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Full length Research paper

Identifying Diesel Utilizing Bacteria in Sediments: A Case Study of Qua River Sediment

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To isolate and quantify diesel utilizing bacteria from Qua river sediments and ascertain their tolerance levels to varying concentrations of diesel was the primary aim of this study. Samples were collected and processed using standard microbiological techniques. Screening tests was then carried out using the vapour phase transfer method and incubated at room temperature (28±2^{0}C). The highest diesel utilizing bacterial count was 9.7 x 10^{3}CFU/g recorded for sample(3) while the least bacterial count was 6.0 x 10^{3}CFU/g recorded for sample one(1). *Pseudomonas sp., Micrococcus luteus* and *Bacillus sp*. were the identified diesel utilizing bacterial isolates. Tests to determine the tolerance of these isolates to 1%, 3%, 5% and 7% diesel in Mineral salt broth proved by optical densities (OD_{600nm}) that *Micrococcus luteus* showed less growth (OD_{600nm}) to 1%(0.279), 3%(0.253) and 5%(0.154) diesel than *Pseudomonas* sp. at1%(0.685), 3%(0.483) and 5%(0.466) and *Bacillus* sp. at 1%(0.509), 3%(0.452) and 5%(0.390) but showed slightly higher growth (OD_{600nm}) at 7%(0.1) than *Pseudomonas* sp. (0.095) and *Micrococcus luteus* (0.093). ANOVA at 5% significance level proved that there is significant difference of diesel concentrations on growth (OD_{600nm}) of these isolates. These results highlight Qua River as a potential source for petroleum bioremediating bacteria.

Key words: diesel-utilizing bacteria, sediments, hydrocarbon degradation, bacterial identification, bioremediation

INTRODUCTION

Sediments are a major part of an aquatic ecosystem superimposed by a permanent water body be it an ocean, fjord, lake or reservoir which usually contain allochthonous and autochthonous organic matter with ability to stimulate a favorable response in aquatic residues (Jian et al., 2022). Biological activities and microbial diversity predominate the sediment region compared to the liquid portion of the water body. Sediments share some properties with soil and yet differ from soil environments for various reasons, many of which favour the population of microorganisms that reside in sediments.

Diesel oil is one of the most complex mixtures comprising of saturated and aromatic hydrocarbons which cannot easily be degraded (Monica *et al.,* 2021). The saturated hydrocarbons dominant in diesel fuels include normal paraffins, isoparaffins and cycloparaffins (naphthenes). Most of the isoparaffins in diesel fuels are mono and dimethyl alkanes, predominantly with branch points near the ends of the alkyl backbone. Cycloparaffins present are mainly the 1- to 3-ringed respectively. The 4- and 5-ring cycloparaffins such as steranes and hopanes are rarely present. Aromatic compounds are mainly benzenes, indans, tetralins, indenes, naphthalenes, biphenyls, acenaphthenes, fluorenes, acenaphthalenes, phenanthrenes, anthracenes and naphthenophenanthrenes. Diaromatic hydrocarbons are generally the most abundant aromatic components in diesel fuels. Trace amount of polycyclic aromatic hydrocarbons such as chryenes, pyrenes, benzanthracenes and perylenes can also be present. The most common sulphur compounds are benzothiophenes and dibenzothiophenes. Minute amounts of nitrogen

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compounds include indoles, carbazoles, quinolines, acridines and phenanthridines. The oxygen compounds mostly present are phenols and dibenzofurans. Formulated diesel fuels also contain small amounts of additives which aid fuel combustion effectiveness, lower fuel usage, decrease engine wear and improve cold weather performance (Ahyan and Mahmut,2021). These additives include cetane improvers, antioxidants, corrosion inhibitors, metal deactivators, dispersants, detergents, lubricity agents, deemulsifiers and cold-flow improvers to enhance storage stability and performance (Chunsan, 2020). Alkanes, cycloalkanes and aromatic compounds that are present in diesel are not easily metabolized (Shen *et al.,* 2023). Hydrocarbons of larger molecular weight exhibit a low biodegradation rate primarily due to their poor solubility in water (Liu *et al.,* 2020; Haijun *et al.,* 2022).

Microorganisms are ubiquitous and easily adaptable bioentities occurring throughout the biosphere owing to their biochemical proficiency (Veler *et al.,* 2020; Viera *et al.,* 2020; Essien *et al.,* 2023). Forhad *et al.,* (2021); Imam *et al.,* (2022) stated that many microorganisms are involved in the degradation of complex toxic components of diesel into nontoxic substances which serve as a source of carbon and energy, hence the presence of these microbial communities in high abundance have a significant effect on the planetary biogeochemical cycle (Ivanova *et al.,* 2023;Mullaeva *et al.,* 2022; Lili *et al.,* 2021). Xu *et al.,* 2018 further stated that the abundance and variety of hydrocarbonoclastic bacteria vary according to the predominant types of petroleum hydrocarbons and local environmental conditions.Due to their microscopic size and metabolic diversity, the bulk of the biomass and chemical activities in sediments is attributed to bacteria in comparison to fungi (Lili *et al.,* 2021). Muhammad *et al.,* (2019) stated that bacteria are widely utilized as agents of diesel fuel bioremediation; continuously proliferating throughout the treatment process. However, Brzeszcz *et al.,* (2024) suggested that a consortium of bacteria with augmented functionalities would do better at diesel fuel biodegradation compared to an undetermined consortium of spontaneously selected degraders.

Employing a consortium offered greater advantages compared to using an individual strain, primarily due to the consortium's enhanced metabolic functions and resilience(Quo *et al.,* 2023; Luo et al. 2021)When *Micrococcus spp* and *Pseudomonas spp* were isolated from diesel engine oil contaminated soil samples after cultural and biochemical characterization, the study revealed that when used individually, *Micrococcus spp* had a slower diesel degradation rate compared to *Pseudomonas spp.* Studies have also shown that *Bacillus* and *Pseudomonas* are good biodegraders of saturated and aromatic hydrocarbons; especially *Pseudomonas* with an exceptional ability to completely degrade resin and asphaltene (Bo *et al.,* 2023). Anjum S. (2022) carried out a research on diesel fuel degradation by both isolates over an estimated period of 25days, and observed that *Pseudomonas spp* was able to degrade 67.57% of oil while *Micrococcus spp* reduced 52.95%. Nevertheless, a combination of both bacterial isolates revealed a higher diesel fuel degradation potential of 89.98% (Sayeda, 2022). This is due to the co-metabolism of the two microorganisms which are individually proficient in diesel oil degradation (Jia *et al.,* 2019; Sun *et al.,* 2021; Bo *et al.,* 2023).

Hydrocarbon utilizing bacteria possess the biochemical ability to degrade hydrocarbons aerobically or anaerobically although the aerobiotic is faster and is thermodynamically favorable (Idongesit *et al.,* 2020; Brzeszcz1 *et al.,* 2023). These microbes occur ubiquitously across marine systems in the water column, sediments, beach sands and marsh mud (Joanna *et al.,* 2023). Microbial hydrocarbon degraders such as *Oceanospirillum, Colwellia, Cycloclasticus, Pseudoalteromonas* (Muhammad *et al.,* 2020)*, Alkanivorax, Alteromonas*, and *Marinobacter* fall within the Gammaproteobacteria. The Betaproteobacteria include *Acidovorax* and *Burkholderia*, *Roseobacter*for Alphaproteobacteria and numerous Deltaproteobacteria as well as Actinomycetes*, Bacillus* and other taxa (Farah *et al.,* 2021).*Rhodococcus* sp. has been reported to be present in samples from arctic soil capable of utilizing a broad range of aliphatics (C_{10} to C_{21} alkanes, branched alkanes and a substituted cycloalkane) present in diesel oil at 5^{o}C (Idongesit *et al.,* 2020). However, *Pseudomonas aeruginosa* is the most naturally occurring potent oil degrading bacteria for environmental pollution abatement owing to its multifarious biochemical makeup (Fanghui *et al.,* 2023).

Various species of *Pseudomonas* have been reported for diesel degradation such as *Pseudomonas stutzeri* and *Pseudomonas fluorescens* (Muhammed *et al.,* 2020). Also, *Staphylococcus* sp. (Essien *et al.,* 2023) and *Bacillus* sp. (Gessesse *et al.,* 2022) have been isolated as diesel degrading microorganisms from a diesel contaminated soil.

Petroleum derived diesel fuel is used by different vehicles, diesel generators and especially transport vehicles. Its manufacturing, transportation, utilization and disposal have the threat to pollute the surrounding environment. Biodegradation is one of the biological processes to remediate these pollutants (Farah *et al.,* 2021). The ability to isolate high numbers of certain oil-degrading microorganisms from petroleum contaminated sites is commonly taken as evidence that these microorganisms are the active degraders of that environment (Farah *et al.,*2021; Gessesse,2022).The aim of the present study is to isolate, identify and quantify the bacterial population capable of utilizing petroleum derived diesel fuel as their sole carbon source. Hence establishing Qua River sediment as a potential source site for diesel remediating bacteria.

MATERIALS AND METHODS

Materials

Materials used for this study are grouped into glassware, equipment, media and reagent. The glassware used for this study were: petri dishes, glass slides, test tubes, measuring cylinders, pipettes, beakers, conical flasks, micro syringe and sample cans. Equipment used for this study was: autoclave, incubator, weighing balance, refrigerator, Bunsen burner, microscope, hot air oven, spectrophotometer and wire loop.
All the culture media used for this study were of analytical grade and were prepared according to the manufacturer's specification. Culture media used include: mineral salt agar, nutrient agar, motility indole ornithine agar, glucose agar, lactose agar, sucrose agar, methyl red Voges Proskauer agar, Simmon's citrate agar, urease agar. Reagents used were: Gram's reagents, acetone, hydrogen peroxide, ethanol, methylene blue, sodium chloride, phenol red.

Description of Study Area

One of the main tributaries of the Cross River Estuary is the Great Kwa River. It originates in Aningeje, Cross River State, Nigeria's Oban Hills and runs south, emptying into the Cross River Estuary at around latitude 4˚45'N and longitude 82˚E. The Great Kwa River empties into the Cross River Estuary at roughly latitude 4˚45'N and longitude 8˚20'E, while the Calabar River joins the main Cross River at a distance of about 8 km2 to the south.
The vast flood plain and wetlands formed by the river system, which is made up of the Great Kwa River and the Cross River, as well as numerous tributaries, discharge into the Cross River Estuary(Eyo and Ekpo, 2013; Iboh *et al.,* 2016).

Sample Collection and Processing

Three sediment samples were collected from the not-too-shallow depths of the river situated behind the University of Calabar Female Hostel Hall 9. The samples were collected 10m apart with the aid of a hand trowel and transferred into wide mouthed glass containers with Teflon lined caps. Collected samples were immediately conveyed to the University of Calabar Microbiology Laboratory. A stock dilution was prepared from the three (3) samples by transferring 1g of sediment into 9ml of distilled water in a test tube followed by a Ten-fold serial dilution which was carried out up to the seventh (7^{th}) level (10^{-7}).

Total Heterotrophic Bacterial Count (THBC)

The Total Heterotrophic Bacterial Count was estimated using the spread plate count method with nutrient agar used as the growth medium. From the last turbid dilution (10^{-4}), 0.1ml was inoculated in nutrient agar plates and incubated at 30^{o}C for 24hours. After 24hours, plates were examined for colonial growth. Colonies were numbered and Colony Forming Units per gram (CFU/g) of sediment was calculated.

Screening for Diesel Utilizing Bacterial Isolates

Mineral salt agar medium was used to screen for diesel utilizing bacterial isolates using the vapour phase transfer technique. This technique enables the isolation of bacteria capable of utilizing diesel fuel as their sole source of carbon. Using the spread plate technique, 0.1ml of the diluted sediment sample was transferred from 10^{-4} dilution level into already prepared mineral salt agar medium in petri dishes for each sample respectively. Diesel oil was sterilized by filtration via the use of filter paper having pore sizes of 0.45µm (Whatman No. 1). The filter paper was saturated with the sterilized diesel oil and placed over the lid of the petri dish. Petri dishes were covered and sealed with masking tape and incubated at room temperature (28±2^{o}C) for 5 days. The total diesel utilizing bacterial colonies were numbered as well as counts for each isolate of distinct colonial morphology.

Purification and Maintenance of Diesel Fuel Utilizing Bacterial Isolates

Diesel fuel utilizing bacterial isolates were subcultured into nutrient agar plates and finally stored in nutrient agar slants in McCartney bottles as stock. The stocked isolates were stored in the refrigerator at 4^{o}C.

Tolerance of Diesel Utilizing Bacterial Isolates to Diesel Fuel

Mineral salt broth was prepared with different percentage compositions of sterile diesel oil (1%, 3%, 5% and 7%) for each isolate respectively. The tubes were inoculated with respective isolates and incubated at room temperature (28±2^{o}C) for 14 days after which optical densities (OD_{600nm}) readings were taken.

Biochemical Characterization and Identification of Hydrocarbonoclastic Bacterial Isolates

Each diesel utilizing bacterial isolate was identified based on their performance in the following biochemical tests: gram staining, catalase test, methyl red voges proskauer (MR-VP) test, motility test, urease test, nitrate reduction test, lactose fermentation, glucose fermentation, sucrose fermentation, oxidase test, citrate test and indole test. Identification of isolates was done using the bergey's manual of determinative bacteriology (Bergey and Holt, 2000).

FIGURE 1: Map showing the location of Kwa/Qua River along the Cross River estuary
SOURCE: (Evans *et al.,* 2017).

Total Heterotrophic and Diesel Utilizing Bacterial Count in Sediment Samples

Table 1: Total heterotrophic and diesel utilizing bacterial count

Sample Code	Total Heterotrophic Bacteria	Total Diesel Utilizing Bacteria
1	1.41 x 10^5CFU/g.	6.0 x 10^4CFU/g
2	1.61 x 10^5CFU/g	8.7 x 10^4CFU/g
3	1.95 x 10^5CFU/g	9.7 x10^4CFU/g

Biochemical Characterization of Diesel Utilizing Bacteria Isolates

Table 2: Morphological and Biochemical identification of the test isolates

Isolate Code	Colony Characteristics	Gram stain	Cell morphology	Endospore	Motility	Catalase	Oxidase	Citrate	Indole	Glucose	Lactose	Sucrose	Methyl red	Voges proskauer	Urease	Nitrate reduction	Organism
C1	1-2mm, circular, entire margin with convex elevation, yellow pigmentation, butyrous and opaque	+	Cocci, Pairs	-	-	+	-	-	-	NA	NA	NA	-	-	-	+	*Micrococcus luteus*
C2	2-3mm in size, irregular shaped with lobate margin, flat elevation, fluorescent green, mucioidal and translucent.	-	Rods, Solitary	-	-	+	+	+	-	NA	NA	NA	-	-	-	-	*Pseudomonas* sp.
C3	3-4mm in size, irregular shaped, lobate margin, flat elevation, creamy, pigmentation/ colour, dry and opaque	+	Rod, Solitary	+	+	+	+	-	-	A	A	A	-	+	+	+	*Bacilius* sp.

Key:-: Negative, +: Positive, NA: No acid produced, A: Acid produced

Table 3: Growth (OD_{600nm}) of diesel utilizing bacterial isolates cultured in different concentrations of diesel after 14days of incubation

Isolates	1% diesel	3% diesel	5% diesel	7% diesel
Micrococcus luteus	0.279	0.253	0.154	0.100
Pseudomonas sp.	0.685	0.483	0.466	0.095
Bacillus sp.	0.509	0.452	0.390	0.093

RESULTS

Tolerance Test of Diesel Utilizing Bacterial Isolates and Statistical Analysis.

The tolerance rate of the diesel utilizing bacterial isolates was determined by their optical densities in 1%,3%,5% and 7% diesel oil after 14 days of incubation (see Appendix III). *Micrococus luteus* showed less growth (OD_{600nm}) to 1% (0.279), 3% (0.253) and 5% (0.154) diesel than *Pseudomonas* sp. (0.685 at 1%, 0.483 at 3% and 0.466 at 5%) and *Bacillus* sp. (0.509 at 1%, 0.452 at 3% and 0.390 at 5%) but showed slightly higher growth (OD_{600nm}) at 7% (0JZ.1) than *Pseudomonas* sp. (0.095) and *Micrococcus luteus* (0.093). Using the growth (OD600nm) values, a bar chart was used to show the growth pattern of the diesel utilizing bacterial isolates as seen in **Figure 1.**

Two-factor Analysis of Variance (ANOVA) to Test for the Effect of Various Concentrations of Diesel on the Growth (OD_{600nm}) of Hydrocarbonoclastic Bacterial Isolates

Two-factor Analysis of Variance (ANOVA) to test the effect of the various concentrations of diesel on the growth (OD_{600nm}) of the hydrocarbonoclastic bacterial isolates at 95% confidence interval reveals that there is significant difference in the growth (OD_{600nm}) of the hydrocarbonoclastic bacterial isolates in various concentrations of diesel as $F_{critical}$=5.4 and $F_{calculated}$ =7.0 at p=0.027 for rows and Fcritical=4.8 and Fcalulated=10.19 at p=0.009 for columns as seen in **Table 4** below. Hence, Growth (OD600nm) is affected significantly by the diesel concentrations and the hydrocarbons bacterial isolates.

DISCUSSION

Most petroleum hydrocarbons encountered in the environment are ultimately degraded or metabolized by indigenous bacteria because of their energetic and carbon needs for growth and reproduction as well as the requirement to relieve physiological stress caused by the presence of petroleum hydrocarbons in the microbial bulk environment (Kleindiest *et al.,* 2015). Many studies have revealed that there is a large number of hydrocarbon degrading bacteria in oil-rich environments such as oil spilled areas and oil reservoirs (Yang *et al.,* 2015), and that their abundance and quantity are closely related to the types of petroleum hydrocarbons and the surrounding environmental factors (Udgire *et al.,* 2015). From the three (3) sediment samples used for this study, the highest Total Heterotrophic Bacterial Count was 1.95 x 10^5CFU/g and the highest diesel utilizing bacterial count was 9.7x 10^4CFU/g. Both counts were obtained from Sample(3) which was collected from more deeper sites than other samples. The least Total Heterotrophic Bacterial Count was 1.41x 10^5CFU/g and the least diesel utilizing bacterial count was 6.0 x 10^4CFU/g. Both counts were obtained from sample(1) which was collected from the shallowest parts of the river (see table 1). From a study on the bacterial degradation of crude oil by Ihsan *et al.,* (2022), the total viable count of *Bacillus subtilis* and *Pseudomonas aeruginosa* were 2.4 x 10^9CFU/g and 2.67x 10^9CFU/g respectively. These counts are much higher than my study counts because the bacterial organisms were isolated from crude oil contaminated sites. Whereas, Qua river is not and has never been contaminated with hydrocarbons. In hydrocarbon contaminated sites, hydrocarbonclastic bacterial counts are much higher due to natural selection.

From this study, *Micrococcus luteus*, *Pseudomonas* sp. and *Bacillus* sp. *w*ere identified as diesel utilizing bacterial isolates. This coincides with a study by Varjani and Upasani (2016), in which *Pseudomonas aeruginosa* NCIM 5514 was able to degrade 60.63% of hydrocarbon (C_8-C_{36}) and Bo*et al.,* (2023) who seco-culture of *Pseudomonas aureginosa* and *Bacillus subtilis* revealed great potential in crude oil degradation particularly at an inoculation ratio of 1:1; the degradation proficiency was 63.05% with the n-alkanes close to total degradation. Again, in a study conducted by Idongesit *et al., (*2020), it was observed that in 6weeks, a microbial consortium of *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* combined at a ratio of 2:1 was able to degrade 90% of hydrocarbons.

Bacillus sp. and *Pseudomonas sp.* showed more tolerance to diesel as expressed by their growth (OD_{600nm}**)**. In a gravimetric analysis for diesel fuel degradation ability of seven bacterial isolates, the results

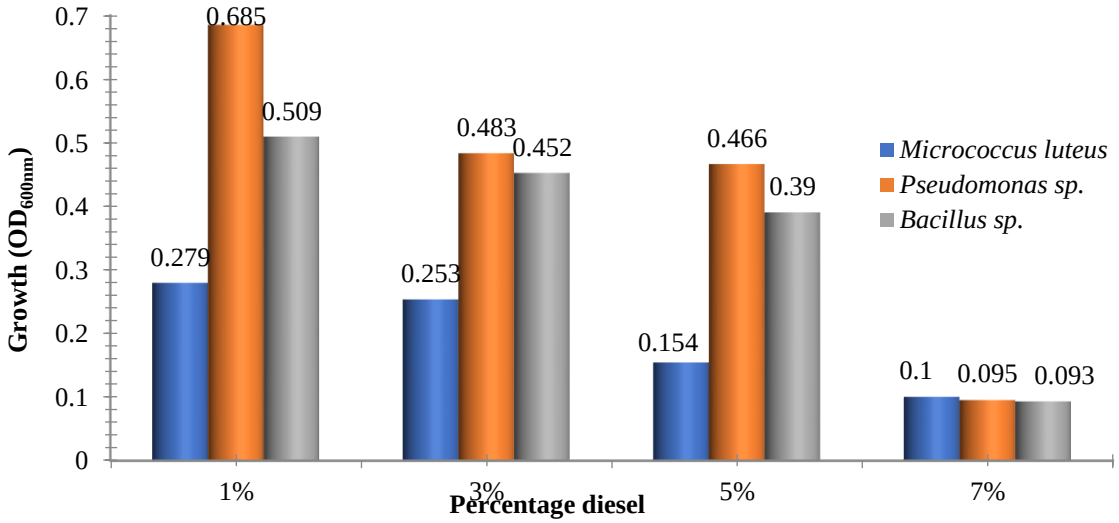

Figure 2: Growth pattern (OD_{600nm}) of hydrocarbonoclastic bacterial isolates exposes to various concentrations of diesel recorded after 14 days of incubation.

Table 4: Two-factor analysis of variance (ANOVA) to test for the significance effect of various concentrations of diesel on growth (OD600nm) of hydrocarbonoclastic species.

Source of Variation	Sum of squares (SS)	Degree of freedom (df)	Mean square (MS)	F ratio	P-value	F critical
Rows (Isolates)	0.1170	2	0.0584	7.0074	0.0270	5.1433
Columns (% diesel)	0.2552	3	0.0851	10.1951	0.0090	4.7571
Error	0.050	6	0.0083			
Total	0.4225	11				

illustrated that in 15days, two isolates of *Pseudomonas spp.* and *Bacillus subtilis* showed degradation rates of 83.6%, 84% and 83% in 5% diesel respectively (Gessesse,2022).In a study, it was stated that *Bacillus* sp. was more tolerant to levels of hydrocarbons in soil due to its resistant endospores (Brzeszcz1, 2023). Similarly in another study, *Pseudomonas pitida* and *Bacillus cereus* degraded crude oil in a crude oil contaminated medium, Growth ability (OD_{660nm}) was measured at the 1st and 7th days after incubation after which results were analyzed, showing that *Pseudomonas putida* had more growth ability in crude oil (Vinothini, Sudhakar and Ravikumar, 2015). Hence the findings of this study further complement the findings of previous research validating the ability of *Bacillus sp.* and *Pseudomonas sp.* to utilize hydrocarbons of petroleum as carbon source.

Micrococcus luteus showed less growth (OD_{600nm}) to 1% (0.279), 3% (0.253) and 5% (0.154) diesel than *Pseudomonas* sp. (0.685 at 1%, 0.483 at 3% and 0.466 at 5%) and *Bacillus* sp. (0.509 at 1%, 0.452 at 3% and 0.390 at 5%) but showed slightly higher growth (OD_{600nm}) at 7% (0.1) than *Pseudomonas* sp. (0.095) and *Micrococcus luteus* (0.093) (see Appendix III). This result corresponds with report that *Pseudomonas* sp. degraded 67.57% of diesel oil compared to 52.95% diesel oil degraded by *Micrococcus* sp. after 25 days of incubation. However, when *Micrococcus* sp. was used for degradation of diesel oil in combination with *Pseudomonas* sp., a greater diesel oil degrading potential was observed this was probably due to the different enzyme system from two different bacterial isolates that acted on the hydrocarbons at a time (Sayeda, 2022). Based on optical density results (OD), *Pseudomonas* and *Bacillus* are considered as having the highest diesel oil degradation potential.

Two-factor analysis of variance (ANOVA) carried out to test the effect of the various concentrations of diesel on the growth (OD_{600nm}) of the hydrocarbonoclastic bacterial isolates at 95% confidence interval reveals that there is significant difference in the growth (OD_{600nm}) of the hydrocarbonoclastic bacterial isolates in various concentrations of diesel as Fcritical=5.4 and Fcalculated =7.0 at p=0.027 for rows and Fcritical=4.8 and Fcalulated=10.19 at p=0.009 for columns as recorded in table 4.

CONCLUSION

The isolation of *Pseudomonas*, *Micrococcus* and *Bacillus* from Kwa river sediments as diesel utilizing Bacteria makes the river a landmark source for microorganisms for bioremediation as these isolated microorganisms have been well noted for their bioremediation potentials. The study recommends that further studies should be carried out at this site and these microorganisms should be engineered to improve their bioremediation potentials.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

Credit Authorship Contribution Statement

Effiom E. Henshaw: Conceptualization, supervision and investigation. **Chioma, B. Ubah and Ujong, E. Confidence:** Methodology, Writing, Review, Original draft and editing. **Ujong, E. Confidence, Bassey O., Inyang, Nsikak, E., Okon, Alice, D., Otoro, Phillip, E., Ita, Raymond Animpuye:** Analysis, review, editing.

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