bioremediation and wetland restoration (Venosa and Zhu, 2005). Aquatic plants, growing in wetlands, are used for food, water quality assessment and as *in-situ* biomonitors and bioremediators (Wersal and Madsen, 2012). They remove suspended solids, nutrients, heavy metals, toxic organics from acid mine drainage, agricultural landfill and urban storm water runoff (Galeotti and McCullough 2008; Prasad, 2008; Forbes *et al*., 2009). Aquatic plants accumulate many pollutants and toxic substances efficiently due to their simple growth requirements and fast growth rates (Shafi *et al*., 2015).Their importance as tools for plant-microbe interaction studies in phytoremediation are recommended since they are cost-effective, readily available and are able to survive adverse conditions (Prasad, 2008).

Endophytes (bacteria and fungi found in plant tissues: roots, stems and leaves) are regarded as harmless occupants which live in mutualism with their host plant, as they enhance plant growth and development, improve plant health and yield, control pathogens, pollutants and plant resistance to stress (Cohen, 2004; Rosenblueth and Martinez-Romero, 2006). The interaction between plants and micro-organisms is important as they may enhance the efficiency of phytoremediation (Weyens, 2009; Amora-Lazcano, 2010). Many endophytes have shown capabilities of degrading petroleum hydrocarbons (Afzal *et al*., 2014; Kukla *et al*., 2014; Lamactud and Fulthorpe, 2018). Using genes from endophytes to enhance remediation in genetically engineered plants is currently being explored (Barac *et al*., 2004; Taghavi *et al*., 2005; Germaine *et al*., 2006; Weyens *et al*., 2009; Afzal *et al*., 2012, 2013).

Research on bioremediation is usually concentrated on plants or bacteria of the rhizosphere (Ryan *et al*.2008; McGuinness and Dowling 2009; Weyens, 2009). Bioremediation of aquatic environments by endophytes from aquatic plants have been less studied hence, this study was carried out to establish and compare the ability of endophytes of aquatic plants from different locations of Bayelsa State, Nigeria, to degrade petroleum products.

MATERIALS AND METHODS

Study Sites

Bayelsa State is situated in the oil-rich Niger Delta Area of Nigeria. It lies in the tropical rainforest belt. Aquatic plants and soils used for the studies were picked from three freshwater soil locations, processed for the endophytes (Pondei *et al*., 2018) and the isolates stored at -80^{0}C until required.

Location one is six-month old petroleum contaminated soil at Oloibiri Oil field (Otuabagi), Ogbia LGA (where oil was first discovered on Sunday 15th January, 1956). It lies at Latitude 4.695°N Longitude 6.35043°E. Oloibiri oil field is made up of 22 Oil Wells, with eighteen owned by Otuabagi community and four by Oloibiri community (Awelewa, 2016). Oil pollution occurred in the community in time past with the oil wells abandoned and no longer in use today, but spills still occur. Aquatic plants and soils used for this study were picked six months after the spill occurred in November, 2013. These were processed for the endophytes (Pondei *et al*., 2018) and the isolates stored at -80^{0}C.

Location two is a twelve-month old petroleum contaminated soil at Ikarama, Yenagoa LGA; Latitude 5.14931°N Longitude 6.45287°E. Nigeria's Agip Oil Company and Shell Petroleum Development Company (SPDC) are situated in Ikarama where incessant oil spills from equipment failure have continued to be the norm (MOEN, 2008; Fatoba *et al*., 2015). Between 18 December 1991 and 23 August 2008, a total of 421 crude oil spills was reported in Bayelsa State, with Ikarama community recording 9 major oil spills (MOEN, 2008; Fatoba *et al.,* 2015). Crude oil spills also occurred in this location in December 2008 and June 2009 (Fatoba *et al*., 2015), and more recently in November, 2013 where the plant and soil samples were collected a year after the spill.

Location three is a non-petroleum contaminated site located at Federal University Otuoke, Ogbia LGA, Latitude 4.8019°N Longitude 6.3189°E. No oil spills or prospecting activities have been recorded in this location but oil slicks are noted on surfaces of standing water.

Characterization of Soil Samples Obtained

Soil samples from the three sites were collected and analysed for their particle sizes (sand, silt and clay) using American Society for Testing Materials (ASTM) D422 and D2487 and physicochemical parameters; pH and electrical conductivity using electronic pH/ conductivity meters (APHA, 1998). Moisture content by simple gravimetric analysis (Eaton *et al.*, 1995), the nitrate content of the sediment was determined using the method of Greweling and Peech (1965), while Bray No. 1 method (Bray and Kurtz, 1945) was used to determine the phosphate content. Total organic carbon was obtained using the Walkley-Black method (1934). The residual oil of soil was extracted using the method of Eaton *et al*. (1995) and the Total Petroleum Hydrocarbon (TPH) analyzed using gas chromatography equipped with single flame ionization detector (GC/FID).

Crude oil, Kerosene and Diesel

Fresh un-weathered Bonny Light Crude (BLC) oil (specific gravity 0.8343) was obtained from Shell Petroleum Development Company (SPDC), Port Harcourt, Nigeria while diesel and kerosene were obtained from Nigerian National Petroleum Corporation, NNPC (commercial marketers) in Yenagoa, Nigeria. Crude oil and diesel were sterilized in the oven at 160^{0}C for 1 hour while kerosene was used directly.

Preliminary Assay on Degradation of Petroleum

To perform and compare microbial degradation of petroleum and some of its fractions on the endophytes, the methods of Wongsa *et al*. (2004) and Hong *et al*. (2005) were adopted with slight modifications. The endophytes were precultured in 10ml LB medium for 18-24 hours at 25°C, diluted serially and then 100µl of dilution 10^{-5}(which was counted) inoculated onto 30-ml screw-capped bottles (to prevent loss of volatile hydrocarbon components) containing 9.9 ml of the minimal salt medium of Okpokwasili and Okorie (1988) made up of $MgSO_4.7H_2O$, 0.42 g/l; KH_2PO_4, 0.83g/l; Na_2HPO_4, 1.25 g/l; KCl, 0.29 g/l; and NH_4NO_3, 0.42 g/l. 100µl (1% v/v) of one of the petroleum products: sterilized Bonny Light crude oil, sterilized diesel and non-sterilized kerosene was added. Cells were incubated for 7 days at 25°C ± 2^{0}C with daily agitation. Petroleum hydrocarbons remaining in the culture medium was determined by Gas Chromatography with flame ion detector (GC/FID). Cultures without bacterial inoculation in sterilized crude oil, sterilized diesel and kerosene were used as controls.

Statistical Analyses

All statistical calculations involving mean values, standard deviations, were determined using Microsoft excel (version 2013) and SPSS (version 20.0). The student t-test for comparison of environmental indices was determined using Microsoft excel version 2010. Analysis of variance (ANOVA) and post-hoc analysis of sources of variations was determined using Duncan's multiple range (DMR); SPSS version 20.0.

RESULTS

Physicochemical Characteristics of the Soils

The physicochemical characteristics of the soils are presented in Table 2. The H^+ ion concentration of the soils were acidic but lowest in Site C (4.97±0.10) while the nitrate and phosphate contents, and conductivity were highest in Site C (7.62±0.28mg/kg, 2.89±0.16mg/kg and 723.51±48.58 µS/cm respectively). The percentage organic carbon content of the soils was between 0.92±0.03 to 2.02±0.07, being more in the petroleum contaminated soils. Expectedly, the total petroleum hydrocarbon (TPH) recorded the lowest average value of 19.98±1.00mg/kg from uncontaminated soil sample from location C (SBC) and highest from petroleum contaminated soil sample from location A (SAC) with 741.08±18.47mg/kg.

Endophytes of the Aquatic Plants

The endophytes and plant species of isolation are presented in Table 1. *P.aeruginosa*, *C. indologenes Pseudomonas*/*P. putida*, *Bacillus* sp. and *P.mirabilis* were common to all the sites. Seven endophytes (*P. rettgeri, P. aeruginosa, C. indologenes Pseudomonas*/*Ps. putida*, *Bacillus* sp., *S. natans* and *P. mirabilis*) were common in the petroleum contaminated sites (Site A and B).

Petroleum degradation by some of the endophytes

The TPH profile of the degradation of crude oil by *P.aeruginosa* in Site A shows the absence of *n*-alkanes C_8 and C_9, low peaks of C_{10}-C_{23}, higher peaks of C_{24}-C_{26}, and some amounts of C_{33}-C_{36} compared to the control. In Site B, high peak of C_{17} was noted while Site C had higher peaks of the markers pristane and phytane which questions the true ability of the organism to degrade crude oil at site C. For kerosene, site A showed almost no carbon peaks (C_{10}-C_{16}). The peaks were higher in site B and lower in site C, indicating that less of the product was degraded by the microorganism in site B. *P. aeruginosa* from all three sites degraded diesel resulting to low peaks.

The endophyte *P. rettgeri* of the petroleum contaminated site A degraded crude oil but with slightly high peaks of C_{20}-C_{22} (especially of C_{21})

indicating the low preference of this group of n-alkanes, and with high peak of the marker, pristane in site B. *S. natans* of site A degraded crude oil with low peaks of pristane and phytane while C_{25}-C_{40} were completely absent, and low peaks of other C-compounds after degradation with the organism from site B (Figure 4). This suggests that *S. natans* of site B could be a better crude oil degrader than that of site A. Kerosene degradation recorded lowest C_8-C_{18} peaks with *P. rettgeri* of site A and the highest with the same organism in site B. Low carbon peaks occurred for diesel degradation in *S. natans* of site B and *P. rettgeri* of site A with the highest.

Comparisons of the degradative potentials of various endophytic bacterial species obtained from the various sites

Comparison of the residual cumulative TPH after crude oil degradation by isolated endophytes showed significant difference ($P<0.05$) in the mean value obtained from site C when compared to the other two sites. There was, however, no statistical significance in the difference of mean values by the common endophytes (Table 3). The mean values of kerosene and diesel degradation by endophytic bacteria showed no statistically significant difference ($P>0.05$) from the activities of the endophytes in the three sites and in the individual culturable endophytic bacteria. The student's t-test comparison of site A and Site B showed no statistically significant difference ($P>0.05$) from the effect of endophytes on the degradation of crude oil. The analysis of variance (ANOVA) also showed no significant difference ($P>0.05$) from the TPH values obtained from the contribution of each common endophytes. Similarly, student's t-test comparison showed no statistical significance ($P>0.05$) in the values of TPH obtained after degradation of kerosene and diesel (Table 4). The analysis of variance (ANOVA) from the values of TPH from individual endophytes in the petroleum contaminated sites (location A and B) had no statistically significant ($P>0.05$) difference.

DISCUSSION

Oil pollution is known to impact negatively on agricultural soils and crops. The hydrogen ion concentration (pH) of all the soils were acidic with the petroleum contaminated soils being slightly less acidic. The acidic nature of soils in the South-South region of Nigeria has been attributed to precipitation and leaching of ions from the soils due to heavy rainfalls or acid rains associated with the region (Fatoba *et al*., 2015; Yakubu, 2017). The slight increase in pH in the petroleum contaminated soils may be attributed to the degradative activities of microorganisms in the contaminated soils.

The organic carbon contents were higher in the petroleum contaminated soils which could be as a result of the introduction of the crude oil in these sites. The phosphate and nitrate concentrations of the petroleum contaminated soils were much lower when compared to the control. This could be due to the long term effect of frequent oil spills and leaching in the region which led to nutrient depletion in the soils. The electrical conductivity of the control soil was also higher compared to the other soils. This further indicates the presence of more nutrients/solutes in the uncontaminated control soil with no history of oil pollution.

The Total Petroleum Hydrocarbon (TPH) of the twelve month-old petroleum contaminated soil was expectedly lower than that of the six month-old petroleum contaminated soil as remediation measures as well as microbial degradation were already in place. However, the hydrocarbon content was still above the minimum value recommended after remediation. Moreso, the location of the spill was less than 50 metres away from residential houses, which placed the inhabitants at high risk as they get their water for household activities by boring the soil around them. Freshwater spills are usually not large as compared to marine spills, but are considered more dangerous to health as they contaminate underground and surface water in populated areas (Zhu, 2001).

Petroleum and its fractions are made up of a vast array of hydrocarbons of various molecular compositions, structures and chain lengths. Microorganisms are able to breakdown some of these hydrocarbons and utilize for their growth and survival and gas chromatography is one of the ways used to determine the residual TPH after degradation. The chromatographic profiles showing the measured *n*-alkane levels/peaks of the various hydrocarbons revealed their utilization by the endophytes. In crude oil degradation, *P. aeruginosa* of the six month-old contaminated site had little peaks of *n*-alkane C_{24}-C_{26} left and in the twelve month-old site, C_{17} was high but all with very low levels of the biodegradation markers, pristane (C_{17+}) and phytane (C_{18+}) resulting to a higher residual TPH value. *P. aeruginosa* of the six month-old site can be said to be a better crude oil degrader than that of the twelve month-old site. Both organisms from the petroleum contaminated sites were better degraders than the organism from the uncontaminated site which recorded higher peaks of n-alkanes, including the biomarkers, and residual TPH. In kerosene degradation the microorganism from the six month-old petroleum contaminated site was best, followed by that from the uncontaminated site. Profiles for diesel degradation were similar, but generally, the Organisms from the uncontaminated site did better.

In crude oil utilization by the other microorganisms of the petroleum contaminated sites, *P. rettgeri* had low TPH and peaks except for C_{20}-C_{22} in site A and pristane in site B. *Sphaerotilus natans* of site B degraded completely longer *n*-alkanes between C_{23}-C_{40}, with very little amounts of the lower *n*-alkanes making them better utilisers of the hydrocarbon. All the organisms degraded kerosene with low peaks but *P. rettgeri* of site A was better while *S. natans* of the twelve month-old site was a better diesel degrader. The occurrence of *P. rettgeri* as both endophytes of only the petroleum contaminated sites is noteworthy. *S. natans* is a filamentous sheathed bacterium of shallow natural freshwater and activated sludge which utilizes numerous compounds for growth and obtains additional energy from the oxidation of iron compounds. The organism can be used as a biosorbent for inorganic pollutants (Gridneva *et al*., 2011). Soils in these study regions contained a lot of iron, especially in the petroleum contaminated sites (unpublished report), which may be the reason for its presence in these sites.

Endophytes of the uncontaminated site degraded the least crude oil compared to the petroleum contaminated sites, which supports the report that prior exposure enables petroleum degrading microorganisms to degrade the hydrocarbons more rapidly. Nonetheless, several studies have indicated endophyte degradation of contaminants without prior exposure (Afzal *et al*., 2014). Statistical analysis showed there were significant differences in crude oil degradation between the petroleum contaminated sites and the uncontaminated site ($p > 0.05$) and no significant difference between the endophytes. Endophytes from the six month-old petroleum contaminated were better crude oil degraders and could have metabolized the simpler alkanes more

preferably because they were readily available and more soluble in water (Macaulay, 2015).

Kerosene is made up of *n*-alkanes, iso-alkanes, cycloalkanes and some aromatic groups in the hydrocarbon range C_8 to $C_{18.}$ Endophytes of the twelve-month site recorded better kerosene degraders than other sites, probably because it is an aged site with well adapted indigenous microbial consortium. The control site was better than the six-month old site probably because of the natural presence of petroleum degraders which used up the shorter chain length alkanes, since they are easier to degrade. Moreso, the endophytes of the six-month old site were probably better adapted to growth in hydrocarbons of varying chain lengths. Endophytes of the Gammaproteobacteria among others have been reported to utilise kerosene, octanol, toluene, naphthalene, or motor oil as sole carbon sources (Lamactud *et al*., 2016). Oboh *et al.* (2006) concluded from their experiments on the utilization of crude oil, kerosene and diesel that bacteria slightly preferred kerosene for growth which was in contrast to the report of Okpokwasili and James (1995). However, comparisons in this study were based on location of the endophytes, and no significant difference occurred in kerosene and diesel utilization with location and the individual endophytes, but in crude oil utilization.

Diesel was degraded almost equally by the endophytes irrespective of the age and condition of site, when the average is considered. Diesel is made up of alkanes in the range C_8-C_{26}, and high amounts of environmentally persistent aromatics, PAHs sulfur, resins and asphaltenes among others. The susceptibility to diesel attack by microorganisms is attributed to its chemical composition of between C_{10} and C_{25} (Atlas and Bartha, 1993) which results to it being easily degraded. de Oliviera *et al.* (2012) reported the degradative ability of endobacteria *Bacillus cereus, Staphylococcus pasteuri* and *Pseudomonas* sp. in petroleum, diesel and gasoline

Although kerosene and diesel fuel are volatile and easily biodegradable they can persist in terrestrial environments when attached to or are buried in oxygen depleted environments such as sediments, soils, groundwater, or marshes (Macaulay, 2015). Diesel degradation by the endophytes seemed to have been easily accomplished because very low profile peaks were obtained (figure not shown for all organisms). However, compared to crude oil and kerosene, the residual TPH amounts were higher in all the sites which could mean that it was the least preferred of the three products.

A wider range of petroleum hydrocarbons is degraded better by the combined or synergistic interaction of microbial species, while single species can only degrade a limited range (Kuiper *et al*., 2004; Barin *et al*., 2014). Using these endophytes in combination will increase the efficiency of phytoremediation. Problems associated with phytoremediation of hydrocarbons in wetlands such as its slow rate of decontamination due to low oxygen levels, accumulation of toxic compounds and release to environment as well as its inhibitory effect to plant growth (Segura *et al*., 2009) can be addressed by using the endobacteria specific for the wetlands.

CONCLUSION

Endophytes are important members in plant-microbe associations in the remediation of environmental pollutants. A lot of studies have indicated the success of plant-rhizosphere partnership in the phytoremediation of crude oil, diesel and other petroleum hydrocarbons but few studies have been done on endophytes of aquatic plants. These endophytes from the aquatic plants studied are potential sources of a vast range of petroleum degradative enzymes/genes since they were all able to utilize crude oil, kerosene and diesel as their sole source of carbon. Using these bacterial species in combination will even enhance their degradative ability. The efficiency of phytodegradation of organic pollutants in wetlands can be enhanced by inoculating the aquatic plants with genetically modified endobacteria which is an area for further studies.

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Table 1: Endophytes of the aquatic plants and their sites of occurrence

Endophytes	**AQUATIC PLANTS**		
	SITE A	**SITE B**	**SITE C**
C.indologenes	*Ageratum conyzoides*	*Cyperus difformis*	*Diplazium sammatii*
P. aeruginosa	*Commelina benghalensis*	*Ageratum conyzoides;*	*Diplazium sammatii*
P. putida	*Chromoleana odorata*	*Chromoleana odorata*	*Panicum laxum*
P. mirabilis	*Kyllinga erecta*	*Solenostemon monostachyus*	*Dissotis rotundifolia*
Bacillus	*Aspilia Africana*	*Chromoleana odorata*	*Dissotis rotundifolia*
P. rettgeri	*Chromoleana odorata*	*Sacciolepsis Africana*	-
S. natans	*Melastomastrum capitatum*	*Kyllinga pumila*	-

Keys; Site A, six month-old petroleum contaminated site; Site B, twelve month-old petroleum contaminated site; Site C, uncontaminated site.

Table 2: Physicochemical characteristics of soil samples obtained

Parameter(s)	SAC	SBC	SC
pH	5.79±0.12	5.52±0.11	4.97±0.10
Nitrate (mg/kg)	2.30±0.09	0.22±0.02	7.62±0.28
Organic Carbon (%)	1.11±0.04	2.02±0.07	0.92±0.03
Moisture Content (%)	6.34±0.13	34.32±1.22	22.69±1.38
Conductivity (µS/cm)	28.42±2.13	130.08±7.66	723.51±48.58
TPH (mg/kg)	741.08±18.47	343.25±8.38	19.98±1.00

Keys: SAC, Soil A, Contaminated; SBC, Soil B Contaminated; SC, Soil C Non-contaminated control.

Table 3: Residual cumulative TPH after degradation of petroleum by endophytes of the petroleum contaminated and uncontaminated sites

Sites	Total Petroleum Hydrocarbon, TPH (mg/L)		
	Crude oil	Kerosene	Diesel
Control	2.02224^{e}5	3.74399^{e}5	2.01026^{e}5
Site A	237.40 ± 176.94^{a}	770.40 ± 564.00	1229.80 ± 369.76
Site B	363.00 ± 209.93^{a}	427.40 ± 223.52	1429.80 ± 663.99
Site C	1634.00 ± 595.67^{b}	701.60 ± 723.05	1477.80 ± 771.40

*Values with different superscript are significantly different at P<0.05 using the Duncan's Multiple Range in post-hoc Analysis of Variance of comparison of mean values
Keys: Site A, six-month old petroleum contaminated site; Site B, twelve-month old petroleum contaminated site; Site C, uncontaminated site (Control)

Table 4: Residual cumulative TPH after degradation of crude oil, kerosene and diesel by endophytes of the petroleum contaminated sites

Site	Total Petroleum Hydrocarbon, TPH (mg/L)		
	Crude oil	Kerosene	Diesel
Control	2.02224^{e}5	3.74399^{e}5	2.01026^{e}5
Site A	205.29 ± 154.54	663.57 ± 495.66	1270.14 ± 452.72
Site B	339.00 ± 190.45	417.00 ± 184.81	1288.86 ± 779.76

*There is no statistical significant difference (P>0.05) in the above distribution
Key: Site A, six-month old petroleum contaminated site; Site B, twelve-month old petroleum contaminated site

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