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Bacterial Community Profile and Phylogenetic Diversity of Water and Surface Sediments in Iko River Estuary, Akwa Ibom State- Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OAM and SPA designed the study. Author OAM carried out the experiment, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SPA and EBEA handled the arrangement of the results while authors OAM, SPA and EBEA managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A large amount of crude oil is spilled annually into the terrestrial and aquatic environments in Nigeria. This tends to upset the natural equilibrium of the marine environment as a microbial habitat. Thus, there is a need to evaluate the effect of exposure to crude oil on bacterial load and diversity using Iko River and Odoro Ikot pond as the study and pristine sites respectively. The bacterial isolates were molecularly identified using the 16S rRNA sequencing protocol. The total heterotrophic bacterial count (THBC) in the surface water (SW), sub-surface water (SSW) and sediment segments of Iko River ranged from 2.23±0.87 to 9.67 ± 0.43 x 10^6 CFU/ML while the THBC in the SW, SSW and sediment segments of the pristine site (Odoro Ikot pond) ranged from 1.87±0.53 to 4.8± 0.04x10^6 CFU/ML. The sediment had a significantly higher ($P<0.05$) THBC than the water segments (SW and SSW) in both Iko River and Odoro Ikot pond. The hydrocarbon utilizing bacteria (HUB) count in Odoro Ikot pond ranged from 0.40 ± 0.01 to 1.10 ± 0.03 x 10^6 CFU/ML while the HUB count in Iko River ranged from 0.53 ± 0.02 to 0.93 ± 0.04 x 10^6 CFU/ML, making Iko River have a higher

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number of total heterotrophic bacteria and hydrocarbon utilizing bacteria than Odoro Ikot pond. The Proteobacteria isolates had the highest bacterial diversity (77%) while members of the Firmicutes phylum had a 23% bacterial diversity. However, higher bacterial count and diversity were obtained from the sediment segment than from the water segments in both Iko River and Odoro Ikot pond implying that the sediment is more favourable for bacterial growth. Although the bacterial profile was affected by exposure to crude oil, there were variations in the phylogenetic diversities obtained from the different water segments attributable to crude oil concentration.

Keywords: Bacterial profile; phylogenetic diversity; 16S rRNA; hydrocarbon; heterotrophic; water and sediment.

1. INTRODUCTION

Water is essential in determining the health condition of people and remains one of the controlling factors for biodiversity and distribution of earth's varied ecosystem [1]. Heterotrophic prokaryotes play important roles in trophic web network structure and dynamics as well as in the remineralisation of organic matter [2,3]. A lot of studies have been carried out in different marine environments which revealed that microbial profile could be consistent in similar marine environments which were being separated by long distances [4-6] but also varies from one marine environment to another [7-9]. Surveys on the oligotrophic open ocean [10], on coastal temperate sediment [11,12] and on marine sediment [13–15] have helped in understanding bacterial population distribution [16,17] as well as understanding marine bacterial diversity global patterns [18].

The estuary is a semi-enclosed coastal body of water which has a free connection with the open sea. It is the coastal adjunct of the micro-ecosystem and lack of constancy due to continuous inflow of water from different sources makes the temperature, salinity, turbidity and nutrient load to fluctuate over a wide gradient of space and time.

Iko River which is located in Iko clan of Eastern Obolo Local Government Area in Akwa Ibom State takes its rise from Qua Iboe River catchment and drains directly into the Atlantic Ocean. The river is of high economic and ecological importance, as it supports the livelihood of a large number of community members who are mainly fishermen. Iko River runs through both mangrove and freshwater swamps with adjoining creeks and tributaries, with communities settling along the river banks.

The devastation done to the river is aggravated by the direct influence of seawater and the inward driving wind from the Atlantic Ocean due to its closeness [19]. Tidal current, stream and wind waves, however, are the primary hydrodynamic factors in seacoast and estuarine environments [20].

2. MATERIALS AND METHODS

2.1 Study Site

The water samples were collected from Iko River in Iko clan which is in the outskirt of Akwa Ibom State, Nigeria. Akwa Ibom State is located in the coastal Southern part of Nigeria, lying between latitudes 4°32′N and 5°33′N, and longitudes 7°25′E and 8°25′E. Iko River has an oil flow station located at the Utapette station (Fig. 1), and sited at different points in the river is an aqua privy used by the rural dwellers (Fig. 2).

2.2 Sample Collection

Three sampling locations (labelled SL1, SL2 and SL3- Fig. 3) were selected from Iko River while one sampling location (SL4) was selected from Odoro Ikot pond. Sediment samples were collected from the sampling areas using a sterile scoop and were then transferred into sterile glass containers for homogenisation. The water samples (both surface and sub-surface) were aseptically collected into sterile plastic containers of I litre capacity. Collection of water samples were done after rinsing at least three times with the water sample to be analysed according to 33. Collected samples were transported to the laboratory in an ice-packed cooler for analyses.

2.3 Microbial Isolation

The media used for microbial isolation were nutrient agar (NA), tryptic soy agar (TSA) and mineral salt medium (MSM).

Samples were serially diluted in sterile distilled water. Prior to the dilution of sediment samples, I g of each sediment sample was weighed using

sterile foil paper and then added into a 9 mL sterile distilled water. Thereafter, 1 mL of the sediment preparation was then serially diluted. Known dilutions of the water and sediment samples were then inoculated in triplicates into the different agar plates. The tryptic soy agar and nutrient agar were used for the total heterotrophic bacteria count while the mineral salt medium was used for the estimation of total hydrocarbon-utilising bacteria. Samples on TSA and NA were incubated at 37ºC for 24 hr while samples inoculated on MSM were incubated at room temperature for 7 days.

Fig. 1. Iko River and the crude oil flow station

Fig. 2. Iko River with a sited aqua-privy for inhabitants of the community

2.4 Bacterial Enumeration, Purification and Maintenance

Enumeration of the emerging colonies was carried out at the end of each incubation period using the colony counter. Discrete colonies were thereafter picked aseptically and sub-cultured on new agar plates for purification. The isolates were later stored on nutrient agar slants prior to their molecular identification using the 16S rRNA sequence.

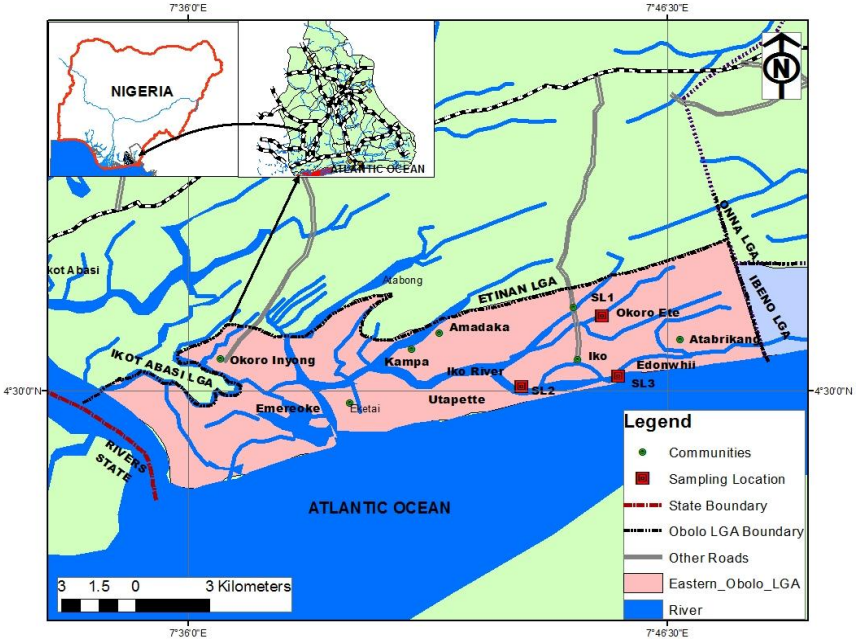

Fig. 3. Map showing Iko River with the sampling locations (SL)

2.5 Amplification of 16S rRNA Genes, Purification, Quantification and Sequencing

The DNA extracted from representative isolates from all the stations were pooled together and used for the polymerase chain reaction (PCR). The DNA extracts were amplified for 16S rRNA genes through the PCR (predenaturation step at 94°C for 1 min followed by 30 cycles of 1 min at 94°C denaturation, 1 min at 55°C annealing and 3 min at 72°C extention, followed by a final elongation step of 72°C for 3 min- final hold), using the 10F forward primer (5^{I} – AGTTTGATCATGGCTCAGATTG- 3^{I}) and the 1401R reverse primer (5^{I} – CGGTGTGTACAAGACCC-3^{I}). Purification of the PCR products was carried out using the GFX kit and quantified in a 1% agarose gel electrophoresis at 80 volts for 25 min using an uncut lambda DNA marker. The quantified genomic DNA were cycle-sequenced using a BigDye Terminator V3.1 Cycle Sequencing Protocol with a 1100R reverse primer (5^{I} – GGGTTGCGCTCGTTG- 3^{I}) while the precipitation of the cycle-sequenced DNA in the microplate was carried out using the EDTA-NaOAc-ethanol precipitation protocol. The partial 16S rRNA gene sequences were determined in an ABI Genetic Analyzer (Applied Biosystems).

3. RESULTS AND DISCUSSION

3.1 Total Heterotrophic Bacterial Count

The result obtained from this study shows that although there were significant differences ($P<0.05$) in some of the mean total heterotrophic bacteria counts (THBC) in the SW, the SSW and sediment, the THBC in the SW from the pristine site (Odoro Ikot station) and Utapette station had no significant difference ($P>0.05$) in their THB mean counts ($4.8 \times 10^6 \pm 0.04$ and $4.83 \times 10^6 \pm 0.33$ CFU/ML- Table 1). However, SW of Okoro station in Iko River had the lowest mean THBC ($4.4 \times 10^6 \pm 0.04$ CFU/ML) while Edonwhii station had the highest mean THBC ($5.6 \times 10^6 \pm 0.53$ CFU/ML). There were significant differences ($P<0.05$) in the THBC mean values of the SSW from Iko River and the pristine site with Okoro station in Iko River having the highest THBC ($4.37 \times 10^6 \pm 0.80$ CFU/ML) while Odoro Ikot Pond SSW had the lowest mean THBC ($1.87 \times 10^6 \pm 0.53$ CFU/ML). The sediment of Edonwhii station in Iko River produced significantly higher ($P<0.05$) THBC ($9.67 \times 10^6 \pm 0.43$ CFU/ML) than the sediments from all other stations, with the pristine site producing the lowest mean THBC ($2.57 \times 10^6 \pm 0.07$ CFU/ML) as shown in Table 1. There were significant differences ($P<0.05$) in the THBC mean values of the samples from the three different segments of Iko River and the pristine site. The result shows that the THBC was highest in the surface sediment ($6.63 \times 10^6 \pm 0.09$ CFU/ML) than in the surface water while the sub-surface water had the lowest THBC ($2.88 \times 10^6 \pm 0.04$ CFU/ML) (Table 2). Although there were significant differences ($P<0.05$) in the mean THBC in the four stations, the THBC mean values from Edonwhii and Utapette stations in Iko River gave the highest average THBC ($5.83 \times 10^6 \pm 0.06$ and $5.53 \times 10^6 \pm 0.09$ CFU/ML) with no significant difference ($P>0.05$) in their THBC mean values (Table 3).

3.2 Total hydrocarbon-utilising Bacteria

The total hydrocarbon-utilising bacteria (HUB) profile showed that there was a significant difference in the HUB count in the sediment sample from Odoro Ikot pond and the HUB counts in the sediment samples from the three stations in Iko River ($P<0.05$). However, there was no significant difference ($P>0.05$) in the HUB counts of the SW and SSW samples from Okoro, Utapette and Edonwhii stations with Okoro giving the highest mean counts of HUB in both the SW and SSW samples (0.83 ± 0.08 and 0.80 ± 0.04 CFU/ML) followed by Edonwhii and Utapette stations (Table 1). The result of the mean HUB count across the three segments as presented in table 2 showed that while the sediment segment had a significantly ($P<0.05$) higher HUB count ($0.89 \times 10^6 \pm 0.04$ CFU/ML), there was no significant ($P>0.05$) difference in the SW and SSW HUB mean values ($0.59 \times 10^6 \pm 0.08$ and $0.58 \times 10^6 \pm 0.02$ CFU/ML). Although there was a significantly ($P<0.05$) higher HUB mean count in Okoro station ($0.82 \times 10^6 \pm 0.01$) than in the other three stations, Odoro Ikot (pristine site), Utapette and Edonwhii stations showed no significant differences ($P>0.05$) in their HUB mean values as shown in Table 3.

3.3 Bacterial Variation and Diversity

The result of the 16S rRNA molecular identification (Table 4) and taxonomic classification (Figs. 4 - 6) of the isolates revealed that a higher bacterial diversity were present in the sediment segments of the two sites (Iko River and pristine site). Among the 22 taxonomically

classified isolates, 17 (77%) were of the phylum Proteobacteria while 5 (23%) were of the Firmicutes phylum (Table 4). When considered at the family level, it was observed that out of the 17 Proteobacteria isolates, 5 (29%) belonged to the Enterobacteriaceae family, 11 (65%) were from the Xnthomonadaceae family while 1 (6%) was of the Pseudomonaadaceae family. The 5 members of the Firmicutes phylum were members of the Enterococaceae family. The majority (3 out of 5) of the 5 members of the Enterobacteriaceae family were isolated from the sediment which produced two different species of *Enterobacter* and one *Klebsiella* specie while the water segment produced two isolates both of which were members of the genus *Klebsiella*. Among the 5 members of the Enterococaceae family, the sediment yielded four isolates, two species of *Enterococcus hirae* and two species of *Enterococcus faecalis* while the water segment (SSW) gave one *Enterococcus faecium* isolate. The members of the Xanthomonadaceae family were equally distributed in the surface water and sediment segments than in the sub-surface water segment while the only Pseudomonaadaceae isolate (*P. aeruginosa*) occurred in the SSW. Precisely, there were 4 (36.36%) Xnthomonadaceae in the SW segment, 3 (27.27%) in the SSW segment and 4 (36.36%) in the sediment. However, among the 22 molecularly identified isolates, 11 (50%) were from the sediment while the SW and SSW gave 5 (23%) and 6 (27%) isolates respectively.

The total heterotrophic bacteria counts obtained from the sampling segments (Sediment, SW and SSW) and the four stations (Odoro Ikot, Okoro, Utapette and Edonwhii) were above the standard value of 1.0×10^2 CFU/mL [21]. There is no clear evidence that the THBC pose public health problem since only certain numbers are opportunistic pathogens [22] and concentration of heterotrophic bacteria does not necessarily indicate health effects with a recommended standard THBC value fewer than 500 CFU/mL [23]. However, they reported that this is not a health-based standard, although it reflects the concern that at concentrations higher than 500 CFU/mL, heterotrophic bacteria can interfere with the recovery of total coliform and *Escherichia coli.* This corroborates with the absence of *Escherichia coli* in this study due to the high THBC above 500 CFU/ml which may have affected *E. coli* recovery from the two sites.

Although *E. coli* was not isolated during the study, the isolation of enterococcal species indicates faecal contamination of the river water. This is due to the high survivability of *Enterococcus* species whose presence indicates pollution of a long time, implying that the sampling was done at a time the *E. coli* in the river must have died off or at a location far from the point of the faecal pollution.

Bacteria isolated from the water and sediment formed distinct groups even though they were collected at the same stations and as expected, the habitat type (water or sediment) is the dominant cause of the differences in microbial communities [24]. The variations in the THBC of Iko River and pristine site in this study corroborates with the report that microbial community composition varies from one environment to another [7-9]. The high THBC obtained in Iko River estuary may be due to high levels of nutrients in both the water column and sediment, making estuaries among the most productive natural habitats in the world [25], and/or due to exposure to crude oil which must have favored the proliferation of hydrocarbon degraders in the environment thereby causing an increase in the bacterial population. The bacterial population in the sediment was significantly higher ($P<0.05$) than that in the SW and SSW segments. This corroborates the report on observed higher bacterial diversity and higher total heterotrophic bacteria count in the sediment samples [24,26]. There were several conditions that might influence the sedimentation of bacterial diversity among which are *in situ* hydrological regime like currents, tides, waves, upwelling, lateral transport, water mixing and exchange, as well as the intensity and dynamics of these activities [27].

There was a significantly higher mean hydrocarbon-utilising bacteria in the sediment than in the surface and sub-surface water. However, the mean HUB counts in all the stations and their segments were lower than the THBC. The mean HUB count obtained from the four locations (Odoro, Okoro, Utapette and Edonwhii) revealed that the Okoro location had a significantly ($P<0.05$) higher count followed by Utapette, Edonwhii and Odoro Ikot stations. This may be due to prior exposure to crude oil in Iko River which must have initiated the presence and action of hydrocarbon degraders in the environment that now resulted to a higher HUB count.

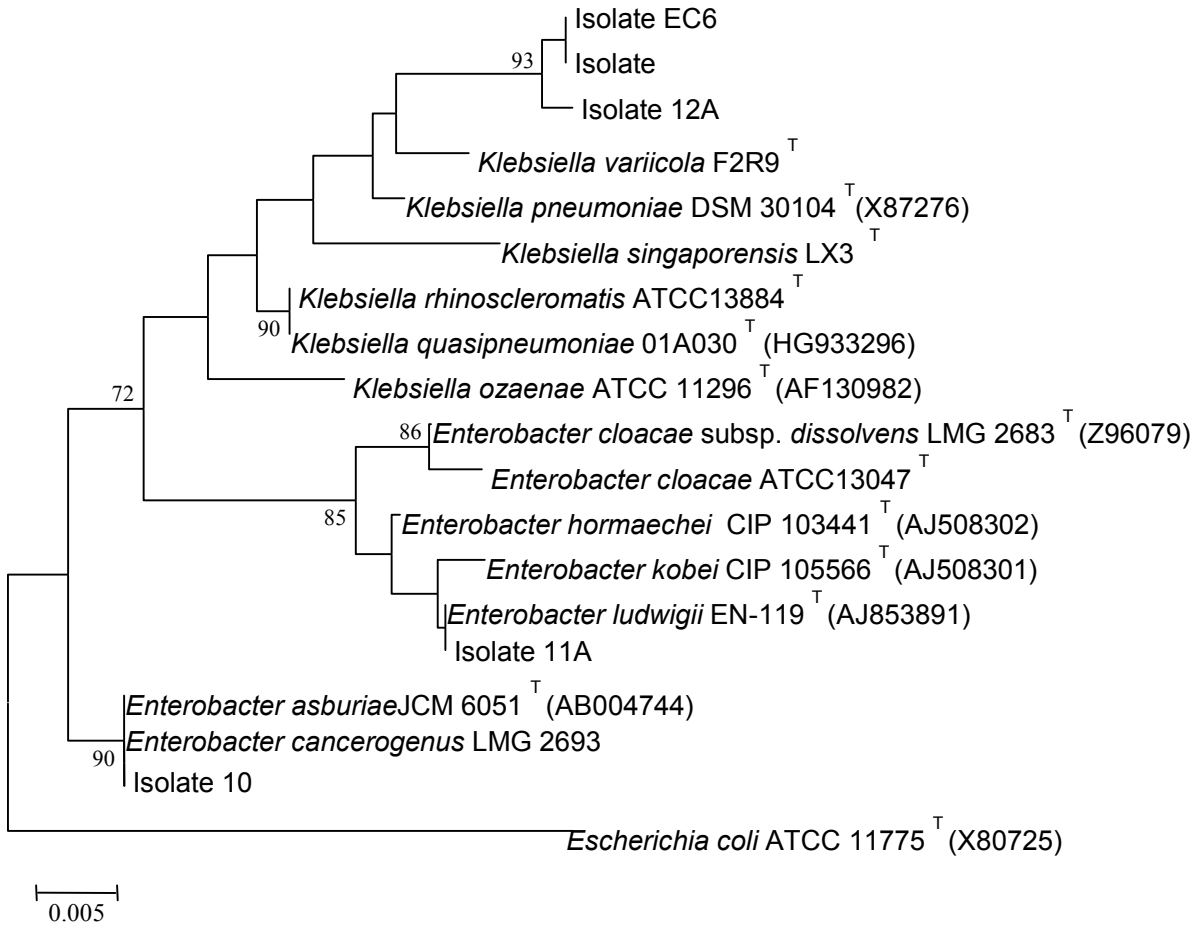

Fig. 4. Phylogenetic tree of the enterobacteria

NB: Bootstrap values less than 70 were removed from the node

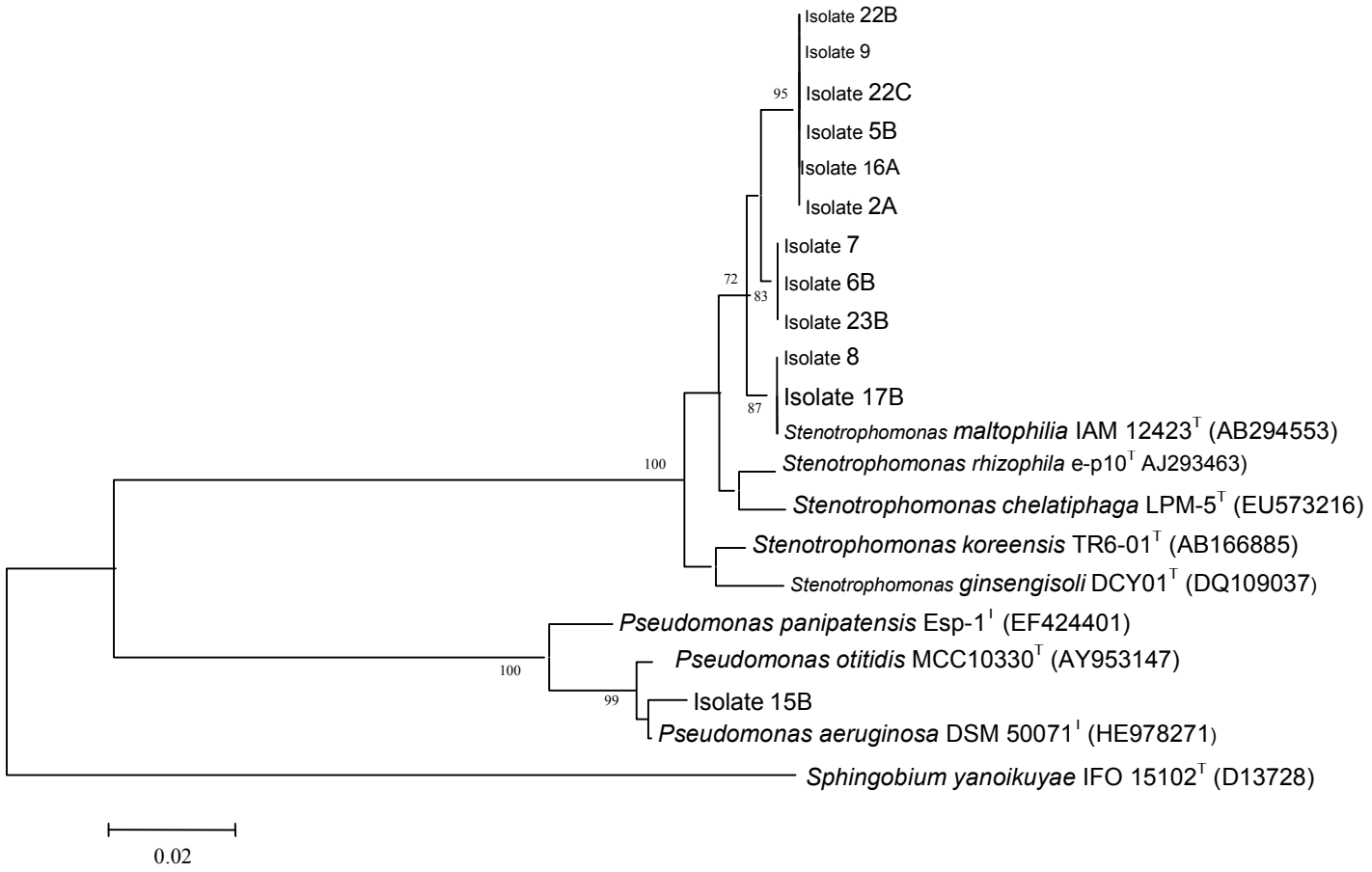

Fig. 5. Phylogenetic tree of the Proteobacteria

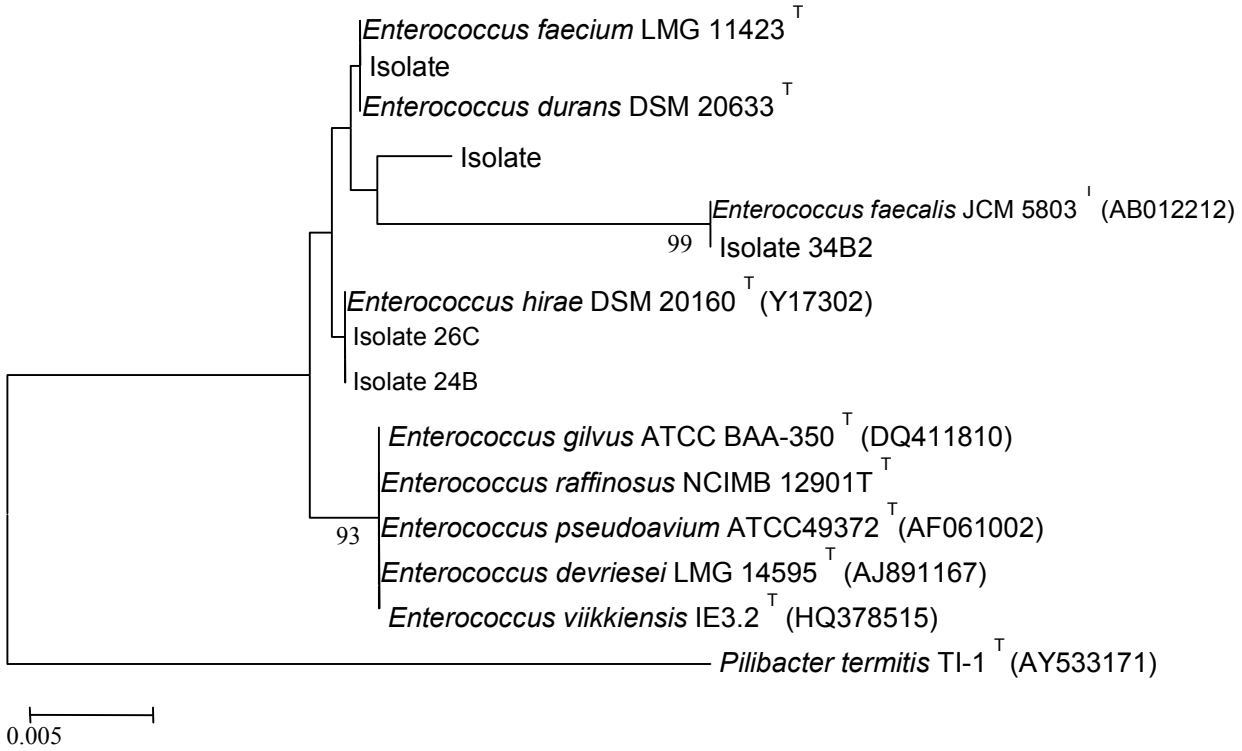

Fig. 6. Phylogenetic tree of the *Enterococci*
NB: Bootstrap values less than 70 were removed from the nodes.

Table 1. Total heterotrophic and hydrocarbon utilising bacteria counts in the sediment and overlying water samples at the various locations

	SW ($X10^6$CFU/ml)				SSW ($X10^6$CFU/ml)				SED ($X10^6$CFU/ml)				
	Odoro	**Okoro**	**Utapette**	**Edonwhii**	**Odoro**	**Okoro**	**Utapette**	**Edonwhii**	**Odoro**	**Okoro**	**Utapette**	**Edonwhii**	**LSD**
THBC	$4.8^{d} \pm 0.04$	$4.4^{e} \pm 0.60$	$4.83^{d} \pm 0.33$	$5.6^{c} \pm 0.53$	$1.87^{n} \pm 0.53$	$4.37^{e} \pm 0.80$	$3.03^{l} \pm 0.43$	$2.33^{g} \pm 0.87$	$2.57^{g} \pm 0.07$	$5.53^{c} \pm 0.92$	$8.73^{b} \pm 0.12$	$9.67^{a} \pm 0.43$	0.26
HUB	$0.4^{c} \pm 0.01$	$0.83^{b} \pm 0.08$	$0.53^{b} \pm 0.03$	$0.60^{b} \pm 0.05$	$0.40^{c} \pm 0.02$	$0.80^{b} \pm 0.04$	$0.53^{b} \pm 0.02$	$0.60^{b} \pm 0.02$	$1.10^{a} \pm 0.03$	$0.83^{b} \pm 0.04$	$0.93^{b} \pm 0.04$	$0.70^{b} \pm 0.09$	0.12

**Means with the same case letter along the horizontal arrays indicates no significant differences (P>0.05)*

Key:
SW = Surface water,
SSW = Sub-surface water,
SED = Sediment,
THBC =Total heterotrophic bacteria count,
HUB = Hydrocarbon utilizing bacteria,
CFU = Colony forming unit

Table 2. Mean total heterotrophic bacteria and hydrocarbon utilising bacteria in sediments and overlying water

	SW ($x10^6$ CFU/ml)	**SSW ($x10^6$ CFU/ml)**	**SED ($x10^6$ CFU/g)**	**LSD**
THBC	$4.91^{b} \pm 0.23$	$2.88^{c} \pm 0.04$	$6.63^{a} \pm 0.09$	1.46
HUB	$0.59^{b} \pm 0.08$	$0.58^{b} \pm 0.02$	$0.89^{a} \pm 0.04$	0.12

**Means with the same case letter along the horizontal arrays indicate no significant differences (P>0.05)*

Table 3. Average total heterotrophic and hydrocarbon utilising bacteria counts in various stations of the river

	Odoro	**Okoro**	**Utapette**	**Edonwhii**	**LSD**
THBC	$3.0^{c} \pm 0.03$	$4.7^{b} \pm 0.04$	$5.53^{a} \pm 0.09$	$5.83^{a} \pm 0.06$	0.53
HUB	$0.6^{b} \pm 0.14$	$0.8^{a} \pm 0.09$	$0.67^{b} \pm 0.01$	$0.63^{b} \pm 0.07$	0.05

**Means with the same case letter along the horizontal arrays indicates no significant differences (P>0.05)*

Table 4. Isolates, stations of isolation and their corresponding names

Isolate	Location	Name of organism
10	SED1	Enterobacter asburiae JCM 6051/ cancerogenus LMG 2693
11A	SED1	Enterobacter ludwigii EN-119
12A	SSW3	Klebsiella variicola F2R9
13	SW2	Klebsiella variicola F2R9
EC6	SED4	Klebsiella variicola F2R9
24B	SED1	Enterococcus hirae DSM 20160
25A	SED1	Enterococcus faecalis JCM 5803
26C	SED1	Enterococcus hirae DSM 20160
27C	SSW2	Enterococcus faecium LMG 11423/durans DSM 20633
34B2	SED4	Enterococcus faecalis JCM 5803
2A	SED4	Stenotrophomonas maltophilia IAM 12423
5B	SSW2	S. maltophilia IAM 12423
6B	SW2	S. maltophilia IAM 12423
7	SSW3	S. maltophilia IAM 12423
8	SED1	S. maltophilia IAM 12423
9	SED4	S. maltophilia IAM 12423
15B	SSW4	Pseudomonas aeruginosa DSM 50071
16A	SED3	S. maltophilia IAM 12423
17B	SSW2	S. maltophilia IAM 12423
22B	SW4	S. maltophilia IAM 12423
22C	SW4	S. maltophilia IAM 12423
23B	SW3	S. maltophilia IAM 12423

Key: S = Sediment, SW = Surface water, SSW = Sub-surface water, 1-3 = Stations in the estuarine coastlines of Iko River (River with crude oil flow station), 4 = Station in the fresh water (Pristine site- Odoro Ikot pond).

Although previous studies on the taxonomic richness of ocean depth yielded mixed results, the bacterial diversity in this study was observed to be higher in the sediment than in the surface water and sub-surface water. However, some studies reported declines in taxonomic richness with increasing ocean depth [28,5,29] while others reported that bacterial communities in the water-column exhibit a clear depth profile with samples declining in similarity from the sea surface to the abyssopelagic waters [7, 28, 30]. In this study, a high diversity of members of the Enterobacteriaceae,and Enterococaceae families were obtained in the sediment followed by the bacterial diversity in the SSW and SW. This observation corroborates the report on the observed higher richness of diversity deeper in the water column [18, 31, 32]. It has been stated that the distribution of abundant bacterial taxa in the surface sediment is indicative of colonisation of the seafloor via the overlying water column [9]. However, there was not much variation in the diversity of members of the Xanthomonadaceae and Pseudomonadaceae families isolated from the three water segments (SW, SSW and sediment) as eleven out of a total of twelve belonged to the same family-Xanthomonadaceae (*S. maltophilia*- IAM 12423) while only one belonged to the Pseudomonadaceae family (*P. aeruginosae*-DSM 50071).

4. CONCLUSION

The result of this study showed that the location of isolation affected the THBC. This was based on the occurrence of significantly higher THBC in the Utapette and Edonwhii stations in Iko River than in the pristine site.

Based on the mean HUB counts recorded in Okoro and Odoro Ikot locations, Iko River gave a higher HUB count than the pristine site. This is due to the bacterial exposure to crude oil in Iko River (which has an oil flow station) which must have led to the presence of hydrocarbon degraders in the environment that now resulted to a higher HUB count that also added up to the THBC in the study site.

Moreover, there was a higher diversity of members of the Enterobacteriaceae and Enterococaceae families in the sediment segment than in the water segment (both surface and sub-surface water) while an equal number of Xanthomonadaceae members occurred in both sediment and surface water segments but less by one in the sub-surface water segment.

From the result of the THBC and HUB mean values, it could be deduced that bacterial exposure to crude oil had an effect on the bacterial community structure based on the high THBC and HUB values obtained from Iko River. Although a higher diversity of members of the Enterobacteriacea,and Enterococaceae were obtained from the study site compared to the pristine site, it was not certain that bacterial exposure to crude affected there diversity since a member of the Pseudomonadaceae family (*P. aeruginosae*- DSM 50071) isolated during this study was from the pristine site while *S. maltophilia*- IAM 12423 specie (family- Xanthomonadaceae) was obtained from both sites.

5. RECOMMENDATION

We recommend that the bacterial diversity across the depth of the river be studied based on the effect of exposure to different concentrations of crude oil- starting from a mere microbial isolation from a crude oil exploration environment to the time of an oil explosion.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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