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Comparative Assessment of Biodegradability Potential of *Pseudomonas putida* and *Bacillus amyloliquefaciens* on Oil Spill Dispersant (Aquabreak and Teepol) in Freshwater

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

To compare and assessment of Biodegradability Potential of *Pseudomonas* putida and *Bacillus amyloliquefaciens* on oil spill dispersant (Aquabreak and Teepol) in Freshwater. Fresh water sample were collected from Biara, Gokana L.G.A, and were transported to the Microbiology Laboratory of Rivers State University, Port Harcourt, Nigeria for analyses while Oil spill dispersant (OSD/Aquabreak) was purchased from Barker and Hughes, all in Rivers state. Nine experimental set up were carried out using *Bacillus* and *Pseudomonas* species as the bio-augmenting organism. Controls were made without organisms. Its bioremediation potential on the pollutants and two types

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of test organisms were monitored for 28 days at an interval of 7day period. The setup was aerated twice a week to provide more oxygen for the organisms to thrive. Analysis of samples were carried out using standard analytical procedures. The resultsphysiochemical property of the water shows that as follows: pH 6.5, Temperature 30.0 ºC, Electric conductivity 15 µs/cm, Total dissolved solid 7 mg/l ,Chlorine 0.1 mg/l, Bromine 0.2 mg/l, Salinity (0.01 mg/l), Dissolved oxygen 1.5 mg/l , Biological Oxygen Demand 0.3 mg/l, Nitrate 0.01 mg/l, Sulphate 4.18 mg/l Phosphate 0.10 to 1.5mg/l, Total Hydrocarbon content 24mg/l. Percentage (%) Ultimate biodegradability of the two oil spill dispersant OSD/teepol and OSD/aquabreak revealed that control set-up recorded the 79.3 and 86.7 % , bioremediation set-up augmented with Bacillus amyloliquefaciens recorded 93.7 and 99.1% while the set-up augmented with P. putida had 98.0 and 94.7% respectively . It was observed that *P. putida degrade Teepol than Bacillus amyloliquefaciens while aquabreak* is more degraded by *Bacillus amyloliquefaciens* than *P. putida.* Nevertheless both dispersant shown high level of degradability by the test organisms. Therefore It is recommended that oil companies and government parastatals carrying out remediation in the Niger Delta should be encouraged and OSD/Teepol and OSD/aqubreak due to their high biodegradation potential.

Keywords: Brackish water; fresh water; aqubreak; teepol.

1. INTRODUCTION

Dispersants are products used in oil spill response to enhance natural microbial degradation, a naturally occurring process where microorganisms remove oil from the environment [1]. All environments contain naturally occurring microbes that feed on and break down crude oil. Dispersants aid the microbial degradation by forming tiny oil droplets, typically less than the size of a period on this page (<100micrones), making them more available for degradation. Wind, current, wave action or other forms of turbulence help both this process and the rapid dilution of dispersed oil. The increase surface area of these tiny oil drop/lets in relation to their volume makes the oil much easier for the hydrocarbon degrading microbes to consume. “In aquatic ecosystems, dispersion and emulsification of oil in slicks appear to be prerequisites for rapid biodegradation. Large masses of mousse, tar balls or high concentrations of oil in quiescent environments tend to persist because of the limited surface areas available for microbial activity. Petroleum fractions containing asphalt components are not degraded quantitatively. The residues, along with polymerization products formed from free radical degradation intermediate with each other, forming tar globules. The tar is a practically oxygenate high molecular weight material resistant to further microbial degradation” [2]. “An ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders in that environment. A number of well-known microorganisms are responsible for the biodegradation of oil dispersants” [3]. “Oil degrading microbes in the water columns tend to work collaboratively to boost a cooperative metabolism to jointly utilize hydrocarbons as a carbon source” [4]. “These microbes could not only partition the existing metabolic pathways within the community, but also improve the degradation capacities by generating mutated new hosts through shuffling hydrocarbon degradation genes among the members” [5]. “The accumulation of microbe community at the oil droplet surface assists the formation of biofilms at the oil-water interface, which results in the formation of microbe-oil aggregates” [6]. “These aggregates also possess a complex microbial network that could rapidly degrade oil dispersed oils” [7].

“The effect of dispersant and dispersant-oil mixtures on the growth of microorganisms also has been reported. The important issue when discussing dispersants is toxicity both of the dispersant itself and of the dispersed oil droplets” [2].

“A key factor contributing to the toxicity of oil spill dispersants meant for environmental release is their degradability. Biodegradability of dispersants is absolutely crucial; otherwise, they get accumulated in the environment and make the secondary cause of water contamination. Modern-day dispersants are much less toxic to sea water than those used in the past. However, concern still exists on their possible toxic effects, on fresh water organisms, especially if dispersants are used near shore waters” [8]. Therefore, this research was aimed to compare and asses the biodegradability potential of

Pseudomonas putida and *Bacillus amyloliquefaciens* on oil spill dispersant *(*Aquabreak and Teepol) in Freshwater

2. MATERIALS AND METHODS

2.1 Collection of Water Samples and Oil Spill Dispersant (OSD)

Fresh water sample was collected with sterile plastic container, The containers was rinsed three times with the water samples to be collected at the site before collection was made and transported in an ice pack cooler to the microbiology laboratory of the Rivers State University, Port Harcourt, Rivers State within 4 hours of collection for analysis [9]. The oil spill dispersants (OSD) used in the study work OSD/ T. Pol and OSD/Aqua break were sourced from Barker and Hughes Nig Ltd.

2.2 Samples Processing

The water sample was processed following the method adopted by Adesemoye et al., (2006). One millilitre (1ml) of the sample were aseptically transferred into 9ml of 1% peptone water as diluents and properly mixed, further ten-fold serial dilution were carried up to 10^{-7} described by Prescott et al., [10].

2.3 Source of Microorganisms

The organisms used in this study were bacteria: *Bacillus amyloliquefaciens* and *Pseudomonas putida*. These organisms were isolated from the fresh water sample. The method described by Holt et al. [11] and Nrior and Inweregbu [12], was adopted. Pure cultures of the organisms were obtained from inoculation and incubation of water samples using nutrient Agar. Pure cultures were obtained by continuous subculturing [13]. Identification of the test organisms was done using conventional and molecular approaches.

2.4 Biodegradation set–up

Three hundred and seventy- six millilitre was measured into fresh sterile 1500ml plastic container which were perforated with spatulas to allow for aeration and were kept at ambient temperature (28±2°C) for 28 days. Contamination of the water with dispersant was done by addition of 4ml of the dispersants and 20ml of the test organisms to make up to a final volume of four hundred millilitres Ogbonna et al., (2007). This was done for both the *Bacillus amyloliquefaciens* and *Pseudomonas putida*. The test organism was not added to the control. Details of the bioremediation set-up is shown in Table 1.

2.5 Sample Analysis

Samples were taken at day 1, 14, and 28 from the respective set-ups. This was to determine the Hydrogen ion concentration (pH) using electrometric pH meter (Jenway 3015 method), dissolved oxygen (DO) and biochemical oxygen demand (BOD) were determined by modified winkler method (APHA, 1998), total dispersants content (oil content) was determined using Gravimetric analysis method, Total Heterotrophic Bacterial Counts, Total Fungal Counts, and Dispersants Utilizing Bacteria [10].

Table 1. Biodegradation set–up

Set-up Code	Water		Test organisms		Dispersant		Final Volume (ml)
	Type	Vol. (ml)	Type	Vol. (ml)	Type	Vol. (ml)	
FW	FW	396	-	-		4	400
FW +TP	FW	376	-	-		4	400
FW+TP+ *Baci.*	FW	376	*Bac*	20	T. Pol	4	400
FW+TP+ *Pse*	FW	376	*Pse*	20	T. Pol	4	400
FW+TP+ *Baci* + *Pse*	FW	376	*Bac* + *Pse*	10 +10	T. Pol	4	400
FW +AQ	FW	396	-	-	Aqua break	4	400
FW+ AQ + *Baci.*	FW	376	*Bacillus* sp	20	Aqua break	4	400
FW+ AQ + *Pse*	FW	376	*Pseudomonas* sp	20	Aqua break	4	400
FW+ AQ + *Baci* + *Pse*	FW	376	*Bac* + *Pse*.	10 +10	Aqua break	4	400

2.5.1 Isolation and enumeration of total heterotrophic bacteria

Total heterotrophic bacteria for each biodegradation set up were enumerated by spread plate method. 0.1ml aliquot was transferred unto well-dried nutrient agar plates and incubated at 37°C for 24 to 48 h. after incubation, the bacterial colonies that grew on the plates were counted and sub-cultured unto fresh nutrient agar plates using the streak plate technique. Discrete colonies on the plates were aseptically transferred into agar slants, properly labeled and stored as stock cultures for preservation and identification (Odokuma and Ibor, 2002).

2.5.2 Isolation and enumeration of total fungal count

The total fungi population in the biodegradation set up (Habitat water sample and oil spill dispersants) were enumerated and isolated by inoculating 0.1ml aliquot of the mixture unto well-dried potato dextrose agar containing antibiotics (Tetracycline) to inhibit bacterial growth. Pure cultures of the fungi isolates were enumerated and transferred unto potato dextrose agar slants as stock cultures for preservation and identification [14].

2.5.3 Isolation and enumeration of Oil spill dispersant (OSD) utilizing bacteria and Fungi

Enumeration of Oil spill dispersant (OSD) utilizing bacteria and fungi was performed by inoculating 0.1ml aliquot of the dilutions unto mineral salt agar plates [14]. Colonies were counted after 48 to 72h of incubation at ambient temperature. The bacterial colonies on the plates after incubation were counted and sub-cultured onto fresh mineral salt agar plate.

2.6 Percentage of Oil Dispersant Biodegradation

The net percentage of total dispersant in the different treatment after 28 days were calculated using the expression given thus:

$$\%\ \mathrm{B.Dnet} = \frac{[PHc - PHt]}{PHc} X \frac{[100]}{1}$$

Where PHc is the initial concentration of the particular dispersant of the experiment on day 1 and PHt is the concentration of the particular dispersant remaining in each experimental set-upafter 28-day study period and % B.Dnet is the net percentage of biodegradation of total dispersant contaminant in water [15].

2.7 Statistical Analyses of Data

All experiments were performed at least in duplicate and the various values were analyzed with SPSS version 25. Results were presented as mean $\pm$SD where necessary. Statistical significance was defined as a *P*-value of less than 0.05 at 95% confidence interval.

3. RESULTS AND DISCUSSION

This study exploits the use of dispersant degrading microbes such as *Psuedomonas putida and Bacillus amyloliquefacie* isolated from the aquatic environment to bioremediation dispersant contaminated water. The results helped to ascertain the types of microorganisms and their abundance in the baseline and contaminated samples, treatments as well as their physiochemical parameters. The morphological and biochemical characteristics of bacteria isolated from fresh water samples showed that three Gram's positive bacteria belonging to the genera: *Staphylococcus, Bacillus, Micrococcus* and eight Gram's negative bacterial species which includes *Escherichia coli*, *Vibrio, Pseudomonas*, *Serratia, Klebsiella*, *Salmonella, Enterobacter,* and *Acinetobacter sp* were isolated and identified from the two water sources. The percentage occurrence of dispersant utilizing bacteria from the water samples is presented in (Fig. 1), the percentage range as follows; *Pseudomonas* sp (31%), *Bacillus* sp (26%), *Staphylococcus* sp (23%), *Micrococcus* sp (15%) and *Serratia* sp (5%). These groups of microorganisms have been found to show appreciable numerical increase in hydrocarbon polluted sites (Saadoun et al., 2008). Results of Physicochemical Characteristics of the fresh water samples are presented in Table 2. The results shows that as follows: pH 6.5, Temperature 30.0 ºC, Electric conductivity 15 µs/cm, Total dissolved solid 7 mg/l ,Chlorine 0.1 mg/l, Bromine 0.2 mg/l, Salinity (0.01 mg/l), Dissolved oxygen 1.5 mg/l , Biological Oxygen Demand 0.3 mg/l, Nitrate 0.01 mg/l, Sulphate 4.18 mg/l Phosphate 0.10 to 1.5mg/l, Total Hydrocarbon content 24mg/l as presented in (Table 2). This results are in agreenment with finding of Song et al., (2016) and Hou et al., (2015). It has been established that the physicochemical features of water is greatly affected when there is oil spill in the

environment, however this is determined by the type of water, petroleum and extent of pollution (Polyaket al., 2018).

This results of changes in the Total heterotrophic bacteria, total fungi, dispersants utilizing bacteria during the biodegradation process over a period of 28 days as presented in (Figs. 2 to 5) shows mild increased compared to the control from day 0 to day 28 however, THB and THF shown slight decrease with increasing time. This observation is in agreement with the report of Okpokwasili and Nnubia [16] that, oil spill dispersants support mild increases (stimulation) and decrease (inhibition) in the growth of specific heterotrophic Bacteria and fungi.

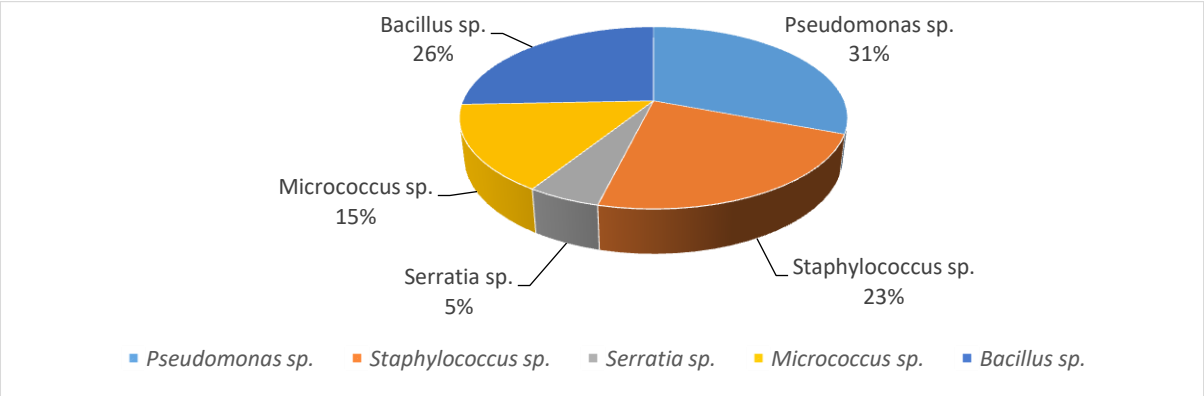

Fig. 1. Percentage Occurrence of Dispersants Utilizing Bacteria

Table 2. Physiochemical Property of the Fresh Water Sample

S/N	PARAMETER	UNIT	FRESHWATER
1	Temperature	^{0}C-	30.0
2	pH	-	6.5
3	Electrical Conductivity (EC)	µS/cm	15
4	Total Dissolved Solids (TDS)	mg/L	7
5	Chlorine	mg/L	0.1
6	Bromine	mg/L	0.2
7	Salinity	mg/L	0.01
8	Dissolved Oxygen (DO)	mg/L	1.5
9	Biochemical Oxygen Demand (BOD)	mg/L	0.3
10	Nitrate	mg/L	0.01
11	Sulphate	mg/L	4.18
12	Phosphate	mg/L	0.10
13	Total Hydrocarbon Content (THC)	mg/L	24

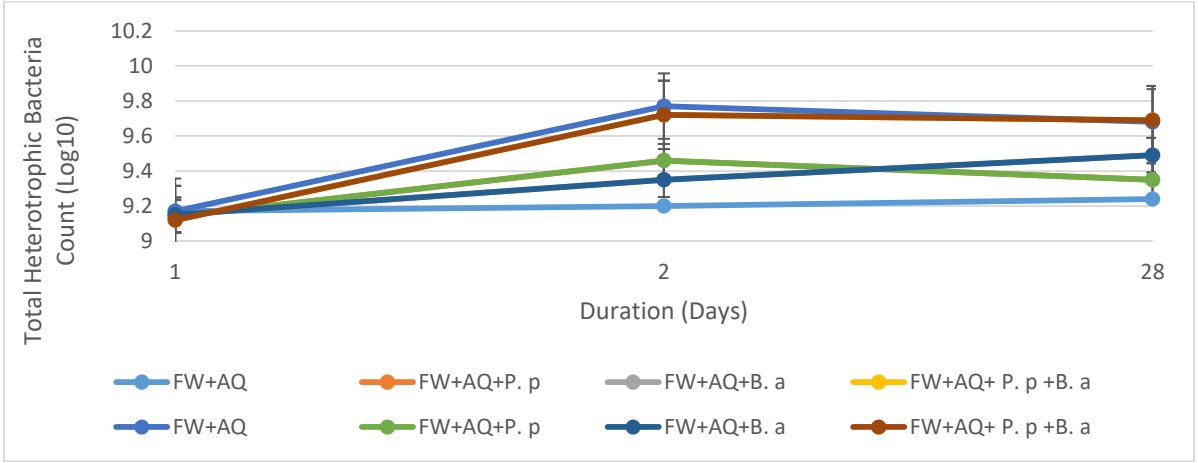

Fig. 2. Changes in Total Heterotrophic Bacterial Counts (Log_{10}) over the Period of 28 Days

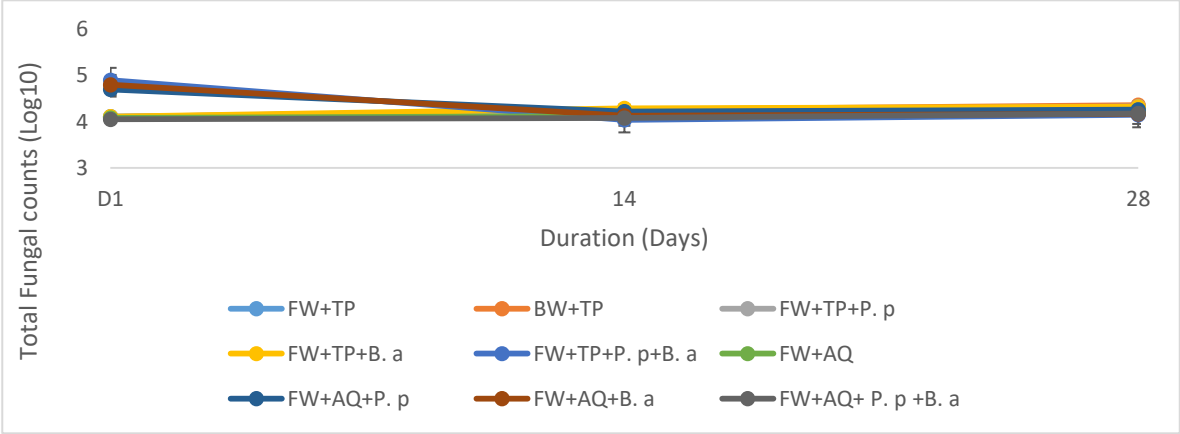

Fig. 3. Changes in Total Fungal Counts (Log_{10}) over the Period of 28 Days

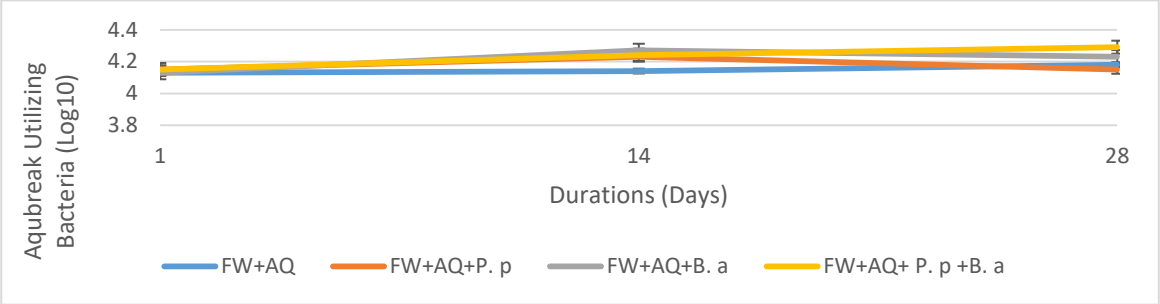

Fig. 4. Changes in Aquabreak Utilizing Bacteria Counts (Log_{10}) over the Period of 28 Days

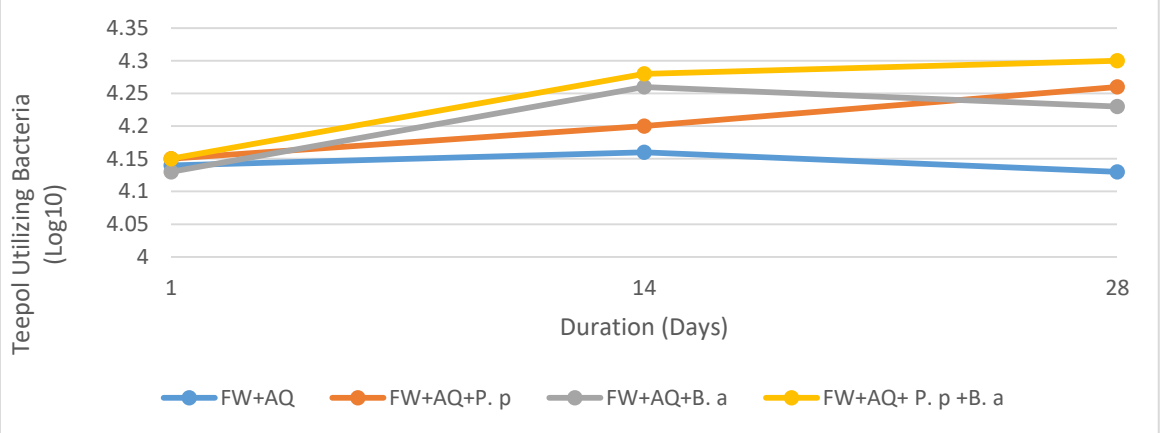

Fig. 5. Changes inTeepol Utilizing Bacteria Counts (Log_{10}) over the Period of 28 Day

The biodegradability of dispersants is the expression with which the living organism present in the water causes its degradation. The results of the physicochemical analyses of the biodegradation set up are represented in Tables 3 to 6. The changes in pH concentration of the respective set-ups (table 3) ranged from 6.5 (Aquabreak) to 8.0 (Teepol) as of day one indications that the samples were all basic, but the biodegradation flask containing aquabreak recorded decrease in pH than the Teepol and this can be a contributory factor to increased total bacteria counts in their biodegradation set up.

Changes in dissolved oxygen over a period of 28 days showed the range of 0.8 - 1.9mg/l for day one, 0.5 -0.8mg/l for the day 28, the mean revealed that control fresh water contaminated with Teepol recorded the lowest 0.7mg/l the set

coded as FW+AQ+*P. p* recoded the highest (Table 4). This is an indication of reduced rate of biodegradation of dispersant by the microbial population. Biochemical Oxygen Demand (BOD) were higher in the set containing the two dispesants than the control (Table 5). There was a sharp and constant decrease in the total oil value from day 1 until the end of the experiment day 28. This decrease in the oil value observed in the biodegradation flasks containing the test samples (test organisms) indicate that biodegradation was taking place (Table 6).

Table 3. Variation in the pH Concentrations during Bioremediation of Dispersant

Set-up Code	Day 1	Day14	Day 28	Mean	STDEV
FW	6.8	4.0	6.5	5.76	1.53
FW+TP	6.5	4.5	6.2	5.73	1.07
FW+TP+*P. p*	6.5	9.7	9.0	8.4	1.68
FW+TP+*B. a*	6.5	9.5	9.5	8.5	1.73
FW+TP+*P. p*+*B. a*	6.5	9.4	8.5	8.1	1.48
FW+AQ	8.0	8.1	7.6	7.9	0.26
FW+AQ+*P. p*	8.0	8.3	8.5	8.2	0.25
FW+AQ+*B. a*	8.0	8.2	7.2	7.8	0.52
FW+AQ+ *P. p* +*B. a*	8.0	7.9	9.0	8.3	0.60

Table 4. Variation in the Dissolved Oxygen (mg/l) Concentrations during Bioremediation of Dispersant

Set-up Code	Day 1	Day14	Day 28	Mean	STDEV
FW	0.8	0.9	0.5	0.7	0.2
FW+TP	1.3	0.9	0.7	0.9	0.3
FW+TP+*P. p*	1.3	0.9	0.8	1.0	0.2
FW+TP+*B. a*	1.3	0.9	0.7	0.9	0.3
FW+TP+*P. p*+*B. a*	1.3	0.9	0.5	0.9	0.4
FW+AQ	1.9	1.0	0.6	1.1	0.6
FW+AQ+*P. p*	1.9	1.5	0.8	1.4	0.5
FW+AQ+*B. a*	1.9	1.2	0.6	1.2	0.6
FW+AQ+ *P. p* +*B. a*	1.0	0.7	0.8	0.8	0.1

Table 5. Variation in the BOD (mg/l) Concentrations during Bioremediation of Dispersant

S/N	Set-up Code	Day 1	Day14	Day 28	Mean	STDEV
1	FW	0.6	1.4	5.2	2.4	2.4
3	FW+TP	0.5	1.9	5.1	2.5	2.3
5	FW+TP+*P. p*	0.5	1.5	4.9	2.3	2.3
7	FW+TP+*B. a*	0.5	1.5	4.2	2.1	1.9
9	FW+TP+*P. p*+*B. a*	0.5	1.2	4.7	2.1	2.2
12	FW+AQ	0.6	1.9	4.6	2.4	2.0
14	FW+AQ+*P. p*	0.6	1.0	4.2	1.9	1.9
16	FW+AQ+*B. a*	0.6	2.0	4.4	2.3	1.9
18	FW+AQ+ *P. p* +*B. a*	0.6	1.8	4.6	2.3	2.0

Table 6. Variation in Total Oil Content (THC mg/l) during Bioremediation of Dispersant

Set-up Code	Day 1	Day14	Day 28	Mean	STDEV
FW	182	68	60	103.3	68.2
FW+TP	2838	2838	588	2088	1299.0
FW+TP+*P. p*	2838	2036	56	1643.3	1431.9
FW+TP+*B. a*	2838	2492	178	1836	1446.2
FW+TP+*P. p*+*B. a*	2838	1970	18	1608.6	1444.3
FW+AQ	914	840	120	624.6	438.6
FW+AQ+*P. p*	914	112	48	358	482.5
FW+AQ+*B. a*	914	174	8	365.3	482.3
FW+AQ+ *P. p* +*B. a*	914	154	10	359.3	485.7

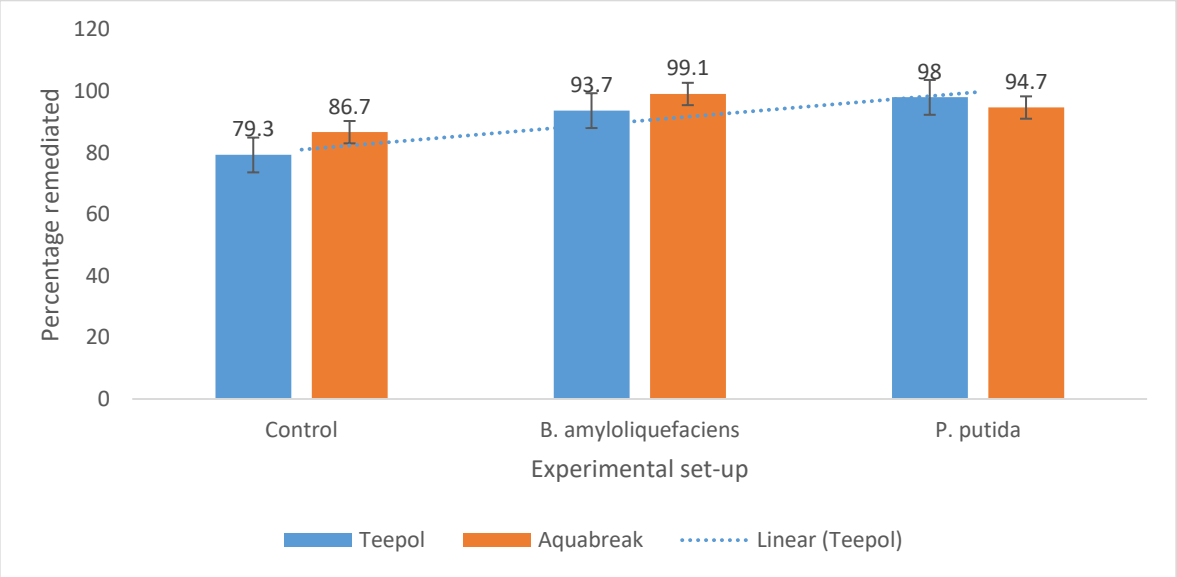

Fig. 6. Percentage (%) biodegradation of Oil spill dispersants in Fresh water ecosystem at day 28

Percentage (%) Ultimate biodegradability of the two oil spill dispersant OSD/Teepol and OSD/aquabreak revealed that control set-up recorded the 79.3 and 86.7 % , bioremediation set-up augmented with Bacillus amyloliquefaciens recorded 93.7 and 99.1% while the set-up augmented with P. putida had 98.0 and 94.7% respectively as presented in (Fig. 6). These shows that *P. putida degrade Teepol than Bacillus amyloliquefaciens while aquabreak is more degraded by* Bacillus amyloliquefaciens than *P. putida.*

According to Saadoun et al., (2008), different environmental conditions as well as the activities of the microbial populations play a role in degradation of the contaminants which was reflected in the results obtained in this study. The depletion in total oil content indicated that dispersant degrading microbes as well as the indigenous bacterial communities in the contaminated water possessd the natural ability to degrade the Dispersants as they use petroleum as their source of carbon and energy. Losses in oil content when treated with the various treatments in our study confirms the findings of Leeraet al., 2018 who observed losses in total petroleum hydrocarbon to natural attenuation. Our result is also in line with the work done by Wemedo et al., [17] where there was a decrease in the TPH levels over a 28 days incubation period from an initial of 74.80mg/kg to 53.03mg/kg. This is due to the lignolytic features of these organisms that produce extracellular enzymes that breakdown the pollutants, releasing and reducing the pH of the medium.

4. CONCLUSION AND RECOMMENDATION

One of the considerations critical for returning environmentally polluted water to its original state is a thorough knowledge of the impact of oil pollution on the parameters for its elimination. In this study, dispersant degrading microbes, their effects on bioremediation of crude oil polluted water was investigated. It was observed that *P. putida degrade Teepol than Bacillus amyloliquefaciens while aquabreak* is more degraded by *Bacillus amyloliquefaciens* than *P. putida.* Nevertheless both dispersant shown high level of degradability by the test organisms. Therefore It is recommended that oil companies and government parastatals carrying out remediation in the Niger Delta should be encouraged and OSD/Teepol and OSD/aqubreak due to their high biodegradation potential.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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