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Bioremediation of Diesel Contaminated Water Using Indigenous Hydrocarbon Degrading Bacteria

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ABSTRACT

Petroleum-based refined products such as diesel, kerosene, petrol are the major source of energy for industry and daily life. Accidental spills and leakages occur regularly during the refining, transport, and storage of petroleum products. Bioremediation of diesel contaminated water was studied using indigenous degrading bacteria isolated from hydrocarbon contaminated soils obtained from automobile mechanic workshops located at Effurun, Delta State. Four (4) bacteria isolates with high diesel biodegradability potential assessed by turbidity measurement were used for the diesel bioremediation study. The bacteria isolates used for the test were identified as, Acetobacter sp, Staphylococcus sp, Marinococcus sp, and Acinetobacter sp. The test microcosms were incubated for two weeks at 28 ± 2oC. Oil & grease concentrations, bacteria count, pH and turbidity were monitored weekly to assess the biodegradation of the diesel. At the end of the test duration, Acetobacter sp. recorded the highest percentage diesel biodegradation (77.57%) for the 5% diesel experimental set up. Similarly, Acinetobacter sp. recorded the highest percentage diesel biodegradation (81.34%) for the 10% experimental set up. Both residual diesel concentrations obtained are below the DPR recommended limit of 10 mg/L for oil & grease in inland waters. Thus, Acetobacter sp. and Acinetobacter sp. could be used to effectively bioaugment the bioremediation process of diesel contaminated waters within a short period of time.

Keywords : Bioremediation, Diesel, Polluted Water, Indigenous, Hydrocarbon Degrading Bacteria

I. INTRODUCTION

Oil is one of the main source of energy, representing approximately 40% of global energy usage. However, the economic advantages and social benefits of oil as the primary power source must be balanced by the negative outcomes of spills associated with petroleum hydrocarbons on terrestrial and aquatic ecosystems (Mos et al., 2008). Globally the annual amount of oil that seeps into the environment have been estimated at 4.5 million barrels per year (Kvenvolden and Cooper, 2003). As a result of industrialization over the last century, vast areas have been left contaminated with high concentrations of a range of hydrocarbons (Sanders et al., 1993).

Diesel oil has often been reported as one of the major hydrocarbon pollutants, as a result of spill incidents, storage tanks and leaking pipelines (Gallego et al., 2001). It consists of many components including aromatic hydrocarbons (23.9 %), cycloalkanes (33.4 %) and n-alkanes (42.7 %). Within diesel, the n-alkanes and aromatic hydrocarbons, which both have relatively low molecular weight, have been reported to be easily degraded through microbial action (Kang and Park 2010). The low molecular weight compounds are usually more toxic than long-chained hydrocarbons, because long-chained ones are less soluble and less bioavailable (Dorn et al., 2000). The typical carbon number in the complex mixture of diesel oil is C8 – C26 (Adam and Duncan 1999).

Diesel fuels vary according to their origin and method of production. In general, they are similar to heating oil, consisting of aliphatic (mostly paraffins including n, iso and cycloparaffins) and aromatic hydrocarbons, including small amounts of organometal constituents such as vanadium and nickel (ATSDR 1995; Van Hamme et al. 2003; Bacha et al., 2007; Zanaroli et al., 2010). Some oils contain heavy residues from distillation and thermal cracking along with a variety of additives (organic nitrates, amines, phenols and polymeric substances) (IARC, 1998). The main purpose of the additives is the performance of the engine and delivery system (e.g., cetane number improvers and lubricity improvers), fuel handling (e.g., antifoam and de-icing additives), fuel stability, and contaminant control (e.g., biocides, demulsifiers and corrosion inhibitors) (Bacha et al., 2007).

The colour of diesel fuels varies from colourless to brown, and the water solubility in 20°C is about 5 mg-1 and Log Kow 3.3 – 7.06 (ATSDR 1995). Diesel fuels are therefore partly soluble in water and possibly bioaccumulative in tissues. Diesel fuels have been observed to cause skin irritation and tumorigenic responses in mice, especially if the fuel contains cracked material (Mckee et al., 1990a; Nessel, 1999). Diesel causes eye and skin irritation in humans, but otherwise its effects on humans are considered to be poorly investigated (Muzyka et al., 2002). Diesel is considered to be harmful and possibly carcinogenic to humans since it contains PAHs that create a risk for human health because of their carcinogenic, mutagenic and teratogenic properties (Bamforth and Singleton 2005; Grant et al., 2007; TTL, 2011).

Diesel spills usually take place during refining, storage and transportation. Major spills, such as pipeline, tanker or storage tank accidents, create an acute impact on the environment. On the other hand, continuous low level inputs are rarely noticed, and may pose a serious threat to the environment as contamination accumulates. Therefore, diesel hydrocarbons create a world-wide problem of contaminated water and soil that require decontamination.

Conventional methods of dealing with oil spills include using dispersants or basically collecting the oil plume or through bioremediation. (Agunwamba, 2004). Bioremediation can be defined as a biotechnological technique using microorganisms to breakdown or neutralize a contaminant from a polluted area. Many strategies of bioremediation can be applied in term of soil / water waste treatment (Iwamoto and Nasu, 2001). These include natural attenuation in which the natural degradation of the pollutant takes place by the water microflora without any human involvement in the degradation process with the exception of monitoring the remediation process (Mills et al., 2003). This strategy has an advantage of not disturbing sensitive ecological habitats. However, the degradation rate of the contaminant might be very slow due to the low population size of the normal flora with the ability to breakdown the contaminant (Yu et al., 2005). A second strategy is biostimulation, which is an accelerated degradation process undertaken through the addition of nutrients to the water to enhance the growth of the indigenous microorganisms and their metabolic activity. While a third strategy is direct release / bio-augmentation, which consists of adding hydrocarbon degrading organisms to the affected site. Bacteria or their extracellular products may be released directly into the contaminated environment (Lee et al., 1994). Bioremediation has many advantages over traditional clean-up methods of oil spills. Major advantages of bioremediation include cost saving and time amongst others. The cost to clean 120 km of shoreline by bioremediation was less than the cost to provide physical washing of shoreline for one day (Las, 1995; Zhu et al, 2001). It is also environmental friendly, unlike chemical methods, no foreign or toxic chemicals are added to the site and do not require any disruption to the natural habitat which often occurs from physical and chemical methods of clean up.

The recovery of soil and water after an oil spill depends on a number of factors including quantity spilled, chemical composition of the crude oil or petroleum product, and the biodegrading potential of the microbial population in the area affected (Sabate et al., 2004). There are many environmental factors which influence the bioremediation process and should be monitored. These include temperature, pH, pollutant type and concentration, nutrients, oxygen availabilities and microorganism concentration on the impacted site. Therefore, there is a need to adjust some environmental conditions in order to stimulate the indigenous

microorganism activity and to obtain the best pollutant removal (Sandro et al, 2005). The aim of this research was to investigate the bioremediation of diesel polluted water using indigenous bacteria isolated from petroleum polluted soils.

II. METHODS AND MATERIAL

A. Collection of Hydrocarbon Contaminated Soils

Hydrocarbon contaminated soil samples were collected from automobile mechanic workshops located in Uvwie and Okpe Local Government Area (LGA), Delta state. Sample A was collected from a workshop at Okuokoko (latitude 5°34'26', longitude 5°46'52'') in Okpe LGA, while sample B was taken from a workshop at Ugbomro (latitude 5°34'33.9'', longitude 5°46'54.7'') in Uvwie LGA, Delta state.

B. Collection of Refined Petroleum Product (Diesel)

Two (2) litres of diesel was bought from St. Luke filling station located at Okuokoko in Okpe LGA, Delta State (5°34'12'', longitude 5°50'26). It was collected in 1L glass bottle.

C. Isolation and Selection Of Diesel Degrading Bacteria

The procedure of Bhattacharya et al., (2015) was adopted for this study. Bushnell-Haas (BH) media with the following composition (g/L): K2HPO4 (1.0 g), KH2PO4 (1.0 g), NH4NO3 (1.0 g), MgSO4•7H2O (0.2 g), FeCl3•6H2O (0.05 g), CaCl2•2H2O (0.02 g), was used as enrichment medium with diesel - 2 % (v/v) added as the sole carbon source to isolate diesel degrading bacteria from the hydrocarbon contaminated soils. Soil samples (10 g) was added to 50 mL BH media in 250 mL Erlenmeyer culture flasks. It was then incubated at 28 ± 2°C at for 7 days. After 7 days incubation, the bacteria cultures were isolated as single colonies on to nutrient agar (NA) media by streak-plate method. The pure bacteria isolates were maintained in slant cultures by preserving at 4°C and sub cultured at 2 weeks interval to maintain its viability.

For the selection of bacteria, the isolated bacteria cultures were screened for effective diesel oil degradation. Fresh overnight cultures suspended in BH medium were used as inoculum. The isolates were aseptically put into the BH medium in culture flasks with 2% (v/v) diesel as carbon source. The flasks were then incubated at 30°C for 7 days. After completion of the incubation period, the growth of the bacteria isolates was measured by turbidity readings (Mounteer, 2006), while the residual oil was measured for evaluating the degradation efficiency of the isolated microorganism. All the experiments were performed in triplicate, and a control devoid of the bacterial isolates was prepared along with the test experiment. Isolates with high turbidity (high growth) and diesel degradability were used for the diesel bioremediation study.

D. Identification of Selected Bacteria Isolates

Screened and selected diesel degrading bacteria were identified by cultural, morphological and biochemical characteristics, following the methods of Buchanan and Gibbons, (2008).

E. Diesel Biodegradability Studies

The diesel contaminated water test solutions were treated with variable culture condition which include, incubation period (7 & 14 days) and oil concentration (5 & 10 % v/v). This was done to study the diesel degradation ability of the selected and screened culture, according to the method of Bhattacharya et al., (2015). Five (5%) (50000 mg/L) and 10% v/v (100000 mg/L) of diesel in 500 mL of BH medium was used for the biodegradation study. In addition, a control devoid of the bacterial isolates was prepared along with the test treatments. The different culture media were incubated for a period of 14 days, at an incubation temperature of 28 ± 2°C. Biodegradation of the diesel at the two test concentrations was assessed and monitored weekly for two weeks, by sampling 100 mL of the culture media and analyzing for physico-chemical and microbiological parameters, which include: Oil & grease, (O&G), pH, temperature, turbidity and total heterotrophic bacteria count (THBC) (APHA, 2009).

F. Biodegradation Monitoring Analysis

Residual Diesel Oil

Residual diesel oil was determined as oil and grease content (API-RP45) in the various inoculated and un-inoculated (control) culture treatments. This was done at the onset of the bioremediation process and weekly for two weeks (0, 7 and 14 days) by extracting a 100 mL of the sample twice with 1:10 ratio of xylene to sample. The combined extract after centrifuging was read in the spectrophotometer at 400nm using Xylene as the reference material. The spectrophotometer had been previously calibrated with crude oil. Readings obtained from the spectrophotometer were traced out on the calibration graph and used to calculate the concentration of Oil and Grease in mg/l (API 1998).

G. Determination of Bacteria Growth

This was determined by turbidity measurements of the inoculated and un-inoculated cultures on day 0, 7 and 14 using a Hach Ratio Turbidimeter (APHA, 2009). Bacteria growth was also determined as total heterotrophic bacteria count (THBC). THBC was carried out by employing the standard plate count technique as follows: Normal saline solution (0.85% NaCl) was prepared and dispensed in 9 mL into McCartney bottles and sterilized at 121oC for 15 mins. One millimetre (1 mL) of the bioremediated diesel contaminated water sample was measured and serially diluted to obtain 10-5 dilution. Nutrient agar was prepared according to the manufacturer's instruction and sterilized at 1210C for 15 mins. The pour plate method was used to culture the prepared inoculum. Aliquots (0.1 mL) of the serially diluted samples were measured into sterile petri-dishes and 15-20 mL of sterilized and cooled nutrient agar was poured into the petri dish. Fungi zone of 0.1 mL was added to the nutrient agar to inhibit fungi growth. The plate was gently swirled to homogenize and evenly disperse the inoculum in the medium. The plates were then incubated at 28± 2oC for 24 h. Bacteria counts was recorded by multiplying colony counts with the reciprocal of the dilution factor and volume of sample used. Result was expressed as CFU/mL

III. RESULTS AND DISCUSSION

A. Isolation and Identification of Diesel Degrading Bacteria From Contaminated Soil

Four bacteria isolates with diesel biodegradative ability were isolated from hydrocarbon contaminated soils from mechanic workshops. The bacteria identified include; Acetobacter sp, Staphylococcus sp, Marinococcus sp, and Acinetobacter sp. Two of the species were Gram negative; Acetobacter sp and Staphylococcus sp., while Acinetobacter sp. and Marinococcus sp. were gram positive.

B. Biodegradation of Diesel Contaminated Water

The biodegradation of diesel for the test period was monitored on Day 0, 7 and 14. Readings for the physicochemical and microbiological properties of the bioremediated diesel contaminated water with the various bacteria isolates for both 5% and 10% concentrations were recorded. For Day 0 readings, the turbidity and microbial counts for both concentrations for the experimental set up with 5% diesel, Turbidity readings ranged from 180 NTU (Isolate C) - 212 NTU (Isolate J). The bacterial counts ranged from 1.33 x 103 (Isolate C) - 2.17 X 103 CFU/mL (Isolate I), while oil & grease concentrations ranged from 25.58 mg/L (Isolate I) – 33.61 mg/L (Isolate C). For the 10% diesel set up, oil & grease values were between 33.20 mg/L (Isolate C) and 34.27 mg/L (Isolate J). Turbidity readings ranged from 107 NTU (Isolate F) to 215 NTU (Isolate I), while bacterial counts ranged from 1.53 x 103 CFU/mL (Isolate J) – 1.87 x 103 CFU/mL (Isolate C) (Table 1). At the end of the experimental period (Day 14) readings obtained for the parameters monitored increased tremendously (Table 2). For diesel oil degradation, there was a progression in the decrease of residual diesel oil determined as oil and grease, from Day 0 to Day 14, indicating increased microbial activity and degradation of the diesel oil.

Table 1. Physicochemical and microbiological properties of bioremediated diesel contaminated water at Day 0

Parameters	Diesel Concentration, mg/L									
	Isolate C (Day 0)		Isolate F (Day 0)		Isolate I (Day O)		Isolate J (Day 0)		Control (Day 0)	
	5%	10%	5%	10%	5%	10%	5%	10%	5%	10%
PH	6.07	6.16	6.18	6.25	6.22	6.27	6.13	6.19	6.35	6.37
Turbidity, NTU	180.00	173.00	257.00	107.00	211.00	215.00	212.00	116.00	140.00	91.60
Oil & Grease, mg/L	33.61	33.70	31.76	34.09	25.88	34.15	26.79	34.27	34.08	34.85
Heterotrophic Bacteria Count, CFU/ml x 10^3	1.33	1.87	2.02	1.73	2.17	1.64	2.19	1.53	1.32	2. 87

Table 2. Physicochemical and microbiological properties of bioremediated diesel contaminated water at Day 14

Parameters	Diesel Concentration, mg/L									
	Isolate C (Day14)		Isolate F (Day14)		Isolate I (Day14)		Isolate J (Day14)		Control (Day14)	
	5%	10%	5%	10%	5%	10%	5%	10%	5%	10%
pH	6.11	6.29	6.09	6.34	6.26	6.38	6.25	6.32	6.27	6.41
Turbidity, NTU	1070	736.00	878.00	1092	906.00	1016	950.00	600.00	398.00	392.00
Oil & Grease, mg/L	7.54	20.57	15.85	6.36	12.85	14.90	10.96	21.63	18.35	22.98
Heterotrophic Bacteria Count, CFU/mL x 10^6	3.62	2.21	2.78	3.15	2.89	2.10	2.57	2.03	0.197	0.148

C. Biodegradation Studies

Effect of diesel concentration on diesel degradation in the test microcosms

As presented in Figure 1, the degradation of diesel hydrocarbon for 5% diesel concentration at the end of the test period (Day 14) were in this sequence; Isolate C – Acetobacter sp. (7.54 mg/L) > Isolate J – Marinococcus sp. (10.96 mg/L) > Isolate I –Staphylococcus sp, (12.85 mg/L) > Isolate F – Acinetobacter sp. (15.85 mg/L) with the control showing the least degradation (18.35 mg/L). The degradation sequence for the 10% concentrations at Day 14 are in this sequence; Isolate F – Acinetobacter sp (6.36 mg/L) > Isolate I – Staphylococcus sp (14.90 mg/L) > Isolate C – Acetobacter sp (20.57 mg/L) > Isolate J - Marinococcus sp (21.63 mg/L) with the control showing the least degradation (21.98 mg/L) (Figure 1).

There is also a progression in the decrease of oil and grease for the 10% diesel test set up from Day 0 to 14 but at a much slower rate than the 5% test set up. This also indicates increased microbial activity and degradation (Figure 1). Isolate C (Acetobacter sp.,) degraded the diesel oil most for the 5% test set up while Isolate F (Acinetobacter sp) degraded the diesel oil the most for the 10% experimental set up. In comparing the amount of diesel degraded by the bacterial isolates in both concentrations of diesel tested, it was observed that the percentage degradation of diesel oil decreased with increasing oil concentration possibly due to the presence of highly persistent aromatic alkanes as reported by Molnar et al., (2005).

D. Effect of incubation time on diesel oil degradation

The effect of increasing incubation period from 0, 7 and 14 days was checked to find out the maximum extent of degradation efficiency at same culture parameters (5% and 10% diesel oil concentration). For the 5% diesel concentration set up, Isolate C which recorded the highest percentage degradation at the end of the incubation period, recorded 71.62% degradation on day 7 and progressed to 77.57% degradation by day 14 (Figure 2). For the 10% diesel concentration set up, Isolate F, which had the highest degradation recorded 46.49% degradation for day 7 and 81.34% for day 14 (Figure 2). Bacterial growth is slower on insoluble hydrocarbon substrates due to less bioavailability, and it is one of the major constraints in bioremediation experiments. Results recorded in this research is in line with that of other researchers (Ghazali et al. 2004; Wang et al., 2011; Abioye et al., 2012) who found that hydrocarbon levels could be significantly reduced with longer incubation period during treatment of diesel oil-contaminated water. Biswal et al., (2009) reported that 40–50% degradation of diesel oil could be achieved within 7 days.

E. Changes in turbidity, residual diesel concentration and bacteria biomass

Bacteria growth can also be determined or measured by turbidity since absorbance (amount of light absorbed by the bacteria cells) is directly proportional to cell concentration. Thus the higher the number of cells, the higher the turbidity (Vasudevan, 2010). The turbidity values at the end of the incubation period (day 14) for the selected isolates were in the various increasing sequence for 5% diesel concentration; Isolate F (878 NTU) > Isolate 1 (906 NTU) > Isolate J (950 NTU) > Isolate C (1070 NTU). Isolate C recorded the highest turbidity value of 1070 NTU, the highest heterotrophic bacteria count of 3.62 x 106 CFU/mL and the lowest residual diesel oil concentration of 7.54 mg/L at the end of the incubation period (day 14).

The control without isolates recorded a turbidity value of 398 NTU (Figure 3). The turbidity readings for the 10% diesel test concentrations were in the following increasing sequence; Isolate J (600 NTU) > Isolate C (736 NTU) > Isolate I (1016 NTU) > Isolate F (1092 NTU). Isolate F recorded the highest turbidity value of 1092 NTU and the highest heterotrophic bacteria count of 3.15 x 106 CFU/mL and the lowest diesel concentration of 6.36mg/L at the end of the incubation period (day 14) (Table 2). The control with 10% diesel oil recorded a turbidity value of 392 NTU. This direct correlation between the turbidity readings and heterotrophic bacterial count of the isolates indicates an increased bacteria growth. The high diesel biodegradation recorded for the selected bacteria isolates is attributed to the biodegradative activities of the bacteria isolate inoculated into the test culture medium in relation to the controls which were uninoculated.

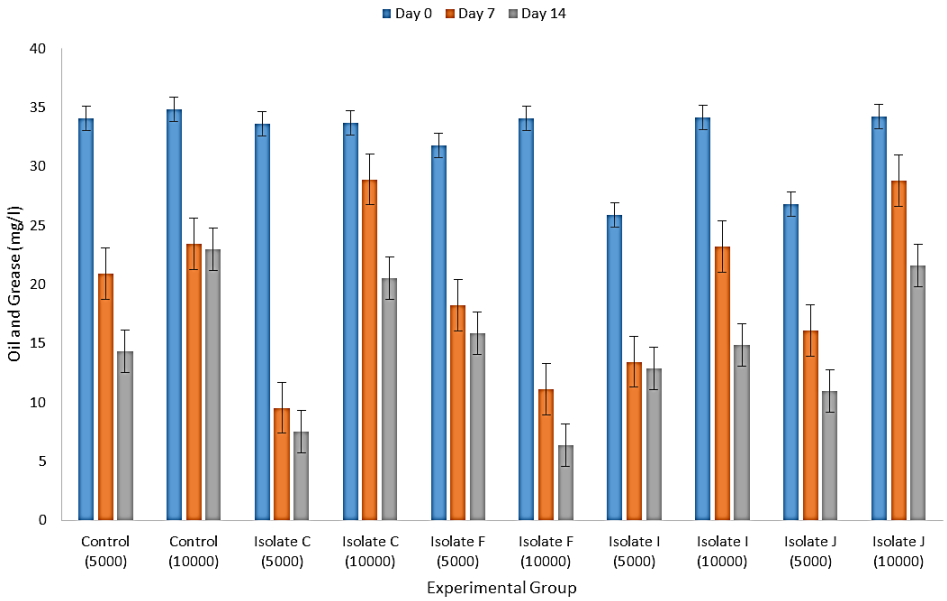

Figure 1 : Mean ± SE changes in Oil and grease concentrations during bioremediation of diesel contaminated water

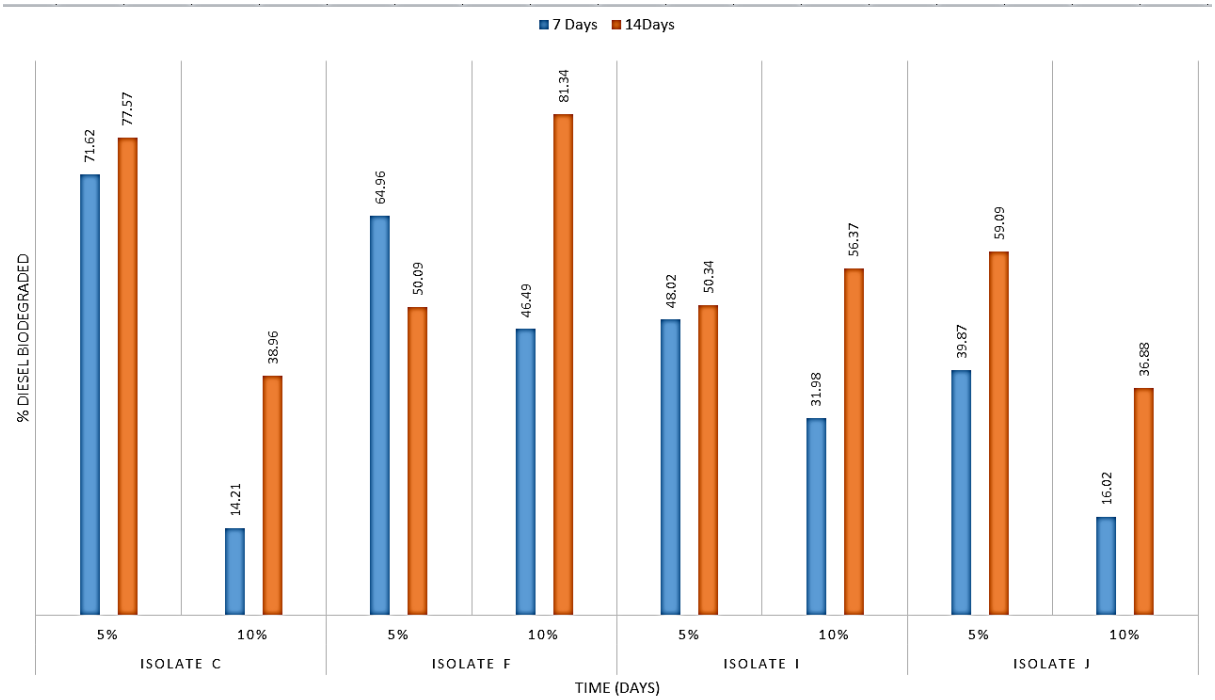

Figure 2: Effect of incubation period on percentage diesel oil biodegradation.

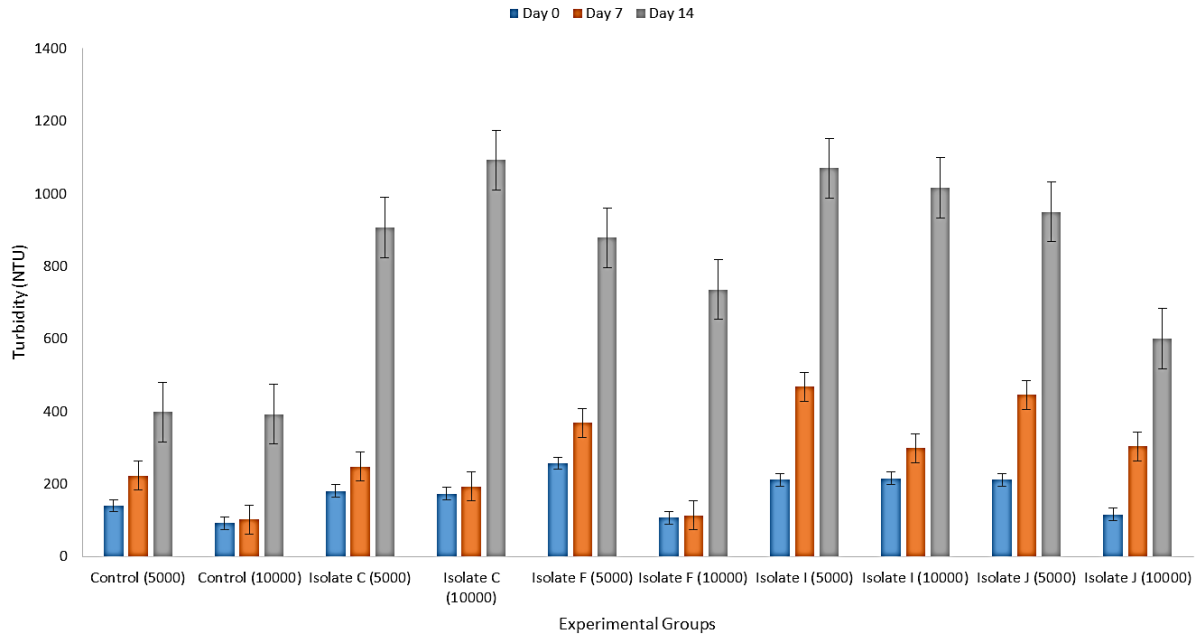

Figure 3: Mean ± SE changes in turbidity during bioremediation of diesel contaminated water.

IV. CONCLUSION

The numerous reports and cases of oil spill though devastating is not a problem but a challenge which has been overcome by environmental scientists and researchers adopting bioremediation technique which is cheap and more environmentally friendly compared to other forms of remediation. Bioaugmentation strategies, which have been successfully utilized for the bioremediation of refined petroleum products by some researchers was successfully utilized in this research to bioremediate diesel contaminated water. With proper manipulation of environmental conditions, diesel concentrations, time and temperature amongst others, indigenous hydrocarbon utilizing microorganisms are readily available in hydrocarbon contaminated soils and

water in the Niger Delta and could be cultivated in a large scale to promptly used to clean up refined petroleum products contaminated aquatic environments.

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