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# Bacterial and Fungal Isolates from Crude Oil Polluted Sediment in Eniong River, Itu, Nigeria

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**ABSTRACT**
The need to know the level of contamination of radioactive elements, especially Microbial populations are crucial for the environment, providing vital ecosystem services such as biogeochemical cycling, decomposition, and degradation, which essential vital for the survival of all life forms on Earth. This study investigated the heterotrophic bacterial and fungal isolates in crude oil polluted sediment from the Eniong River using standard conventional analytical and culture-dependent techniques. The mean total heterotrophic bacterial (THBC) and mean total fungal counts (THFC) in sediment samples from the three stations of Eniong river sediment was 9.3 x $10^4$CFU/g and 2.4 x $10^4$ CFU/g, respectively. The heterotrophic bacterial count was highest in location A (upstream) and lowest in location C (downstream) while the lowest fungal count was obtained in location B (midstream) and highest in location C (downstream). The results revealed diverse bacterial and fungal assemblages and ecosystem's richness in microbial diversity. Twelve (12) bacterial species from the genera *Staphylococcus, Salmonella, Shigella, Escherichia, Bacillus, Klebsiella, Micrococcus, Aeromonas* and *Pseudomonas* were isolated. The eight (8) fungal species belonging to genera *Aspergillus, Penicillium, Rhizopus, Geothrichium, Candida* and *Mucor* were obtained. *Bacillus cereus* was the most prevalent bacterial isolate, accounting for 16%, while the least isolated bacteria were *Aeromonas* sp. and *Salmonella* sp. (4%). The most isolated fungi was *A. niger* (31.8%), while *A. fumigatus* (13.6%) was the least occurring fungal isolates. The findings clearly demonstrated that the sediment of the Eniong River provided a unique ecological niche that supports a variety of microorganisms.

**Keywords:**
Polluted,
Sediment,
Diversity,
Ecosystem,
Eniong River.

## INTRODUCTION

Introduction of crude oil into aquatic environment as a result of tanker accidents and offshore oil exploration has resulted into significant water pollution (Bostrom *et al.*, 2015). Once crude oil spills into the water ecosystem, it goes through natural processes like emulsification, dispersion, evaporation, photo-oxidation, biological degradation and sedimentation (Bostrom *et al.*, 2015). It is very slow to recover crude oil from the aquatic ecosystem as compared to its accumulation. However, part of the hydrocarbon goes down to the sediment while some are either adsorbed on suspended solids or interacts with surfactants whereas others are dispersed to the bottom of sediment of the ecosystem (Ramseur *et al.*, 2010; Gong *et al.*, 2014). These processes underscore the complications that arise in the management of oil spillages in the aquatic ecosystems.

Majority of the study on petroleum spillage on biological degradation most of the time are targeted at hydrocarbonoclastic bacteria from aquatic sediments (Syakti *et al.*, 2019), while some researches have suggested that water sediment bacteria have the ability to degrade crude oil (Dwinovantyo *et al.*, 2016). Petroleum contamination of the aquatic sediments changes microbial dynamics, thereby improving the potentials of the oil degrading microbes (Chikere *et al.*, 2017). However, it is important to note that the already existing hydrocarbonoclastic microbes that are present in the aquatic sediment are mostly fungi and bacteria which include *Aspergillus, Candida, Cunninghamella,*

*Fusarium, Penicillium, Trichoderma Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter and Bacillus,* (Safdari *et al*., 2018; Varjani, 2017). It is imperative to note that microbial communities in aquatic sediment system utilize crude oil aerobically in the sediment surfaces and anaerobically under the subsurface of sediments.

Water environments significantly affected by oil pollution is the most important factor that contributes to environmental deterioration of the system. Pollution of the environment is caused as a result of unwholesome waste disposal, agricultural practices and atmospheric fallout (Jain *et al.*, 2010). It is therefore proper to ensure that a total biological and chemical analysis of pollutants is carried out before they are disposed into the environment in order to prevent the deterioration of the ecosystems. It is important to note that potential toxic elements, persistent organic pollutants and high density of pathogenic microorganisms (Saber *et al.*, 2014; Hoballah *et al.*, 2015) underscores the significance of establishing procedures or measures based on field results on the effect of high concentrations of pollutants on water systems. There is a need to update available information on the microorganisms presented in polluted sediment, focusing on bacterial and fungal isolates from crude oil-contaminated sediment. Therefore, the study determined the bacterial and fungal isolates from crude oil polluted sediment in Eniong River, Itu, Nigeria.

## MATERIALS AND METHODS

### Study Area

The study area is the Eniong River, a humic freshwater ecosystem on the south-eastern coast of the Niger Delta, Akwa Ibom State, Nigeria. This tributary of the Middle Course of the Cross River is located at latitudes between $05^{o}$ $15^{1}$ and $56.0^{11}$ N and longitudes $05^{o}$ $12^{1}$ N and $05^{o}$ $054^{11}$ E. The Eniong River serves as a crucial natural resource in the region, supporting diverse biological communities.

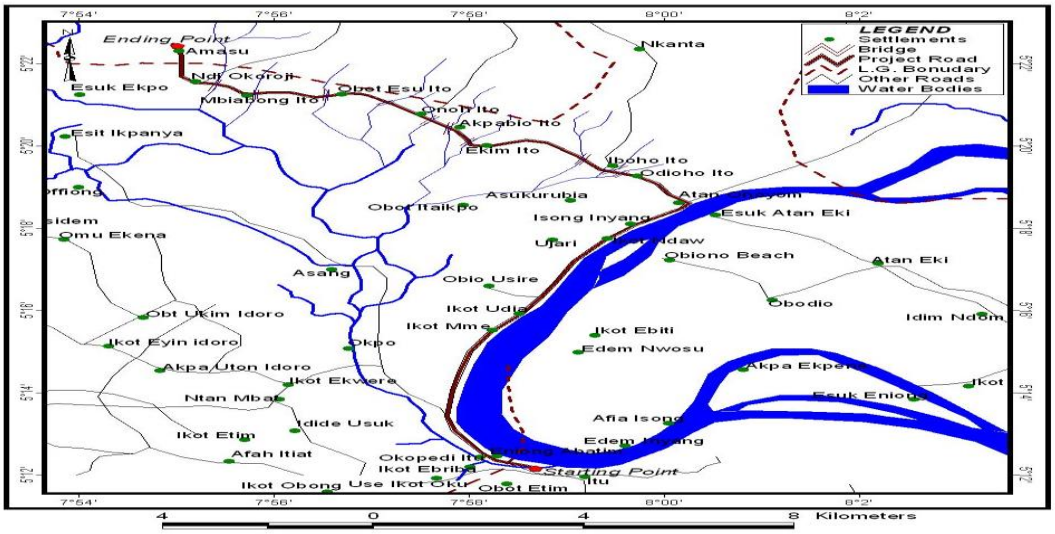


Figure 1: The middle course of the Cross River, showing the location of the humic freshwater ecosystem of Eniong River.

### Sterilization of Glasswares

All glass wares used were thoroughly washed with detergent and rinsed with clean water. The glass wares such as test tubes, conical flasks, Petri dishes, beakers, pipettes, Durham tubes and McCartney bottles were sterilized in the hot air oven at $180^{o}$C for an hour. Wire loop was flamed to redness before and after used.

### Collection and Preparation of Samples

Sediment samples were collected from three designated stations along the river: A (Upstream), B (Midstream), and C (Downstream). The sediments were aseptically collected with an Eckman sediment grab into 95% ethanol-sterilized plastic containers and promptly transported to the Microbiology laboratory within 24 h and subsequently stored in a refrigerator at 4°C until analysis was needed (Essien and Udosen, 2000).

### Determination of Densities of Heterotrophic Bacteria and Fungi

The total heterotrophic bacteria bacterial counts (THBC) and total fungal counts (TFC) of the sediment samples were determined by the pour plate techniques using nutrient agar (NA) and Sabourand dextrose (SDA) agar, respectively. The sediment samples were homogenously mixed and carefully sorted to remove stones and extraneous debris. Each sample, weighing 10 grams (10 g), was then mixed with 90 mL of distilled water ($dH_2O$)

in a conical flask and shaken for 1 min. One millilitre (1 mL) aliquot from dilutions of $10^{-3}$ was plated in triplicate onto sterile NA and SDA plates. To selectively inhibit fungal and bacterial growth, 500 µL of Griseofulvin was added to NA plates and Chloramphenicol to SDA plates. The culture plates were swirled and allowed to solidify, and the NA plates were incubated aerobically at 37 °C for 24-48 h and the SDA plates at 28 ± $2^0$C for 3-5 days. After incubation, the microbial growths were observed, and the colonies were counted and expressed as colony-forming units (CFU) per gram of the sediment sample.

**Densities and Isolation of Pollution Indicator Bacteria from Eniong River Sediment**

The bacteriological culture media employed for the determination of densities and isolation of pollution indicator bacteria from Eniong River sediment samples were MacConkey agar (MCA), Eosin methylene blue agar (EMB), Mannitol Salt Agar (MSA), and *Salmonella-Shigella* agar (SSA) (Merck, Germany). These media facilitated the enumeration and isolation of total coliform coliforms (TCC), faecal coliform counts (FCC), *Staphylococcus aureus* counts, and *Salmonella-Shigella* counts (SSC), respectively (Essien and Udosen, 2000; Odeyemi *et al*., 2023). Each sample, weighing 10 grams (10 g), was then mixed with 90 mL of distilled water ($dH_2O$) in a conical flask and shaken for 1 min. Serial dilutions were made to obtain $10^{-1}$, $10^{-2}$, and $10^{-3}$, and then 1 mL of aliquots from dilutions of $10^{-3}$ was pour-plated onto sterile MCA, EMB, MSA, and SSA in triplicate. Then the inoculated plates were incubated aerobically at 37 °C for 24-48 h. After incubation, the discreet colonies on the positive plates were counted and recorded accordingly (Akinjogunla and Divine-Anthony, 2013; Odeyemi *et al*., 2023).

**Purification and Identification of Bacterial Isolates**

The bacterial colonies were subcultured onto freshly prepared plates of NA and incubated at 37°C for 24 h. Pure cultures of isolates were streaked onto NA slants and SDA plates, incubated at 37°C for 24 h for bacteria, and stored in the refrigerator at 4°C for characterization and identification. Identification of bacteria by conventional biochemical tests. Bacteria were identified using Gram staining and conventional biochemical tests such as catalase, coagulase, Vogues Proskauer, methyl red, indole, urease, motility, citrate, hydrogen sulphide, oxidase, opsochin, spore, fructose, mannitol, maltose, galactose, lactose, glucose, and sucrose. The characteristics of the bacteria were evaluated using Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

**Purification and Identification of Fungal Isolates**

Colonies from the primary plates were aseptically picked with a sterile inoculation needle and transferred onto a freshly prepared sterile SDA plate with a streaking method and incubated at 28 ± $2^0$C for 72 h. Discrete colonies were aseptically transferred and stocked in the plates and incubated for at 28 ± $2^0$C for 3-5 days. Pure colonies were stored in the refrigerator until needed for characterization and identification Fungal isolates were identified based on their colony growth pattern, conidia, morphology and pigmentation (Akinjogunla and Eghafona, 2012). A few drops of lacto phenol in cotton blue was placed on clean grease free slide. A small portion of the mycelium was picked with a sterile inoculating loop. The portion was placed in the lacto phenol cotton blue droplet on the slide and emulsified out with a sterile wire loop. The slide was covered with a cover slip and viewed under a light microscope using the 40x objective lens. The structure of the mycelium, spore structure and fruiting bodies were identified with the help of Standard Colour Atlas of Diagnostic Microbiology by Watanabe (2002).

## RESULTS AND DISCUSSION

**Densities of Bacteria and Fungi in Crude Oil Contaminated Sediment from Eniong River**

The range of total heterotrophic bacterial counts (THBC) obtained in sediment samples from three different sampling locations (A, B and C) varied from 8.5 x $10^4$ CFU/g to 1.0 x $10^5$ CFU/g, with a mean count of 9.3 x $10^4$ CFU/g (Fig. 2). The highest THBC was obtained in location A (upstream), while the lowest THBC was obtained in location C (downstream). The result of the total fungal count (TFC) ranged from 1.8 x $10^4$ CFU/g to 3.0 x $10^4$ CFU/g with a mean count of 2.4 x $10^4$ CFU/g. Location B (midstream) had the lowest TFC, while Location C (downstream) had the highest TFC. (Fig. 3).

**Densities of Pollution Indicator Bacteria from Eniong River Sediment Ecosystem**

The densities of pollution indicator bacteria such as total coliform, faecal coliform, *Salmonella* spp., *Shigella* spp., and *S. aureus* in sediment samples collected from three distinct stations in Eniong River ecosystem are presented in Figs. 4-7. The results showed that the total coliform count (TCC) ranged from 1.5 x $10^4$ to 4.7 x $10^4$ (CFU/g) with a mean count of 2.9 x $10^4$CFU/g (Fig. 4), while the faecal coliform count (FCC) ranged from 1.0 x $10^4$ to 3.4 x $10^4$ (CFU/g) with a mean count of 2.3 x $10^4$ CFU/g (Fig. 5). The highest and lowest TCC and FCC were obtained at station A and C, respectively. *Salmonella-Shigella* count (SSC) ranged between 3.1 x $10^4$ and 4.0 x $10^4$ (CFU/g) with a mean count of 3.7 x $10^4$ CFU/g (Fig. 6). *S. aureus* count (SAC) varied from 1.2 x $10^4$ to 2.2 x $10^4$ (CFU/g) with a mean count of 1.7 x $10^4$ CFU/g (Fig. 7). The highest and lowest *Salmonella-Shigella* counts were obtained from the sediment samples from location B and C, respectively.

**Morphological and Biochemical Characteristics of Bacteria Isolated from Crude Oil Contaminated Sediment from Eniong River**

The morphological and biochemical characteristics of bacteria isolated from crude oil-contaminated sediment from the Eniong River are presented in Table 1. The bacteria isolated were *Staphylococcus epidermidis, Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Klebsiella* spp., *Micrococcus* spp., *Aeromonas* spp., *Bacillus subtilis*, and *Pseudomonas aeruginosa.*

**Morphological and Microscopic Characteristics of Fungi Isolated from Crude oil Contaminated Sediment from Eniong River**

The morphological and microscopic characteristics of fungi isolated from crude oil-contaminated sediment from the Eniong River are presented in Table 2. The fungi isolated belongs to genera *Aspergillus, Penicillium, Fusarium, Rhizopus, Geothrichium* and *Candida* (Table 2).

**Distribution of Total Heterotrophic Bacterial Isolates from Eniong River Sediment**

The distribution and occurrence of heterotrophic bacterial species in Eniong River sediment samples collected at three different stations are presented in Table 3. The results showed that the most encountered bacterial species was *B. cereus* (16%), followed by *B. subtilis* (14%), and *Micrococcus* sp. (12%), while the least encountered were *Aeromonas* sp. and *Salmonella* sp. (4%). Also, location A had the highest number of bacterial species (44%), while locations B and C had equal occurrences of bacterial isolates (28%).

**Distribution of Fungal Isolates from Eniong River Sediment**

The percentage distributions of fungi isolated from Eniong River sediment are presented in Table 4. The result showed that the most isolated fungus was *Aspergillus niger* (31.8%), followed by *Fusarium* sp. (18.2%) and *Aspergillus fumigatus* (13.6%), with *Candida* sp. and *Geothrichium sp* being the least occurring fungal isolates.

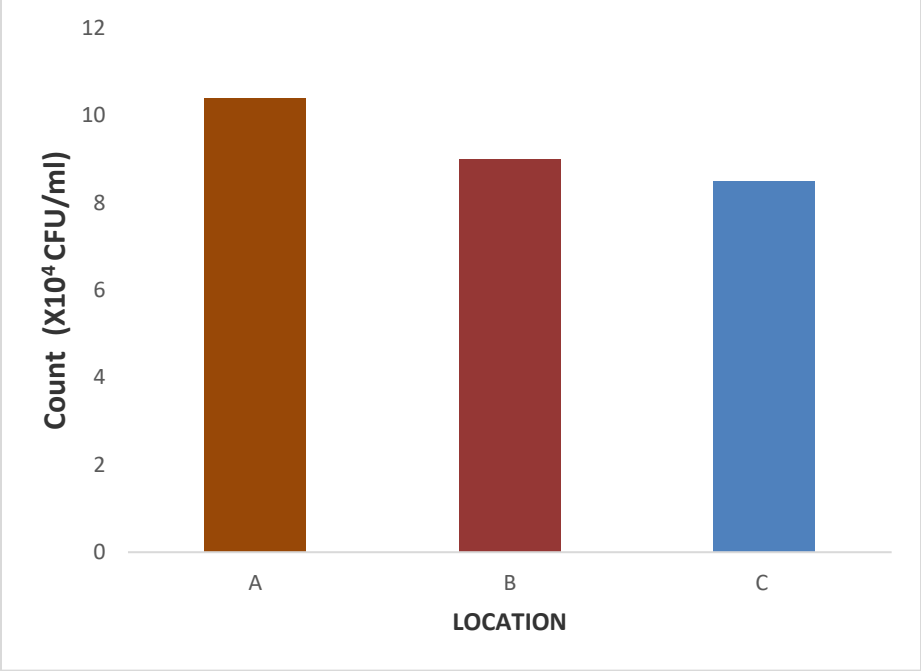


Figure 2: Total Heterotrophic Bacterial Counts of Sediment Samples from Eniong River

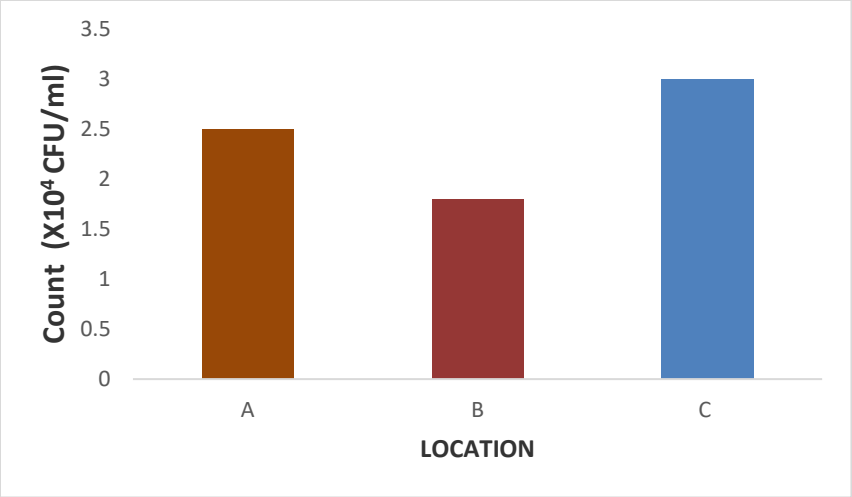


Figure 3: Total Fungal Counts of Sediment Samples from Eniong River

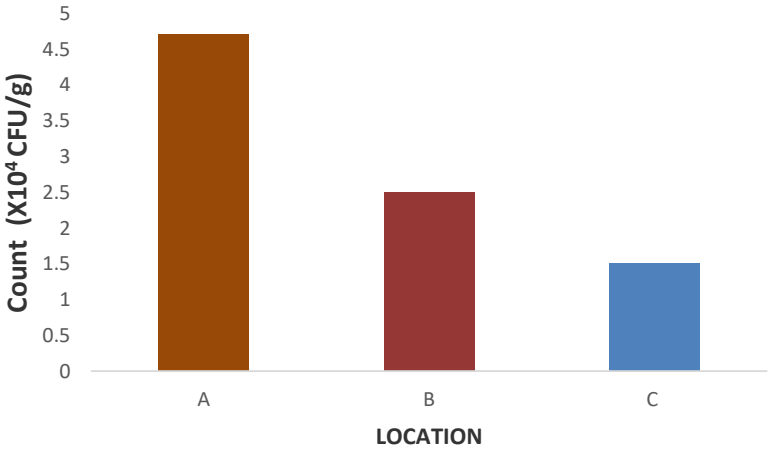


Figure 4: Total Coliform Count of Sediment Samples from Eniong River

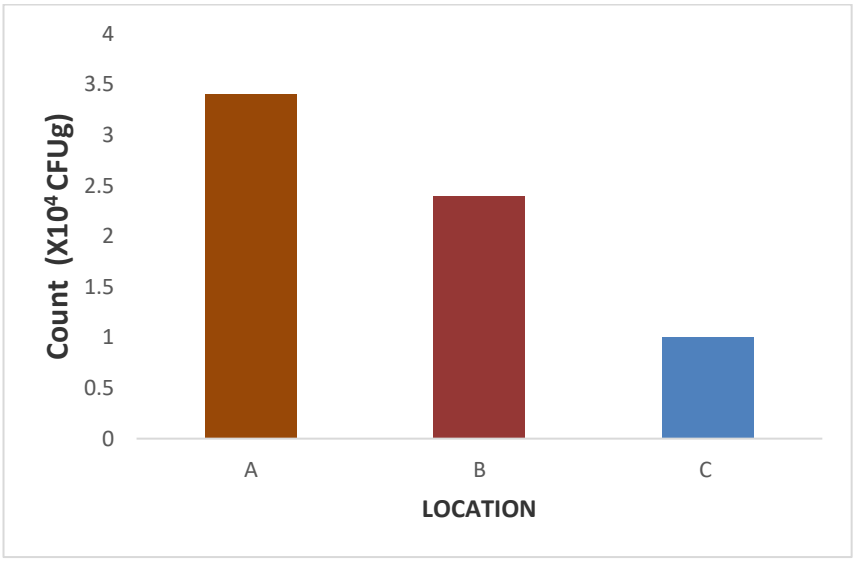


Figure 5: Faecal Coliform Count of Sediment Samples from Eniong River

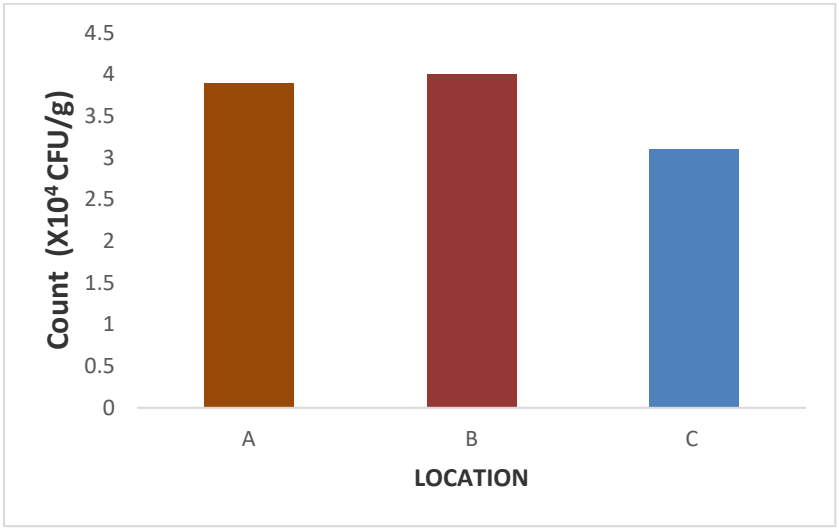


Figure 6: *Salmonella-Shigella* Count of Sediment Samples from Eniong River

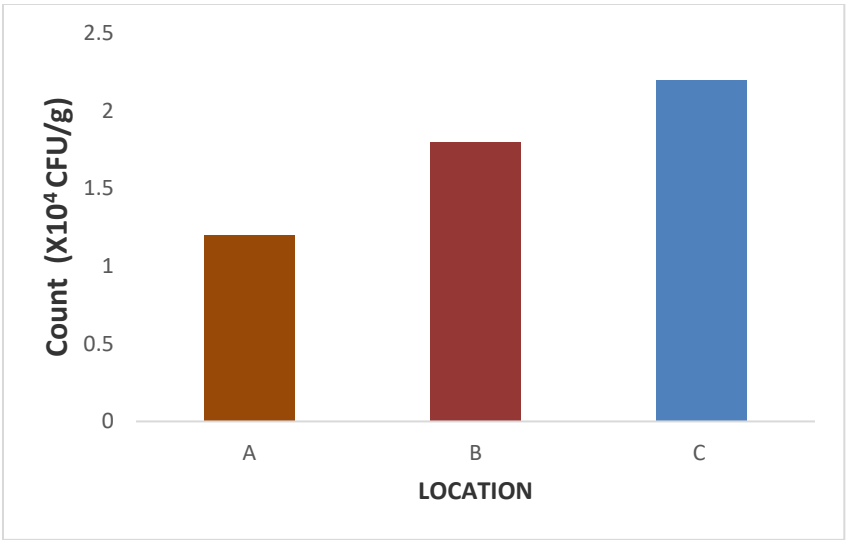


Figure 7: *Staphylococcus aureus* Count of Sediment Samples from Eniong River

**Table 1: Morphological and Biochemical Characteristics of Bacteria Isolated from Crude oil Contaminated Sediment from Eniong River**

| S/N | Gram | Citrate | Catalase | Coagulase | Spore | Indole | MR | VP | Oxidase | Motility | Urease | Glu | Suc | Lac | Man | Probable Organism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | + | - | + | - | - | - | - | + | - | - | + | + | + | + | + | *S. epidermidis* |
| 2. | - | - | + | - | - | - | + | - | - | + | - | + | - | + | + | *Salmonella spp* |
| 3. | - | - | + | - | - | - | + | - | - | - | - | - | - | - | + | *Shigella spp* |
| 4. | + | + | + | + | - | - | + | - | - | - | + | + | + | + | + | *S. aureus* |
| 5. | - | - | + | - | - | + | + | - | - | + | - | + | - | + | - | *Escherichia coli* |
| 6. | + | + | + | - | + | - | - | + | - | + | - | + | - | - | - | *Bacillus cereus* |
| 7. | - | + | + | - | - | + | - | + | - | - | + | + | + | + | + | *Klebsiella spp* |
| 8. | + | - | + | - | - | - | + | - | + | - | + | + | + | - | + | *Micrococcus spp* |
| 9. | - | + | + | - | - | + | - | + | + | + | - | + | - | - | + | *Aeromonas spp* |
| 10. | - | + | + | - | - | - | - | - | + | + | - | - | - | - | - | *P. aeruginosa* |
| 11. | + | + | + | - | + | - | - | + | + | + | - | + | + | - | + | *Bacillus subtilis* |

Key: Methyl red: MR; Voges Proskauer: VP; Glucose: Glu; Sucrose: Suc; Lactose: Lac: Mannitol: Man; Positive: +; Negative: -

**Table 2: Morphological and Microscopic Characteristics of Fungi Isolated from Crude oil Contaminated Sediment from Eniong River**

| S/N | Morphological/cultural characteristics on SDA | Microscopic characteristics | Suspected organism |
|---|---|---|---|
| 1. | Colonies exhibited white mycelia growth with dark spots which later increases with time. The margin of the fungi was whitish while the colonies were yellowish. | Non-branched conidiophores with bulb end carrying conidia like sunrays. Fruiting body (cleistothecia) present. | *Aspergillus niger* |
| 2. | Colonies are whitish-green with margins within | Long erect conidiophores with round conidia. Cleistothecia present. | *Penicillium chrysogenum* |
| 3. | Yellow at first but quickly becoming brown to yellow green with radial grooves cottony and powdery colony | Chains of round conidia with conidial heads in compact columns. Presence of uniseptate phialides that concentrate on the upper surface of the vesicle. Cleistothecia present. | *Aspergillus fumigatus* |
| 4. | White colonies resembling cotton but with less aerial mycelia growth. | Hyphae are septate. Produces rod-like and slightly bent macroconidia. | *Fusarium* spp. |
| 5. | Fluffy and whitish hyphal that turns brown after three days. Aerial mycelia growth is observed with the reverse plate showing yellow. | Hyphae is non-septate, sporangiospores are ovoid and directly opposite the branched rhizoid. | *Rhizopus* spp. |
| 6. | White spongy colony with creamy underside, filamentous and slowly growing not filling the plate | Presence of septate hyphae with many anthroconidia that are rectangular in shape. | *Geothrichium* spp. |
| 7. | Creamy colonies that are smooth, convex and opaque with a yeasty odour | Presence of blastoconidia with small rounded clusters and pseudohyphae. Cleistothecia present. | *Candida* sp. |
| 8. | White cotton colony with white underside, mucoid and rapidly filling the plate | Single and branching sporangiospores with round sporangium at the tip. | *Mucor* spp. |

Key: Sabouraud dextrose agar: SDA

**Table 3: Distribution of Total Heterotrophic Bacterial Isolates from Eniong River Sediment**

| SN | Bacterial Isolates | Upstream A | Midstream B | Downstream C | Total | Occurrence % |
|---|---|---|---|---|---|---|
| 1 | *Staph. epidermidis* | +3 | +2 | -0 | 5 | 10.0 |
| 2 | *Salmonella sp.* | +1 | +1 | -0 | 2 | 4.0 |
| 3 | *Shigella sp.* | +1 | +1 | +1 | 3 | 6.0 |
| 4 | *Staph. aureus* | +2 | +1 | +1 | 4 | 8.0 |
| 5 | *Escherichia coli* | +2 | +2 | +1 | 4 | 8.0 |
| 6 | *Bacillus cereus* | +4 | +1 | +3 | 8 | 16.0 |
| 7 | *Klebsiella sp.* | +1 | +1 | +1 | 3 | 6.0 |
| 8 | *Micrococcus sp* | +3 | -0 | +3 | 6 | 12.0 |
| 9 | *Aeromonas sp* | +2 | -0 | 0 | 2 | 4.0 |
| 10 | *Pseudomonas. aeruginosa* | -0 | +3 | +2 | 5 | 10.0 |
| 11 | *Bacillus subtilis* | +3 | +2 | +2 | 7 | 14.0 |
| 12 | Species richness | 22(44.0%) | 14(28.0%) | 14(28.0%) | 50 | 100 |

**Table 4: Percentage distribution and occurrence of fungi isolated from Eniong River sediment**

| SN | Fungal isolate | Upstream A | Midstream B | Downstream C | Occurrence % |
|---|---|---|---|---|---|
| 1 | *Aspergillus niger* | +3 | +2 | +2 | 31.8 |
| 2 | *Penicillium chrysogenum* | +2 | 0 | 0 | 9.1 |
| 3 | *Aspergillus fumigatus* | +2 | +1 | 0 | 13.6 |
| 4 | *Fusarium* sp. | +1 | +2 | 0 | 18.2 |
| 5 | *Rhizopus* sp. | +2 | 0 | 0 | 9.1 |
| 6 | *Geothrichium* sp. | +1 | 0 | 0 | 4.5 |
| 7 | *Candida* sp. | +1 | 0 | 0 | 4.5 |
| 8 | *Mucor* sp. | +2 | 0 | 0 | 9.1 |
| 9 | Species richness | 14(63%) | 4(18%) | 2(9%) | 100 |

**Discussion**

The exploitation of microorganisms' ability to detoxify or break down organic contaminants into non-harmful components for the biological remediation of environments contaminated with substances like crude oil has been reported (Mbachu *et al*., 2014). In aquatic sediment, bacterial communities respond to hydrocarbon pollution through aerobic processes in surface sediments and anaerobic processes in subsurface sediments.

The result of the study revealed that in the sediment ecosystem of the Eniong River, the mean THBC in the polluted sediment sample was higher than the mean TFC. These findings are consistent with the previous findings by Agu *et al.* (2015) in Kwata, Awka South, Nigeria, and by Imarhiagbe and Onwudiwe (2022) in Benin City, Nigeria, indicating a trend where bacterial populations outnumber fungal populations in polluted sediments. This predominance of bacteria over fungi may be attributed to the nutrient composition of the sediment, coupled with the preponderance of indigenous microbial sediment flora and the existence of some toxic substances that hinder the growth of many fungi. In addition, the study showed that the sediment from upstream and downstream locations had the highest THBC and TFC, respectively, when compared to other locations. The variance in microbial diversity and distribution across these locations may be due to variations in oil pollution concentrations at the respective locations (Nakatsu, 2007; Green *et al.*, 2015). Sediment samples showed bacterial counts exceeding WHO drinking water standards, indicating substantial organic contamination likely from human and animal activities, including runoff and sewage (Uzoigwe and Agwa, 2012). The microbial population within the Eniong River sediment is continually evolving due to spillages of crude oil and other petroleum products, biomass, and the ecosystem of the sediment. This change affects the diverse microbial group establishment in an attempt to remedy the petroleum product spillage (Mishra *et al*., 2001).

The bacterial isolates encountered in the crude oil-contaminated sediment in our study were *B. cereus, B. subtilis, Micrococcus* sp., *S. epidermidis, P. aeruginosa, E. coli, Salmonella* sp., *Shigella* sp., *Klebsiella* sp., *S. aureus,* and *Aeromonas* sp. This corroborated the findings of Agu *et al.* (2015) and Imarhiagbe and Onwudiwe (2022). The detection of enteric bacteria such as *Salmonella*, *Shigella*, and *E. coli* suggests contamination from faecal and municipal waste, posing potential health risks for people using the water (AOAC, 2005, Akinjogunla *et al*., 2023; Odeyemi *et al*., 2023). Similarly, the presence of bacteria such as *S. aureus*, *Salmonella* sp., *Shigella* sp., and *B. subtilis* in sediment samples underlines their public health significance due to their toxin-producing capabilities and potential for causing diseases like diarrhoea and nausea (APHA, 2005; Akinjogunla *et al*., 2021). *Pseudomonas aeruginosa*, when exposed to hydrocarbons, can evolve to utilize these compounds as energy and carbon sources. This adaptability makes them key players in the microbial degradation of hydrocarbons, offering a promising solution to pollution issues through bioremediation (Kumar and Philip, 2006). *Pseudomonas aeruginosa* effectively breaks down toxic substances found in crude oil-polluted sediment and water by metabolizing crude oil, transforming environmental pollutants into non-harmful substances. This showcases its potential in environmental clean-up, especially in the ex *situ* bioremediation of crude oil-contaminated sites (Akhundova and Atakishiyeva, 2015).

In our study, *A. niger, A. fumigatus, P. chrysogenum, Fusarium* sp., *Rhizopus* sp., *Geothrichium* sp., *Candida* sp., and *Mucor* sp. were the eight fungal species obtained from the crude oil-polluted sediment samples, with *A. niger* being the most abundant fungal species. These findings are similar to those of Okigbo and Okafor (2022), in which *A. niger* was reported as the most pronounced fungal species from sediment samples from aquatic ecosystems. Similarly, our findings substantiated the reports of Sanyaolu *et al.* (2012) on the isolation of *Aspergillus* spp. from crude oil-polluted samples.

**CONCLUSION**

The study of fungal and bacterial isolates from the Eniong River has provided valuable insights into the microbial

ecology of crude oil-polluted sediments and highlights the importance of microorganisms in the natural attenuation of environmental pollutants. This work also laid the foundation for future biotechnological applications aimed at mitigating and reducing pollution and improving the condition of Nigeria's aquatic environments.

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