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Effects of Four Crude Oil Degrading Bacteria Strains on the Physicochemical Parameters of Water from River Erhioke, in Kokori, Delta State, Nigeria

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ABSTRACT: This study investigated the effect of four crude oil degrading bacteria strains (*Pseudomonas putida*strainAY001, *Alcaligenes aquatilis* B61, *Winkia neuii* B12 and *Enterococcus casseliflavus* AAOO-1) on the physicochemical parameters of water from River Erhoike, in Kokori, Delta State, Nigeria using appropriate standard methods. Results obtained show that The pH ranged between 6.3 and 7.3, the dissolved oxygen ranged between 5.80±0.10 and 10.00±0.20, the highest total petroleum hydrocarbon degradation rate was 70% and results showed that .*Pseudomonas putida* strainAY001 which had 70% crude oil degradation rate ranked the best among others, making it good indigenous bacteria with a great potential to perform bioremediation of hydrocarbon polluted environment

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Keyword: Bioremediation; physicochemical; properties; parameters; petroleum.

Crude oil is a major source of environmental pollution, as highlighted by the Intergovernmental Panel on Climate Change (IPCC, 2021), with frequent accidents leading to ecological and social disasters (Das & Mukherjeen, 2007). These events have drastically influenced the biodiversity, distribution, and contamination levels of microorganisms in the environment (Qingguo *et al*., 2020). In the last twenty years, there has been a growing public awareness regarding the environmental impacts of oil extraction activities. The harmful effects of crude oil and its derivatives on plants, animals, humans, and entire ecosystems are extensively documented. (Fowzia and Fakhruddin, 2018). The presence of polycyclic aromatic hydrocarbons (PAHs) in soil and water poses a significant threat due to their persistence as environmental pollutants. Traditional remediation methods, such as volatilization, photooxidation, chemical oxidation, and bioaccumulation (Zhao *et al.*, 2008), often fail to remove PAHs efficiently (Prince and Ronald, 2005), and these methods can be unsafe and costly compared to microbial bioremediation. Bacteria have long been acknowledged as crucial agents in the degradation of hydrocarbons in the environment, as they are ubiquitous and capable of living independently (Dasgupta *et al.*, 2013). Although petroleum hydrocarbons are essential energy sources for industry and daily life, they also contribute significantly to environmental pollution (Mehdi *et al.*, 2008). The complex composition of petroleum can

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lead to various toxic effects, from acute lethality to chronic harm, depending on the exposure levels, doses, and affected organisms (USEPA, 2013). Certain petroleum components can bioaccumulate in vulnerable aquatic organisms and transfer through the food chain (Orisakwe *et al.*, 2004). Research suggests that microbial bioremediation, involving microorganisms such as bacteria, fungi, and microalgae, is the most effective method for transforming pollutants into less harmful and less mobile forms, or for completely mineralizing hydrocarbon contaminants into water, carbon dioxide, and microbial biomass (Imam *et al.*, 2019). Bacteria play a crucial role in the biodegradation of hydrocarbons, acting as the primary agents in breaking down these pollutants (Abena *et al*., 2019). Over time, certain microorganisms have adapted to thrive in hydrocarbon-contaminated environments, using the hydrocarbons as their exclusive sources of carbon and energy. These microorganisms can enhance their metabolic capabilities through mechanisms like horizontal or vertical gene transfer and mutations, acquiring genes that encode enzymes involved in hydrocarbon degradation (Gessesse *et al.*, 2019). Certain bacteria have shown a broad capacity for degrading petroleum hydrocarbons. For example, *Dietzia sp.* DQ12-45-1b can utilize n-alkanes ranging from C6 to C40, among other compounds, as sole carbon sources (Wang *et al.*, 2011). Similarly, *Achromobacter xylosoxidans* DN002 has been effective in degrading various monoaromatic and polyaromatic hydrocarbons (Ma *et al.*, 2015). However, it is important to note that very few bacteria can degrade the entire range of petroleum hydrocarbons. In practice, most bacteria specialize in degrading specific components of petroleum hydrocarbons, leaving other components unaffected (Varjani *et al.*, 2017). This variation in degradative capability can be attributed to differences in catalytic enzymes among different indigenous bacteria, leading to a wide range in their effectiveness in oil-contaminated environments. Conducting bioremediation projects can be costly; therefore, the first step should involve in situ bioremediation to ensure safety before implementation in the open field. Consequently, it is necessary to study the microbiological and physicochemical parameters affecting hydrocarbon degradation and the potential of various indigenous bacteria to determine the most effective strains for achieving optimal bioremediation outcomes on polluted sites. This paper aims to evaluate the effects of four crude oil-degrading bacterial strains (*Pseudomonas putida* strain AY001, *Alcaligenes aquatilis* B61, *Winkia neuii* B12, and *Enterococcus casseliflavus* AAOO-1) on the physicochemical parameters of water from River Erhoike, located in Kokori, Delta State, Nigeria.

MATERIALS AND METHODS

Collection of sample: The hydrocarbon contaminant used in this study, Escravos blend crude oil, was sourced from Heritage Energy Operational Services Limited located in Kokori, Delta State. The bacteria utilized in this research were indigenous strains isolated from the crude oil-contaminated River Erhioke in Kokori, Delta State, Nigeria.

Physicochemical analysis: The medium's pH was taken by pH meter, while other physicochemical parameters like chemical oxygen demand (COD), biochemical oxygen demand (BOD), Total organic carbon, Total dissolved solids, etc were carried out according to standard methods.

Enumeration of bacteria population: The total viable counts were measured using the standard plate count method, and the colonies were counted using a colony counter. To prepare the sample, a series of dilutions was performed on the crude oil-polluted water: 1 ml of the water sample was added to 9 ml of distilled water in a test tube, resulting in an aliquot. This process was repeated for four dilutions.. Using the pour plate method, a 0.1 ml aliquot from the third and fourth dilution factors was transferred into sterile glass Petri dishes. Then, 20 ml of molten nutrient agar, at 45°C, was carefully poured into the dishes under sterile conditions. The plates were gently swirled to ensure even distribution of the medium before being left to solidify. Afterward, the plates were placed upside down and incubated at 37°C for a duration of 24 hours. The growth on the plates was subsequently observed and recorded.

Preparation of bacteria inoculum: Nutrient broth (NB) was prepared following manufacturer's instructions, thereafter they were inoculated with the bacterial strains, and this was followed by an incubation at 37°C temperature for a duration of24 hours to facilitate the growth of bacteria. Series of dilutions were carried out on each bacterial culture to ascertain the cell count per milliliter aliquot. A volume of 0.1 ml from the suitable dilution was applied to spread plates for further use.

Experimental design: Replicate 1000ml glass bottles of the four bacterial strains containing mineral salt medium ((gl ^{1}): NaCl, 10.00, MgSO4.7H2O, 0.42; KCl, 0.29; KH2PO4, 0.83; Na2HPO4.H2O, 1.25; NaNO3, 0.42; Agar, 15.0; and distilled water (pH 7.2), with 1% crude oil were prepared for all the four bacteria strains tested. All the experimental set-up

were manually agitated thrice daily to promote air circulation and uniform spreading of crude oil for 28 days.

*Determination of the Total Petroleum Hydrocarbon (TPH):*The degradation of crude oil in the experimental setup was analyzed weekly over four weeks using Gas Chromatography with Flame Ionization Detection (GC-FID) at Multi-environmental Consultant Limited in Ikorodu, Lagos. The extraction of Total Petroleum Hydrocarbons (TPH) was performed using a MARS 6 microwave-assisted system from CEM in Matthews, NC, following EPA Method 3546 (2007), with modifications based on the manufacturer's technical guidelines.

A solvent mixture comprising acetone (HPLC grade) from Merck in Darmstadt, Germany, and dichloromethane (HPLC grade) from Scharlau in Barcelona, Spain, in a 1:1 ratio, was employed (30 mL). Extraction was carried out at 150 °C for 15 minutes, utilizing a power range of 100–300 W. Subsequently, the extract was filtered using GE Healthcare equipment in Chicago, IL, and transferred to an evaporation flask.

The GC-FID analysis was conductedon an Agilent Technologies 7890A GC system/5975C inert MSD with TripleAxis-Detector. Helium was employed as the carrier gas, with an average linear velocity of 1.0 mL/min (Roy *et al*., 2018). The initial oven temperature was set at 80 °C for 4 minutes, followed by a programmed increase to 250 °C at a rate of 5°C/min, where it remained for 10 minutes Yan *et al.* (2013). The injector, transfer line, and ionization source temperatures were maintained at 200 °C, and the electron impact ionization was set at 70 eV Yan *et al.* (2013). Identification of detectable crude oil components, represented by the highest peaks on the gas chromatograms, was achieved by aligning their retention times and mass spectrum profiles with reference data in the mass spectral library (Roy *et al*., 2018).Various compounds such as nonane, hexadecane, tetracosane, pentadecane, etc were individually analyzed to establish their retention times, serving as references for identifying crude oil components. The percentage of degradation for different hydrocarbons in crude oil was assessed by comparing their peak areas with those of corresponding peaks in the control.

RESULTS AND DISCUSSION

Weekly total viable bacteria count showing the various bacteria growth phase: (lag, log, stationary and death phase). Decline/death is seen at the last week (week 4) for most of the bacteria. The total hydrocarbon utilization count showing the initial increase in the first and second week and decline in the third week. The pH of the watersamples with 1% (%/v) crude oil polluted water. The pH ranged between 6.3 and 7.2. The highest pH 7.2 was recorded at week 1 from *Alcaligenes aquatilis* B61. The optical density of 1% (w/v) crude oil polluted water shown in table 1, it ranged between 0.06 at week 1 and 0.52 at week 3. *Pseudomonas putida strain* AY001 showed the highest optical density at week 3. The dissolved oxygen of 1 % (w/v) crude oil polluted water shown in table 2, it ranged between 5.05±0.15 and 8.10±0.60 for the four isolates. *Winkia neuii* B12 had the highest dissolved oxygen. Figure 4 showing the Chemical oxygen demand of 1% (w/v) crude oil polluted water: a continous decrease is also observed from week 1 to week 4 in comparison with the control, *Enterococcus casseliflavus AAOO-1* had the least COD.Figure 3 showing the Biochemical oxygen demand of water samples with 1% (w/v) crude oil polluted water. A sharp decrease was observed in comparison with the control from week1 to week 4 and *Enterococcus casseliflavus AAOO-1* showed the least biochemical oxygen demand.

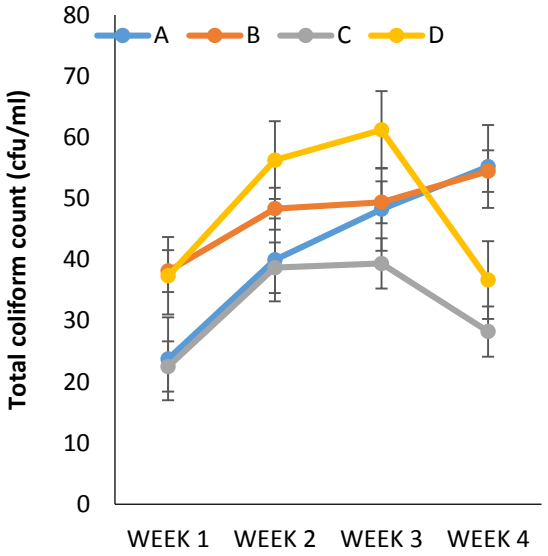

Fig. 1: Total viable bacteria count of 1% crude oil polluted water
Key: A-*Pseudomonas putida strain* AY001, B-*Alcaligenes aquatilis* B61; C-*Winkia neuii* B12,D-*Enterococcus casseliflavus* AAOO-1

Total organic carbon of 1% (w/v) crude oil polluted water: a slight decrease is observed against the control. The Oil and grease content of 1% (w/v) crude oil polluted water is shown in Figure 5 with majority of the bacteria showing continuous decline bacteria in the oil and grease content. Isolate B - *Alcaligenes aquatilis* B61showed the most degraded oil and grease content.

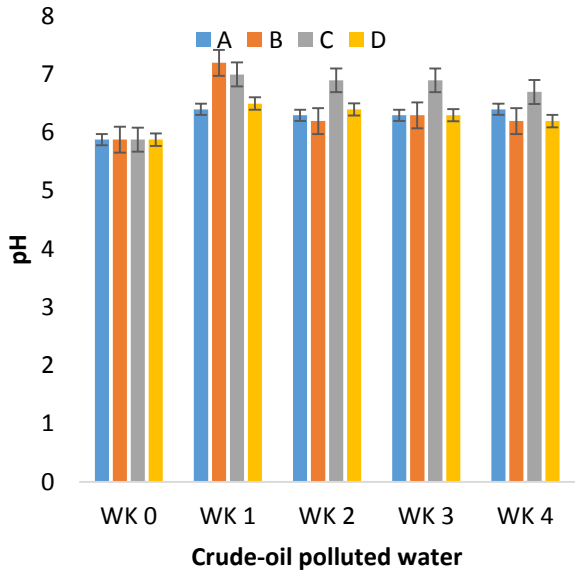

Fig. 2: pH of water samples with 1% (w/v) crude oil polluted water
Key: A-*Pseudomonas putida strain* AY001; B-*Alcaligenes aquatilis* B61; C-*Winkia neuii* B12,D-*Enterococcus casseliflavus* AAOO-1

Table 1: Optical density of 1%(w/v) crude oil polluted water

Isolate	WEEK 1	WEEK 2	WEEK 3	WEEK 4
Control	0.057±0.11	0.057±0.20	0.057±0.20	0.057±0.20
A	0.06±0.35	0.43±0.35	0.52±0.41	0.37±0.85
B	0.09±0.25	0.192±0.22	0.27±0.04	0.38±0.40
C	0.14±0.50	0.20±0.16	0.27±1.13	0.33±0.60
D	0.10±0.10	0.15±0.32	0.45±0.25	0.25±0.45

Key: Values are presented as mean±SE of duplicates, values in the same column carrying same superscript are not different significantly ($p<0.05$) according to new Duncan's Multiple Range test.
A-Pseudomonas putida strain AY001; B-Alcaligenes aquatilis B61; C-Winkia neuii B12, D-Enterococcus casseliflavus AAOO-1

Table 2: Dissolved oxygen of 1 %(w/v) crude oil polluted water

Isolate	WK 1	WK 2	WK 3	WK 4
Control	9.95±0.15	10.00±0.20	10.0±00.20	10.0±00.20
A	7.75±0.35	5.35±0.55	5.50±0.40	5.55±0.95
B	5.95±0.25	5.50±0.20	7.75±0.05	5.65±0.45
C	8.10±0.60	5.05±0.15	6.35±1.15	6.20±0.70
D	5.80±0.10	5.45±0.35	5.55±0.15	5.65±0.45

Key: Values are presented as mean±SE of duplicates, values in the same column carrying same superscript are not different significantly ($p<0.05$) according to new Duncan's Multiple Range test.A-Pseudomonas putida strain AY001,B-Alcaligenes aquatilis B61; C-Winkia neuii B12, D-Enterococcus casseliflavus AAOO-1

The Carbon-dioxide evolution of water samples with 1% (w/v) crude oil polluted water is shown in Figure 7 with continuous decline in the CO_2evolution from week 1 to week 4 across all the four samples. The Total petroleum hydrocarbon of 1% (w/v) crude oil polluted water is shown in Figure 8 with *Pseudomonas putida* AY001 showing the highest rate of degradation of 70%, others also showed continuous decrease from week 1 up till week 4

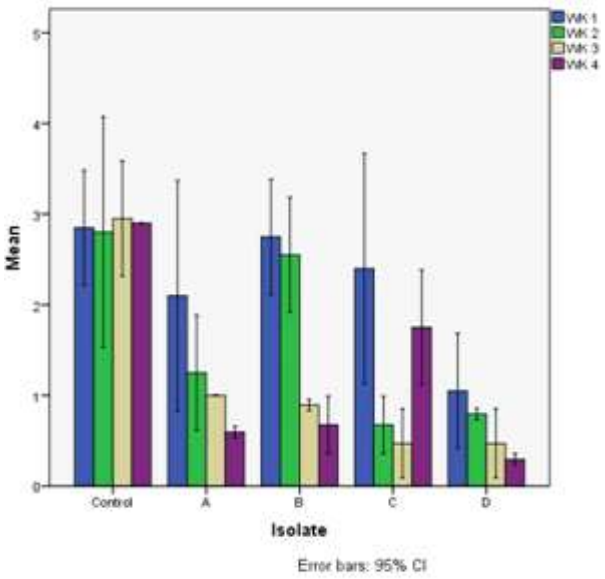

Fig.3: Biochemical oxygen demand of water samples with 1% (%/v) crude oil polluted water
Key: *A-Pseudomonas putida strain* AY001, *B-Alcaligenes aquatilis* B61, *C-Winkia neuii* B12, *D-Enterococcus casseliflavus* AAOO-1

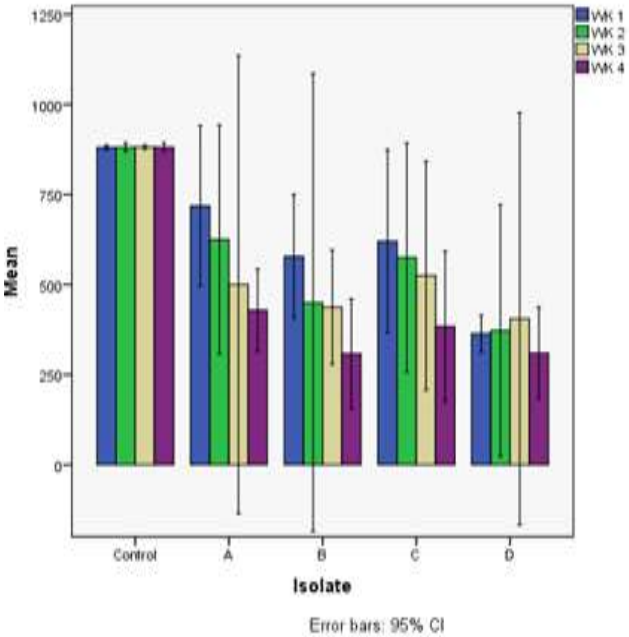

Fig. 4: Chemical oxygen demand of 1% (w/v) crude oil polluted water
Key: *A-Pseudomonas putida strain* AY001, B-*Alcaligenes aquatilis* B61; *C-Winkia neuii* B12, *D-Enterococcus casseliflavus* AAOO-1

Table 3: Total organic carbon of 1% (w/v) crude oil polluted water

Isolate	WK 1	WK 2	WK 3	WK 4
Control	11.54±0.01^{a}	11.52±0.02^{a}	11.53±0.02^{a}	11.53±0.02^{a}
A	10.04±0.03^{b}	9.93±0.02^{c}	9.770±.02^{d}	9.74±0.05^{c}
B	10.07±0.01^{b}	10.05±0.01^{b}	10.06±0.01^{b}	10.00±0.00^{b}
C	10.07±0.00^{b}	10.09±0.02^{b}	9.72±0.02^{d}	9.84±0.05^{c}
D	10.02±0.01^{b}	9.95±0.01^{c}	10.00±0.00^{c}	10.00±0.00^{b}

Key: Values are presented as mean±SE of duplicates, values in the same column carrying same superscript are not different significantly ($p<0.05$) according to new Duncan's Multiple Range test.; A-Pseudomonas putida strain AY001, B-Alcaligenes aquatilis B61; C-Winkia neuii B12, D-Enterococcus casseliflavus AAOO-1

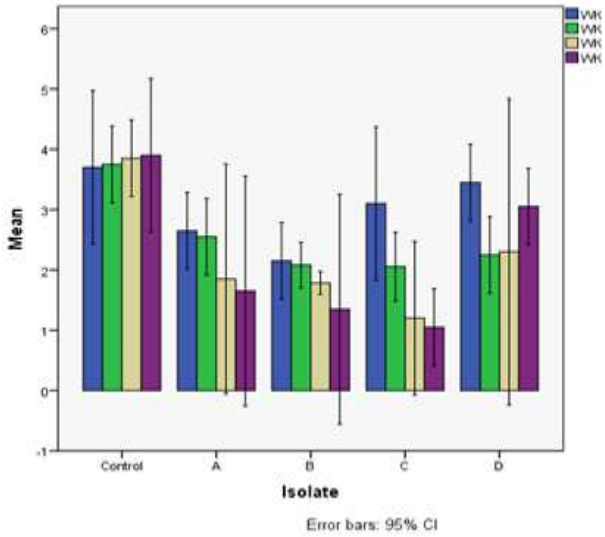

Fig. 5: Oil and grease content of 1% (w/v) crude oil polluted water
Key: *A-Pseudomonas putida strain* AY001, *B-Alcaligenes aquatilis* B61; *C-Winkia neuii* B12, *D-Enterococcus casseliflavus* AAOO-1

The variation (increase and decrease) in the total viable count of 1% crude oil samples from week 1 to week 4, is a reflection of different bacterial growth phase (lag, exponential, stationary and death) Bate *et al.* (2023). The total viable count ranged between 21 X 10^{-3} n the first week and 57 X 10^{-4} in the fourth week. This also demonstrates the bacteria's consumption of the crude oil substrate (Omenna *et al.* 2023).

The optical density ranged between 0.06±0.35in week 1 and 0.43±0.35 in week 2 completely higher than the control 0.057±0.11. The bacterial strain exclusively relied on crude oil hydrocarbons as its primary carbon and energy source, as indicated by the noticeable increase in culture cell density following incubation (Mira *et al.*, 2022). The statistical evaluation showed a significant difference ($P<0.05$) in the observed growth at 1% crude oil concentration and the control group, demonstrating high growth rate. The varied value of Total Dissolved Solids (TDS) was a result of the biodegradation of oil and grease by bacteria, leading to the amount of dissolved solids to vary Mandonna (2022). The dissolved oxygen decreased from week 1 to week 2 for all the water samples, though some later increased in third week and decreased eventually, as seen in sample B inoculated with *Alcaligenes aquatilis B61* which increased from 5.95 ppm in week 1 to 5.05 ppm in week 2 and later increased to 7.75 ppm and declined to 5.65 ppm at the last week. The highest dissolved oxygen occured in the first week with isolate *C Winkia neuii* strain B12, having the highest DO of 8.10 ppm and the lowest recorded in week 2 at 5.05 ppm.

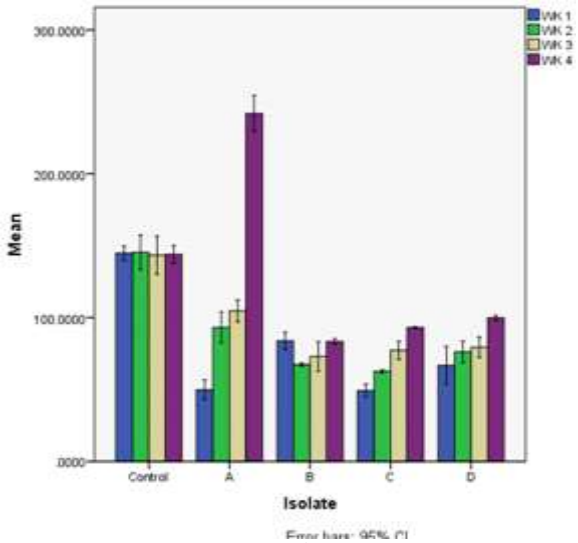

Fig. 6: Dissolved organic matter of water samples with 1% (w/v) crude oil polluted water
Key: *A-Pseudomonas putida strain* AY001, *B-Alcaligenes aquatilis* B61; *C-Winkia neuii* B12, *D-Enterococcus casseliflavus* AAOO-1

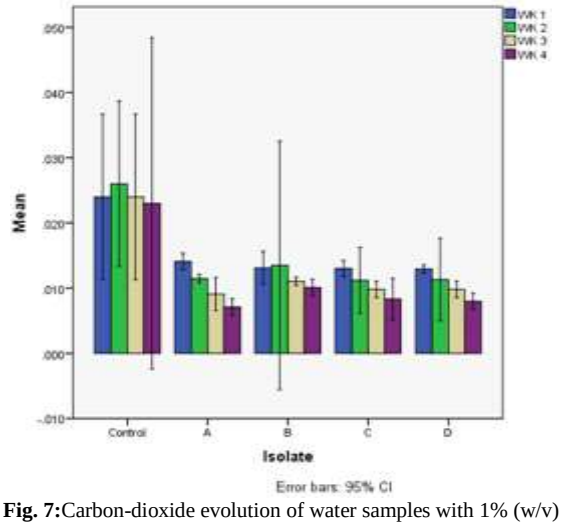

Fig. 7: Carbon-dioxide evolution of water samples with 1% (w/v) crude oil polluted water
Key: *A-Pseudomonas putida strain* AY001, *B-Alcaligenes aquatilis* B61; *C-Winkia neuii* B12, *D-Enterococcus casseliflavus* AAOO-1

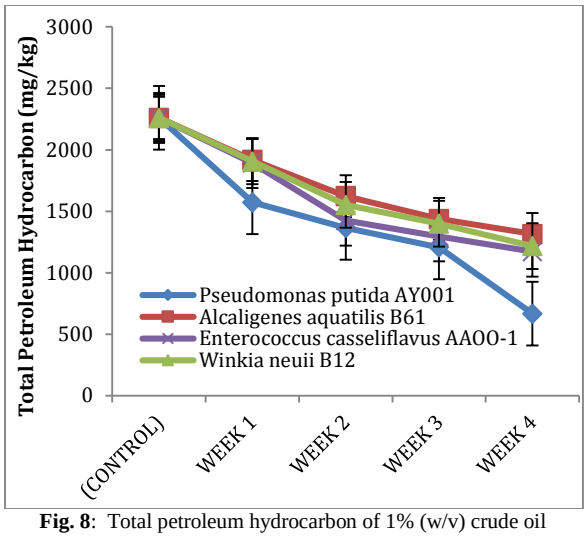

Fig. 8: Total petroleum hydrocarbon of 1% (w/v) crude oil polluted water

This is in relation to the work of Hefni *et al* 2007who observed that the levels of dissolved oxygen (DO) exhibited a decrease from the first week to the second week, followed by an increase during the third week, and then experienced another decrease by the end of the fourth week, and could be attributed to oxygen being utilized by microorganisms to degrade organic matter.

Biochemical oxygen demand reduced drastically from week 1 to week 4 as compared to the control. The reduction in BOD could be attributed to the activities of the microbes in the contaminated water which converted the oil into less toxic substances such as CO_2 and H_2O. This is in agreement with the work of Onoh *et al.,(*2022) who also observed decrease in BOD in the course of bioremediation. A continuous weekly decrease is observed in the chemical oxygen demand (COD) of the set-up; this could be due to the fact that microorganisms responsible for breaking down the crude oil may have consumed a significant portion of the biodegradable elements in the crude oil leading to a decrease in COD over time, this is in line with the work of Maxwell *et al.,*(2023) who observed a 60% reduction in the chemical oxygen demand of their 20 days long bioremediation experiment. The total organic carbon showed a slight decrease in comparison to the control for sample A and B from week 1 to week 4- the last week, its related to the crude oil bioremediation experiment of Okwonna and Otaraku (2022), but sample C and D showed a differed trend, there was a slight increase from week 1 to week 2 followed by a decline at the third and fourth week respectively. The rise in total organic carbon for sample A and B may result from the breakdown of hydrocarbons through microbial activity. Crude oil, which is recognized for its significant carbon content, could contribute to this increase (Mekonnen *et al*., 2024). The decrease of the TOC was in line with the work of Wang *et al.,* 2022 who observed a decrease in the TOC in their experiment. The decrease in total organic carbon during crude oil bioremediation can be attributed to several factors. First, microbial populations metabolize the organic carbon available in crude oil as the principal energy and nutrients, leading to its degradation (Ławniczak *et al.,* 2020). Second, some microorganisms involved in bioremediation may convert organic carbon into biomass as they grow and reproduce (Ayilara and Babalola 2023), third processes such as volatilization, absorption, and mineralization can also play a role in the reduction of total organic carbon by transforming it into different forms or removing it from the system (Clarisse *et al.,* 2023). Overall, the combination of microbial activities and abiotic processes drives the decrease in total organic carbon during crude oil degradation Prartono *et al*., (2022). There was a drastic decline up to 40% in the oil and grease content as compared with the control, except for organism D this conforms to the research of Isukul *et al.,* 2023 who noticed a very sharp decline in the oil and grease content of their 3 weeks spent oil degradation work. Increase in carbon dioxide CO_2 evolution indicated the existence of biodegradation (Pandolfo *et al.,* 2023).Priyamvada and Fairbanks 2015 observed a 1600% increase in CO_2 evolution in the first week of their crude oil bioremediation experiment followed by a constant production of carbon dioxide. Fig. 8 displays the rate of Total Petroleum hydrocarbon removal (TPH). The initial TPH concentration was 2259.05mg/kg, and on the fourth week (last week), the concentration had been reduced to 662mg/kg, 1209mg/kg, 1503mg/kg, 1509mg/kg concentration for *Pseudomonas putida* Strain AY001, *Alcaligenes aquatilis* Strain B61, *Winkianeuii* strain B12, and *Enterococcus casseliflavus* strain AAOO1 respectively. This result showed that *Pseudomonas putida* Strain AY001 performed best among the four tested bacteria from week 1 to week 4 by degrading the crude oil by 70%, compared to others, 40%, 50% and 30%. Saidu *et al.,* 2022 observed that *Pseudomonas putida* strain C15a among other species of bacteria tested, utilized 98.3% crude oil, Choudhury *et al.,* 2022 reported that *P. putida*7525 strain was able to degrade 91.90% phenol within 50 days. The reason for the excellent performance of *p. putida* includes their relatively large and flexible genome, which enables them adapt to a new hydrocarbon-rich environments Lu *et al*., 2024 and their biofilm forming ability Amina and Puhm *et al.,* 2022.

Conclusion: The findings of this study suggest *Pseudomonas putida* strain AY001 is a good

indigenous bacteria with a great potential to perform bioremediation of hydrocarbon polluted environment, this is seen in the activity of the bacteria against others in the physicochemical characteristics and the total petroleum hydrocarbon removal rate.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

Data Availability Statement: Data are available upon request from the corresponding author.

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