Received: 18 March 2023 | Revised: 25 November 2023 | Accepted: 27 November 2023

Research Article
Journal of Research and Review in Science
13-26, Volume 10, December 2023
DOI: 10.36108/jrrslasu/3202.01.0160

ORIGINAL RESEARCH

Degradation of crude oil by the microbial populations of Lagos and Ologe Lagoon waters

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Abstract:

Introduction: hydrocarbon contamination of surface waters as a result of anthropogenic activity poses threat to ecosystems and counter their beneficial uses. Some indigenous microbial communities have the potential to purify such waters unaided.

Aims: To show the biodegradative potential of microbial communities in Lagos and Ologe Lagoons during minimal pollution with crude oil.

Materials and Methods: The total heterotrophic bacteria and hydrocarbon utilising bacterial and fungal populations were estimated from Lagos and Ologe lagoon water samples contaminated with 1% crude oil over 42-day incubation period by plate count and vapour-phase transfer techniques. Residual hydrocarbons were determined by Gas chromatography.

Results: The predominant bacterial genera identified from the lagoons include *Enterobacter, Klebsiella* and *Proteus,* while *Escherichia,* and *Morganella*. *Aspergillus* and *Mucor* were the predominant fungal genera in both waters. The hydrocarbon degradation rate in the Lagos Island microcosm was 65.391±0.370 mg/l/d, degradation rate constant 0.05±0.01 /d, half-life 9.559±0.093 /d and percentage degradation of 95.315 ± 0.134. Corresponding values in the Ologe water were 61.190± 8.542 mg/l/d, 8.725 ±0.389 /d, 0.055±0.003/d and 96.345±0.488 respectively. There was almost complete disappearance of the various fractions of the oil in the two samples. The microbial communities from both lagoons effectively utilised majority of the hydrocarbon fractions after 42 days where 66.890±1.075 and 100±000 were recorded for benzene, toluene had 100±000 percent degradation, anthracene 96.755±0.119 and 99.726±0.026, and pristane had 91.674±0.222 and 99.943±0.015 while phytane had 96.44±0.058 and 99.670±0.104 respectively.

Conclusion: Efficient biodegradation of moderate contamination crude oil could be achieved by indigenous microbial flora present in Lagos and Ologe lagoon waters.

Keywords: Biodegradation; Gas Chromatography; Hydrocarbons; Lagoon; Microcosms

All co-authors agreed to have their names listed as authors.

1. INTRODUCTION

Crude oil is a complex mixture consisting of hundreds of different hydrocarbon molecules, primarily composed of aliphatic and aromatic hydrocarbons, along with nitrogen, sulphur and oxygen containing compounds called asphaltenes and resins [1]. The importance of petroleum products in global economy has resulted in continuous distribution of large amounts of crude oil all over the world, which has led to pollution of the environment. Generally, crude oil gains access into the environment through leak of oil tankers, cleaning of tankers by merchants, leaks during exploration, refining, storage and transportation [2]. There are also small-scale releases, which at times may pass unnoticed, particularly in developing countries like Nigeria [3]. The sources of spills are multifarious, the consequences for human health and environment are dire, encompassing human, animal and plant toxicity, shift in community diversity, rapid depletion of inorganic nutrients, carbon-nitrogen imbalance and persistent oxygen anomaly, and in the short or long term unsavoury socioeconomic outcomes [4,5,6]. Spills on land often affect limited area, as there is more of vertical movement than horizontal, making way for delimitation of specific area for remediation purpose, which may be in- situ or ex-situ in terms of strategy. However, at sea and in other large water bodies like lagoons and lakes, horizontal spreading potentiated by the low density of oil and water waves expands the sphere of concern beyond the immediate precincts of the spill and onto shorelines and beaches [7]. According to Ifabiyi [8], freshwaters have capacity for self-restoration through the processes called self-purification. The processes of self-purification may be in the form of dilution of polluted water with influx of surface or ground water or through hydrologic or biological processes including coagulation, volatilization, sedimentation and precipitation of colloids, material exchange, and photosynthesis [9]. Water self-purification process may also occur through biological uptake of pollutants. Factors such as temperature, water current, amount of inorganic substance in water, presence of aquatic weeds may determine the self-purification capability of freshwater. Also, within the oxygen-driven zone self-purification is reported to take place [10]. Within this zone, bacteria utilise wide range of organic compound such as humic substances probably as electron acceptor donor for anaerobic respiration and in turn could lead to release of nutrients from the sediment under hypoxic condition [11]. Microorganisms are important producers and decomposers and play a chief role in maintaining fresh water ecosystem health [12]. Many abiotic and biotic factors interplay to eliminate hydrocarbons from aquatic environments. The most important among these factors is biodegradation by microorganisms, particularly autochthonous populations [13].

Several oil degrading microflora have been reported to play important roles in the biodegradation of oil polluted sites. These include species in the genera *Achromobacter, Bacillus, Enterobacter, Escherichia, Gordona, Mycobacterium, Pseudomonas, Rhodococcus, Serratia* and *Staphylococcus* [3, 13, 14 15, 16]. Bioremediation procedures involving the exploitation of degradative ability of microorganism, via bioaugmentation, nutrient application and alteration of environmental factors, has become a veritable approach to clean up of aquatic environments and marine ecosystems [17, 18, 19, 20, 21].

The increased human pressures on the environment have resulted in biological consequences in Lagos coastal waters, especially the Lagos Lagoon and its drainage path to sea. The Lagos Lagoon is a wide expanse of estuarine water extending from Lagos harbour to the Niger Delta in South-West Nigeria. It is highly polluted at the harbour by seepages from the oil discharge terminals and also receives an unquantifiable amount of spent lubricating oils from the adjoining drainage systems [22,23]. On the other hand, the Ologe Lagoon, which is an inland lagoon, is a major source of water to Badagry creek, which opens into the Atlantic Ocean via the Lagos Harbour [24,25]. It is a major receptacle of treated and untreated wastes from the industrial estates around it. The fate of petroleum pollutants in environmental waters is predictable through environmental modelling and laboratory simulative microbiological degradative experiments. Such study could be observation under relevant natural conditions or nutrient-based assessment [26,27]. Thus, this study was aimed at providing information on biodegradative capacities of microbial communities of Lagos and Ologe lagoons under condition of small-scale pollution with crude oil.

2. MATERIALS AND METHODS

Description of sampling sites

Water samples were collected from two different waterbodies in Lagos State, namely Lagos Island and Ologe lagoons. The coordinates of the sampling sites were: Lagos Island lagoon - 6.46443 ºN and 3.8765 ºE and Ologe lagoon - 6º27'N 3º01'E and 6º30'N 3º07'E respectively. Ologe lagoon is a large expanse of water body located in Lagos, Nigeria. It drains rivers such as Owo and Ore.

Sample collection

Water samples (1 liter) were collected from three different sampling points using sterile sample bottle and pooled together in a sterile 3 L sample bottle for each of the lagoons. The samples were transported to the laboratory for microbiological and physicochemical analysis.

Physicochemical analysis of Lagoon waters

The temperature of the water sample was determined on- site using thermometer (ADWA- 121), pH was determined using pH meter (Jenway 3051, Staffordshire, England), the biological oxygen demand, sulphate, nitrate and total nitrogen were determined as described by Chopra and Kanwar [28]. The heavy metal contents were estimated using atomic absorption spectrophotometer (AAS) (Perkin Elmer A Analyst 200 using air acetylene flame) [29].

Microcosm study

Replicate flasks containing 50 ml of water samples and 0.5 milliliter of crude oil (Escravos Light) were prepared. The flasks were plugged with cotton wool and placed in a shaker at 120 rpm at room temperature (28 ±2^{O}C) and changes in microbial populations monitored for 42 days. At an interval of 7 days, flasks were randomly removed from the lot to determine the total heterotrophic bacteria and fungi counts and the total hydrocarbon utilisers.

Enumeration and characterization of water microflora

The total heterotrophic bacteria (THB) and total heterotrophic fungi (THF) counts were enumerated using spread plate technique. These was carried out by plating 0.1 ml aliquot of appropriate dilutions of the water samples on nutrient agar and potato dextrose agar fortified with Nystatin (50 µg/ml) and Streptomycin (1 mg/100 ml) respectively. All plates were incubated aerobically at room temperature (28 ±2°C) and counted after 24 h and 48 h for bacteria and fungi respectively. In the same way, the populations of hydrocarbon utilisers were estimated on mineral salts medium (MSM) described by Habe *et al.* [30]. The medium contained (in g/L) K_2HPO_4 (0.38 g), KH_2PO_4 (0.6 g), $MgSO_4.7H_2O$ (0.2g), NH_4Cl (1 g), $FeCl_3$ (0.05 g) and agar (20 g).

Sterile trace elements solution (1.0 ml/l) described by Bauchop and Elsden [31] was aseptically added to the medium after sterilization. The pH of the medium was adjusted to 7.2 and 5.6 respectively for bacterial and fungal estimations. The MSM was also fortified with nystatin (50 µg/ml) for total hydrocarbon utilising bacteria (THUB) and 10 µg/ml of streptomycin for total hydrocarbon utilising fungi (THUF). Sterile crude petroleum served as the sole carbon and energy source and was made available to the cultures through vapour-phase transfer [32]. All plates were incubated at room temperature and the colonies were counted after 5 – 7 days. The percentage of hydrocarbon-utilisers relative to the heterotrophic population for each time point was subsequently determined.

Identification and characterization of isolates

Predominant bacterial isolates in the microcosms were characterized and identified based on their colonial, morphological and biochemical characteristics according to the taxonomic schemes of Cowan and Steel [33]. Identification was complemented by phenotypic typing using Microbact 12 E rapid test kit according to the manufacturer's specifications (Oxoid Limited, Basingstoke, England). Purified dominant fungal isolates

were identified using their microscopic, cultural and morphological characteristics as described by James and Natalie [34].

Gas chromatographic analysis of residual hydrocarbons

Extraction of residual oil

Residual oil in the microcosm setup before (Day 0) and after (Day 42) were analysed as described by Adebusoye *et al.* [35]. The degradation rate constant was determined by fitting the residual oil data to the kinetics model [36].

$y = ae^{-kt}$ where *y* is the residual crude oil in culture (mg/l), *a* is the initial crude oil in culture (mg/l), k is the degradation constant (/day), and t is the time (day). Half- life (t1/2) was then calculated as:

$$Half - life = \frac{In(2)}{k}$$

3. RESULTS

Physicochemical characteristics of Lagoon water

The physicochemical characteristics of Lagos and Ologe lagoons are shown in Table 1. The biological oxygen demands (BOD) of the samples were 14.00 and 16.00 ppm, while the total organic carbons (TOC) were 0.04 and 0.07 ppm. Five heavy metals; nickel (Ni), zinc (Zn), chromium (Cr), cadmium (Cd) and Iron (Fe) were investigated, Cd was not detected in the samples, whereas Ni also not detected at Lagos Island lagoon was detected in a low concentration in Ologe lagoon. The concentration of iron was 3.71 and 2.99 ppm from Lagos Island and Ologe waterbody. Sulphate concentration in the samples were 12.00 and 9.00 ppm, well below the FEPA limit of 500 ppm. The two waters were however rich in nitrate and phosphate, with phosphate levels of 15 and 11 ppm respectively, which are far higher than the permissible levels of 5 ppm permissible limits of FEPA.

Hydrocarbon degrading communities of microcosm

The predominant hydrocarbon degrading members of the microcosm from Lagos lagoon were *Enterobacter cloaca*, *Klebsiella pneumoniae* (2 strains) and *Proteus mirabilis,* while *Escherichia coli*, *Morganella morganii*, *Enterobacter aerogenes* and *Klebsiella oxytoca* the dominant hydrocarbon degraders from Ologe microcosm. Hydrocarbon degrading fungal isolates from both microcosms were predominantly of the genera *Aspergillus* and *Mucor*.

Microbial population dynamics of the microcosm

The changes in population densities of bacteria and fungi in the two microcosms over 42-day incubation period are shown in Figure 1. From Lagos Island microcosm, the total heterotrophic bacterial population (THB) increased from an initial value of 4.50 $x10^6$ to 3.95 $x10^7$ cfu/ml at day 35 and dropped to 2.65 $x10^7$ cfu/ml at the end of the incubation period. Equally, total hydrocarbon utilising bacteria (THUB) had initial population density of 1.76 x 10^6 cfu/ml, increased steadily to 3.32 $x10^7$ at day 21, and final population growth value of 1.21 $x10^7$ at day 42. The population of total hydrocarbon utilising fungi (THUF) followed the same trend as THF. In Lagos Island lagoon, the population density was 1.50 x 10^3 and dropped sharply to $1x10^1$ cfu/ml at the end of 42 d of incubation. Ologe microcosm had the total heterotrophic bacteria population of 4.25 x 10^6 cfu/ml at day 0, followed by steady increase till day 35 before a declined to 1. 86 x 10^7 cfu/ml at the end of the 42 days incubation period. The total hydrocarbon-utilising bacteria (THUB) from Ologe microcosm, cell increase was observed from initial value of 1.84 x 10^6 to 3.42 x 10^7 at day 14, then 2.56 $x10^7$at day 21 then 2.72 $x10^7$, 4.86 x 10^7 at day 35 and 42 respectively. The total hydrocarbon-

utilising fungi (THUF) from Ologe Lagoon microcosm had initial value of 6 x 10^3 and decreased steadily to 1x10^2 cfu/ml at the end of the 42-day incubation period.

Table 1. Physicochemical parameters of water samples

Parameters (mg/L)	Lagos Island	Ologe	FEPA/National environment protection (effluent limitation) regulation
BOD	14.00	16.00	80
Hardness	ND	ND	NA
Zn	0.10	0.120	< 1
K	0.09	1.230	NA
Na	2.61	2.00	NA
Fe	3.71	2.99	20
Ni	ND	0.01	< 0.05
Cr	0.01	0.03	<1
Cd	ND	ND	<1
SO_4^{2-}	12.00	9.00	500
NO_3^-	15.00	13.00	20
PO4-	15.10	11.00	5
N	4.00	5.00	NA
NO_2-	ND	ND	20
TOC	0.04	0.07	NA

Key: ND not detected NA not available

Kinetics of petroleum hydrocarbon degradation

The kinetics of degradation of crude oil by the lagoon microcosms are shown in Table 2. The results showed that the microbial communities in the two water samples utilised the crude oil effectively as source of carbon and energy. At the end of the 42-day incubation period, Lagos Island microcosm had degradation rate of 65.391±0.370 mg/l/d, degradation rate constant of 0.050±0.001 /d, degradation half-life of 9.559±0.093/d and overall percentage degradation of 95.315±0.134%. Ologe water microbial communities had overall rate of degradation of 61.190±8.542 (mg/l/d), degradation rate constant of 0.055±0.003 (/d), degradation half-life of 8.725±0.389, and overall percentage degradation of 96.345± 0.488 %.

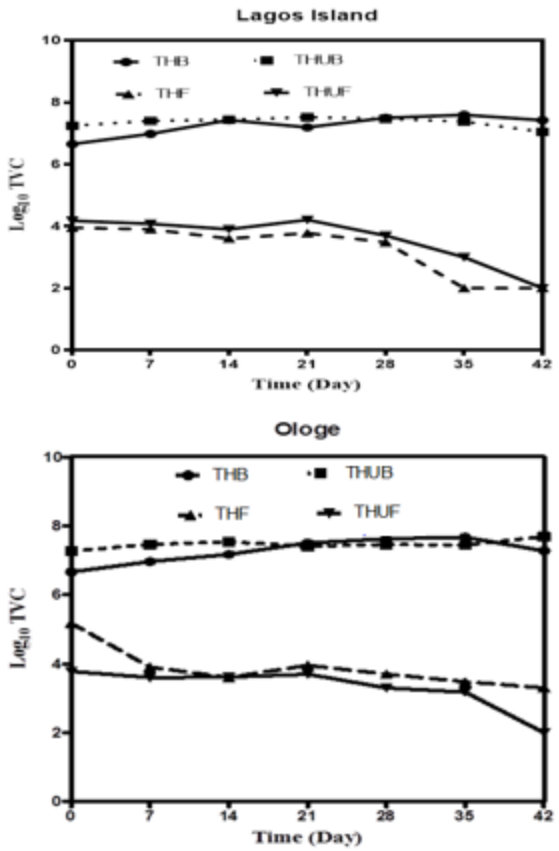

Figure 1: Population dynamics of different microbial communities in the Lagos Island, and Ologe Lagoon water microcosm during 42 days of incubation with crude oil at room temperature (28 ±2°C). THB: Total heterotrophic bacteria (●); THUB: Total hydrocarbon utilising bacteria (■); THF: Total heterotrophic fungi (▲); THUF: Total hydrocarbon-utilising fungi (▼). Data points represent the means of three replicate determinations

Table 2: Kinetics of degradation of Escravos crude oil by the microbial communities in microcosms from Lagos Island and Ologe

Parameters	Lagos Island	Ologe
Percentage degradation (%)	95.315±0.134	96.345±0.488
Degradation rate (mg/l/d)	65.391±0.370	61.190±8.542
Degradation rate constant (/d)	0.050±0.001	0.055±0.003
Degradation half-life (d)	9.559±0.093	8.725±0.389

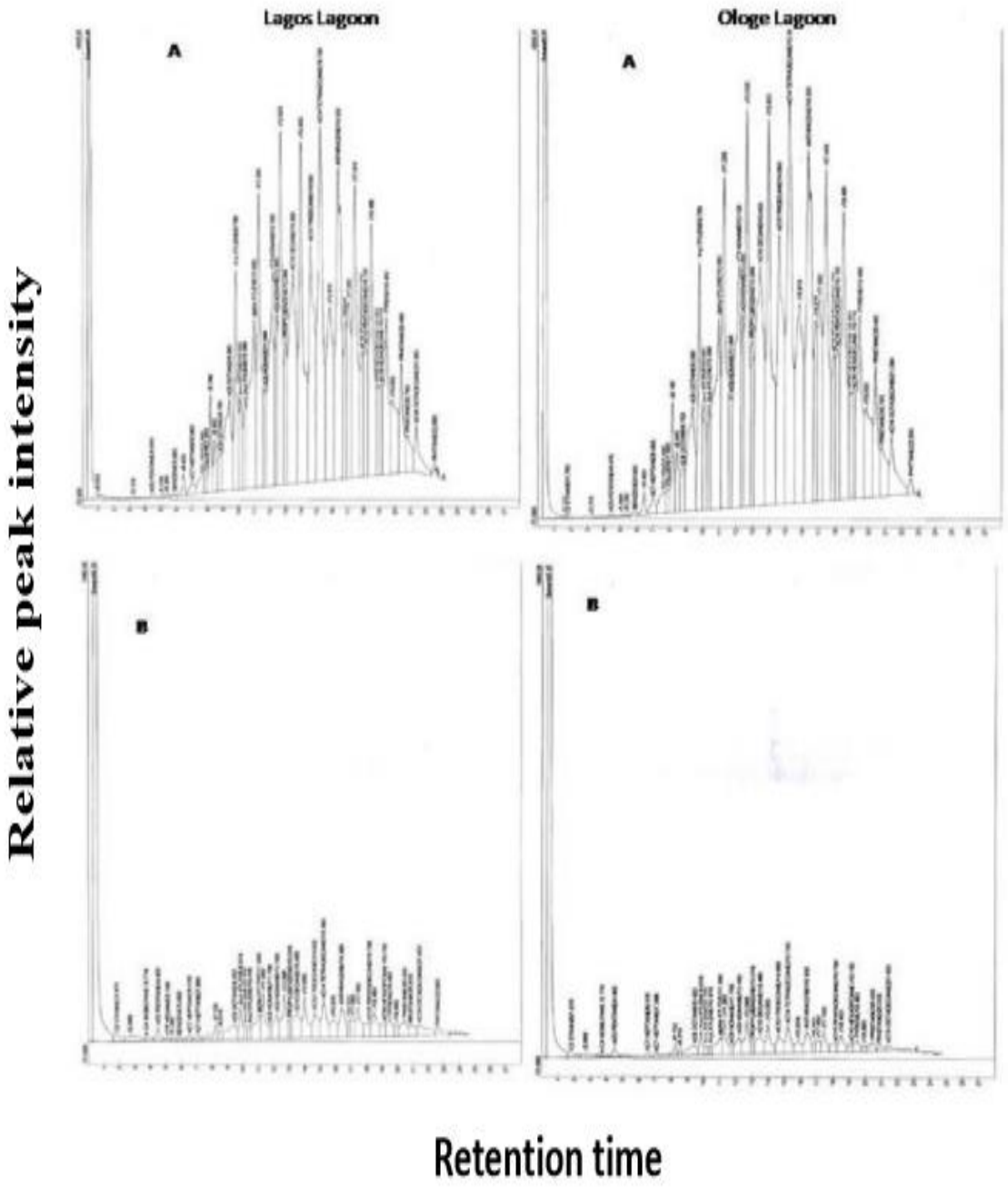

Figure 2: GC-FID revealed near complete disappearance of petroleum hydrocarbon fractions following 42 days incubation at room temperature (28 ± 2°C). Gas chromatographic traces of n-hexane extracts of crude oil from culture fluids of Lagos Island microcosm at Day 0 and Day 42 are represented in panel A1 and panel A2 respectively. Panels B1 and B2 show Gas chromatographic traces of n-hexane extracts of crude oil from culture fluids of Ologe microcosm at Day 0 and Day 42 respectively.

Percentage of crude oil fractions degraded by microbial communities after 42 days of incubation at room temperature.

The percentage removal of aliphatic and aromatic hydrocarbon fractions by microbial communities from Lagos Island and Ologe water samples respectively is shown in Table 3. Benzene recorded 66.890 ± 0.75%, heptane 89.380 ±0.509%, toluene 100%, n-p xylene 96.636 ± 2.184%, decane 96.243 ± 0.530%, Tetradecane 96.698 ± 0.011%, pyrene 97.702 ± 0.117% and phytane 96.440 ± 0.058% respectively. For Ologe microcosm over 98% removal of hydrocarbon fractions were observed with octane, n-p xylene, alpha xylene, nonane, tetradecane, pentane and phytane.

Table 3. Percentage of crude oil fractions degraded by microbial communities after 42 days of incubation at room temperature.

Oil Fraction	Lagos Island	Ologe
Benzene	66.890±1.075	100±0.000
Heptane	89.380±0.509	99.941±0.026
Toluene	100.00±0.000	100±0.00
Octane	66.750±0.495	99.725±0.034
n-p Xylene	96.636±2.184	99.969±0.016
Alpha Xylene	95.356±0.061	99.959±0.003
Nonane	94.260±1.992	99.963±0.004
Propyl Benzene	97.952±2.316	99.968±0.004
Decane	96.243±0.530	99.967±0.003
Tridecane	96.440±0.581	99.970±0.002
Tetradecane	96.698±0.011	99.971±0.002
Anthracene	96.755±0.119	99.726±0.026
Pentadecane	94.820±2.305	99.952±0.007
Hexadecane	92.387±0.222	99.946±0.013
Pyrene	95.702±0.117	99.968±0.006
Pristane	91.674±0.222	99.943±0.015
Octadecane	84.540±1.866	100+0.00
Phytane	96.440±0.058	99.670±0.104

4. DISCUSSION

Lagos is a megacity in Nigeria that harbours 75% of the industrial outfits in Nigeria [37]. These industries include textiles, chemical, bottling, breweries, paints, metals, petrochemicals, paper and sawmills factories. Many of these industries discharge their mainly untreated waste into canals and drainages, which later find their ways into nearby waterbodies. This has made Lagos and Ologe lagoon some of the waterbodies at the receiving ends of these pollutants. It is important to analyse the physicochemical properties of these water bodies because its influences on the type, number and metabolic activities of microflora in any ecosystem [38]. Determination of biological oxygen demand of any waterbody is important because it measures directly the amount of oxygen utilised by microorganism in decomposing the organic matters in wastewater. The biological oxygen demand of both water bodies falls within the acceptable limit of Nigeria federal environmental protection agency (FEPA) regulation [39].

So also, the fact that most other parameters such as nitrate, phosphate, iron, zinc, and sulphate were within the permissible limit of FEPA standard indicated that the pollution levels in the water may not grossly affect negatively. This is important because high concentration of certain nutrients, such as nitrogen, may trigger eutrophication, algal bloom on surface water, and increase in plant growth [40]. Thus, there is reduction in oxygen contents of the waterbody and therefore reduced ability of water to support biological life [41], just as high concentration of heavy metals could vitiate the role of microbial community in the biogeochemical cycle. The high phosphate value obtained may be an important factor in efficient mineralization of inorganic nutrient deficient carbon sources such as petroleum hydrocarbons. The slight variation in the physicochemistry of the two lagoons may be due to the differences in the nature of pollution they received. Hydrocarbon degraders have been isolated from diverse environment including water that had low or non-detectable level of petroleum hydrocarbon pollution, perhaps because of the fact that hydrocarbons occur naturally as parts of many biological systems including plant, animal and bacterial cells and tissues [42]. The dominant bacteria and fungi found in the microcosms in this study belong to the genera that have been commonly reported as hydrocarbon degraders from diverse environments both within tropical Africa and elsewhere [43, 44, 45, 46]. It is equally not surprising that moulds, including genus *Aspergillus and Mucor*, were part of the microbial communities encountered over 42 days enrichment on crude oil as they are ubiquitous and have been shown to also exhibit adaptation to hydrocarbon degradation [47]. Filamentous fungi are particularly relevant due to their expression of extracellular enzymes with broad enzymatic activities against varied pollutants including high molecular weight and extremely recalcitrant hydrocarbons [48].

Diverse organisms including yeasts and non-enteric organisms such as *Bacillus, Alcaligenes, Pseudomonas* with degradative capabilities on petroleum hydrocarbons and related compound such as cyclohexanol and polychlorinated biphenyls (PCB) had been shown to play important role in the biological weathering taking place in Lagos lagoon [49,50,51]. The predominance of the enteric group such as *Escherichia*, *Enterobacter*, *Klebsiella* and *Proteus* encountered in this study could be because of high level of disposal of waste, especially fecal materials and other forms of contamination resulting from human activities. This is in consonance with the findings of Ajao and Fagade [52], who also noted that the lagoon served as the ultimate sink for the disposal of untreated domestic sewage, which explains the high frequency of the enteric group in the two-lagoon water. Khanafer *et al.* [53], in the United Arab Emirate showed that coliform of the genus *Enterobacter*, *Escherichia* and *Klebsiella* isolated from domestic sewage exhibited significant hydrocarbon degradation capability. Degradation of petroleum hydrocarbons by mixed microbial populations is often more efficient than a single pure microbial strain because with mixed culture cometabolism of substrate may occur making the organisms show more degradation competence on various hydrocarbon fractions [54,55, 56,57]. From the foregoing, it can be inferred that the two lagoons water represent a reservoir of microorganism that have adapted to hydrocarbon pollution in the water bodies. This more so as a recent study revealed that Ologe Lagoon and part of the Lagos lagoon harboured potential hydrocarbon-degrading biosurfactant producing bacterial populations [58]. The increase in population densities of the THUB and THUF with corresponding decrease in the residual oil concentration suggests that the microbial communities were able to utilise the crude oil as their source of carbon. The downward trend in populations of both THF and THUF in both microcosms after 21 days could be due to them being outcompeted after an initial role in breakdown of complex compounds to simpler metabolites that were more available to the bacteria population.

This trend correlates well with the observed increase in populations of bacteria in the later days of incubation (from Day 21). Petroleum hydrocarbon degradation by mixed culture had been reported with varied efficiencies on 1% crude oil [59] but our study recorded 95.315± 0.134% and 96.345 ±0.488% for Lagos Island and Ologe microbial communities. Studies had shown that individual organisms only metabolize limited range of hydrocarbon. As revealed by GC fingerprints, there was almost complete removal of various hydrocarbon fractions after 42-day exposures; this unquestionably signifies the competence of the microbial communities towards degradation of petroleum hydrocarbon. Rahman *et al.* [60], described the ability of mixed bacterial culture of *Micrococcus* sp., *Bacillus* sp., *Corynebacterium* sp., *Flavobacterium* sp., and *Pseudomonas* sp. to degrade crude oil after 20 days of incubation and 78% overall degradation was achieved. Al-Wasify and Hamed [61] also reported 85% degradation of Egyptian crude oil by mixed population of bacteria present in oil-polluted water as compare to lower value of 74.3-77.8% recorded by

the individual bacterium present. Greater than 90% degradation of benzene, heptane, toluene, octane (Ologe microcosm), n-p xylene, nonane, propyl benzene and decane by lagoon water microflora may suggest that these hydrocarbon fractions could be less toxic and readily available to be used as carbon and energy source [3,62, ,63].

Aside lower fractions that were effectively utilised by lagoon microbial communities, higher hydrocarbon fractions such as tridecane, tetradecane, anthracene, pentadecane, hexadecane, pyrene, pristane, octadecane and phytane were also degraded above 90% after 42 d exposure. It is however noteworthy that percentage degradation of most hydrocarbon fractions was higher in the Ologe lagoon microcosm. This is most likely due to the differences in nutrients consortia composition. T he almost complete disappearance of pristane and phytane, isoprenoid fractions usually adopted to measure the efficiency of biologically mediated hydrocarbon degradation when compared with heptadecane and octadecane respectively, signifies the competence of this organism towards mineralization of petroleum hydrocarbon [64].

5. CONCLUSION

This study demonstrated that efficient degradation of various components of crude oil in Lagos and Ologe Lagoon waters could be achieved by self-purification activities of the microbial communities present in the lagoons at moderate pollutant concentration of about 1%, meaning that these waters could effectively resolve small-scale petroleum pollution without intervention. Such ability suggests that the consortia in the lagoons were genetically endowed and metabolically primed for hydrocarbon utilisation, attributes which are useful in designing bioremediation programme for compartments with similar characteristics. A deeper insight into this using a multiplicity of molecular and systems biology approaches is the subject of an ongoing study.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for- profit sectors.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHORS' CONTRIBUTIONS

OSO participated in experimental design, collation and interpretation of data, manuscript preparation, and overseeing execution of experimentation; MOF participated in interpretation of data and manuscript preparation; AOA participated in interpretation of data and manuscript preparation; IMO, AOO, FOA and ETO participated in sample collection, experimentation and data collation. All authors read and approved the final manuscript.

ETHICAL APPROVAL

Neither animal nor human was used in part or whole during this study, as regards the procedures performed during the present investigation. Therefore, this article does not contain any studies with human participants or animals performed by any of the authors.

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