Original Research Article

Enhanced Biodegradation of Degreaser Using *Pseudomonas* and *Bacillus* Species in Fresh Water Ecosystem

`A B S T R A C T

Aim: The aim of this study is to enhance the biodegradation of degreasers used in upstream sectors of Nigeria Petroleum Industry using bio-augmenting organisms such as: Pseudomonas and Bacillus species in freshwater Ecosystem.
Study Design: This study employs experimental designs, statistical analysis of data and interpretation.
Place and Duration of Study: Freshwater sample for this research was collected from Asarama Andoni, in Rivers State, Nigeria.
Methodology: The experimental set-up was carried in 500ml conical flask with two species of bacteria, two types of degreaser and fresh water sample giving a total of 8 set-up including controls. The *Pseudomonas* and *Bacillus* species used in this study were isolated from the freshwater ecosystem and identified using standard microbiological methods. The bioremediation potential of the respective test organisms were monitored for 28 days at a constant interval of 7 days using the following Physiochemical parameter; Total dissolved Solid, Hydrogen concentrations ions and Total Hydrocarbon Content. While the following Microbiological parameters; Total heterotrophic Bacteria, Total Heterotrophic Fungi, Hydrocarbon Utilizing Bacteria, and Hydrocarbon Utilizing Fungi were monitored. Five species of bacteria: *E. coli sp, Micrococcus, Citrobacter, Bacillus, and Pseudomonas* species and four fungal species: *Penicillium, Mucor, Aspergillus and Rhizopus* species were isolated and identified as hydrocarbon utilizing bacteria and fungal organisms respectively.
Results: The percentage of degradability of the respective set-ups ranged from Control (Rigwash) (3.29%) < Pseudomonas *sp.* + Rigwash (27.56%) < Pseudomonas + Bacillus + Rigwash< (31.57 %), Bacillus *sp.*+ Rigwash (37.57%) Control 2 (Aquabreak) (9.45%) < Pseudomonas *sp.*+ Aquabreak (26.77%) < Pseudomonas + Bacillus + Aquabreak (31.32%)< Bacillus *sp.*+ Aquabreak (32.46%).
Conclusion: The results revealed that *Bacillus* species have more degradability potential than *Pseudomonas* species for both Aquabreak and Rigwash not only that but also the result indicates that Rigwash has low biodegradation potential in fresh water Ecosystem.

Keywords*:* Aquabreak, Degreaser, *Pseudomonas*, *Bacillus*, fresh Ecosystem. Rigwash, Enhanced biodegradability

1. INTRODUCTION

Many substances known to have toxic properties are regularly introduced into the environment through human activity. These substances range in degree of toxicity and danger to human health. Many of these substances

either immediately or ultimately come in contact with or are sequestered by soil [1]. Conventional methods to remove, reduce, or mitigate toxic substances introduced into soil or ground water via anthropogenic activities and processes include pump and treat systems, soil vapor extraction, incineration, and containment. Utility of each of these conventional methods of treatment of contaminated soil and/or water suffers from recognizable drawbacks and may involve some level of risk.

Bioremediation offers an alternative method to detoxify contaminants and is being used as an effective means of mitigating hydrocarbons, halogenated organic solvents and compounds, non-chlorinated pesticides and herbicides, nitrogen compounds, metals and radionuclides [2].

Bioremediation technology exploits various naturally occurring processes which include; natural attenuation, biostimulation, and bioaugmentation. Natural attenuation occurs without human intervention other than monitoring but rather relies on natural conditions and behavior of soil microorganisms that are indigenous to soil [3]. Biostimulation also utilizes these indigenous microbial populations to remediate contaminated soils and consists of adding nutrients and other substances to soil to catalyze natural attenuation processes. Bioaugmentation involves introduction of exogenic microorganisms (sourced from outside the soil environment) capable of detoxifying a particular contaminant, sometimes employing genetically altered microorganisms [4,5].

During bioremediation, Microorganisms are known to be ubiquitous and also adapt to the environment for effective competition for available nutrient utilize chemical contaminants in the environment as an energy source and, through oxidation-reduction reactions, metabolize the target contaminant into useable energy for microbes [6]. The by-products (metabolites) released back into the environment are typically in a less toxic form than the parent contaminants. For example, petroleum hydrocarbons can be degraded by microorganisms in the presence of oxygen through aerobic respiration. The hydrocarbon loses electrons and is oxidized while oxygen gains electrons and is reduced. The result is formation of carbon dioxide and water [7]. When oxygen is limited in supply or absent, as in saturated or anaerobic soils Environment, anaerobic (without oxygen) respiration prevails. Generally, inorganic compounds such as nitrate, sulfate, ferric iron, manganese, or carbon dioxide serve as terminal electron acceptors to facilitate biodegradation [8,9].

However the introduction of degreaser alters the environment causing a selection of those microorganisms capable of degrading petroleum [3]. According to (Nrior and Odokuma, 2015) Degreasers are chemical substances used for the removal of water insoluble substances such as grease, oil, paint, from auto engine parts. A degreaser can either be oil-based or water-based. Oil-based degreasers are usually toxic and flammable. Even small amounts entering surface or groundwater can result in serious pollution. Degreasers can be used in a variety of industries such as aircraft, automotive, nuclear power plants, pharmaceutical, paint, printing, transportation, optics, marine and semiconductor. They can also be used in domestic cleaning, e.g. floors, tiles cleaning, etc. Many oil-based degreasers readily evaporate and contribute to smog or ground level ozone. Water based cleaners are generally safer for the user and the environment. They are less toxic than oil based degreasers and small amounts can be broken down in sewage treatment facilities. In the biodegradation process, it is pertinent that the only carbon source should be petroleum products. This otherwise would slow

UNDER PEER REVIEW

down the biodegradation rates as the microorganism will turn to alternative carbon sources as a source of energy thus leaving behind the hydrocarbon. More so, hydrocarbon degrading microorganisms require nitrate and phosphate for growth, limitation of these substrates affects the rate and extent of degradation of petroleum in soil environment [10]. The aim of this research work was to evaluate the Enhanced Biodegradation of Degreaser Using *Pseudomonas* and *Bacillus* Species in Freshwater Ecosystem.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The freshwater used in this study was collected in a sterile four (4) litres plastic container from Asarama Andoni, in Rivers State, Nigeria.

2.2 Isolation of the Test Organisms

The test organisms (*Bacillus* and *Pseudomonas* species) were isolated from the fresh water using standard microbiological methods (spread plate method) as described by Prescott, *et al.,* [11] An aliquot (0.1ml) of the fresh water sample was aseptically inoculated into properly dried nutrient agar plates in duplicate, spread evenly using flamed bent rod and incubated at 37^0C for 24 hours, after incubation, the bacterial colonies that grew on the plates were sub-cultured unto fresh nutrient agar plates using the streak plate technique to obtain pure culture of the bacterial isolates as adopted by [12].

2.3 Characterisation and Identification of Test Organisms

Bacterial isolates were characterised on the basis of their colonial morphology, microscopic and biochemical characteristics and by making reference to the identification manual by [13]

2.4 Preparation of Broth culture and Standardization of Inoculums

Five colonies from the pure culture of each isolate were inoculated into nutrient broth in 500 ml conical flask separately, and incubated at 37°C for 18 to 24 hours. After incubation, an aliquot of 0.1ml was inoculated on a pre dried nutrient agar to determine the total viable counts of the broth culture. Turbidity of the bacterial suspension (i.e overnight nutrient broth with population density) was adjusted to match that of 0.5 McFarland Standard (30 to 300 colonies) by making a dilution of 1:100 in sterile nutrient broth [14].

2.5 Bioremediation set-up.

The experimental set-up was carried in 500ml conical flask with two species of bacteria, two types of Chemical and fresh water sample giving a total of 8 set-up including controls. The flask were coded with number from 1-8. Set 1 contain~~s~~ed 396ml of freshwater and 4ml of Aquabreak without organism which served as control for Aquabreak while set-5 ~~is~~ was a control for Rigwash it contain~~s~~ed 396ml of freshwater and 4ml of Rigwash without organism.

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2.6 Monitoring of the bioremediation Potential

The bioremediation potential of the respective test organisms were monitored for 28 days at a constant interval of 7 days using the following Physiochemical parameter; Total dissolved Solid (TDS), Hydrogen concentrations (P^H) and Total Hydrocarbon Content (THC). While the following Microbiological parameters; Total heterotrophic Bacteria, (THB) Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) were also monitored.

2.7 Physiochemical Parameters

2.7.1 Determination of Total Hydrocarbon Content (THC) in Liquid Sample

a) 20ml of sample was measured into a 150ml flask
b) 4ml of Extracting solvent (Chloroform) was added and shaken for 2minutes and poured into a separating funnel.
c) The extract was filtered through a glass funnel stocked with cotton wool and anhydrous Sodium Sulphate.
d) Absorbance was measured @ 420nm.

2.7.2 Percentage (%) Biodegradation Evaluation

The percentage (%) biodegradation rate was calculated from the formula adopted by Nrior *et al.,* (2017) as follows:

Step 1:

Amount of total oil and grease remediated equals to Initial concentration of THC (Day 0) minus final concentration of pollutant at end of experiment (last day).

Step 2:

Percentage (%) bioremediation equals to amount of oil and grease remediated divided by initial concentration of pollutant (Day 0 or 1) multiplied by 100.

Thus; $B_c = I_c - F_c$

$B_x = I_c - I_o$

Where, B_c=Amount of oil and grease degraded

I_c = Initial concentration of oil and grease (Day 0)

F_c = Final concentration of oil and grease at end of experiment (Last day)

I_o = Initial concentration value of Control at day 0

B_x = Actual amount of oil and grease in test medium

$$\textbf{\% Bioremediation} = \frac{B_c}{B_x} \times 100$$

2.7.3 Determination of pH and Total Dissolved Solid (TDS)

The pH and Total Dissolved Solid (TDS) were determined using Multiple Hannah meter as follows:

a) 20ml of liquid sample was measured into 100ml beaker
b) The electrode meter was immersed into the sample
c) The pH and electrical conductivity were recorded for each sample.

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2.8 Microbiological Parameters

2.8.1 Total Heterotrophic Bacteria (THB)

Total heterotrophic bacteria were enumerated using spread plate method. An aliquot (0.1ml) from 10^{-5} dilution from each of the set-ups were aseptically transferred unto properly dried nutrient agar plates in duplicate, spread evenly using flamed bent glass rod and incubate at 37^0C for 24 hours as described by Prescott *et al.,* [11]. After incubation, the bacterial colonies that grew on the plates were counted and average taken. Total Heterotrophic Bacteria (THB) Counts was then taken and express as colony forming unit per millilitter using the below equation as adopted by Nrior and Kpormon (2018)

$$\text{THB (cfu/ml)} = \frac{\text{Number of Colonies}}{\text{Dilution } (10^{-4}) \times \text{Volume plated (0.1ml)}}$$

2.8.2 Total Heterotrophic Fungi (THF)

The total Heterotrophic fungi in each of the set-ups were enumerated using spread plate method. An aliquot (0.1ml) of the dilution of 10^{-2} dilution was aseptically transferred unto properly dried Sabouraud Dextrose Agar plates containing antibiotic (250 Tetracycline) to inhibit bacterial growth, in duplicate, spread evenly using bent glass rod and incubate at 35^0C for 3 days, spores of fungal isolates that grew on the plate were counted and expresses as colony forming unit per milliliter using the below equation:

$$\text{THF (cfu/ml)} = \frac{\text{Number of colony}}{\text{Dilution } (10^{-2}) \times \text{Volume plated (0.1ml)}}$$

2.8.3 Hydrocarbon Utilizing Bacteria and fungi (HUB and HUF)

An aliquot of 0.1 ml from 10^{-2} dilution of the respective set-ups were ~~in~~ inoculated into Mineral salt agar that was formulated as adopted by Nrior and Odokuma [15] for isolation of both hydrocarbon utilizing bacteria and fungi, in duplicate using spread plate techniques. Sterile filter papers placed in the cover of the Petri dishes were saturated with 1ml of crude oil. The plates were then incubated inverted at 28°C for 5-7 days [16]. The filter paper saturated with crude oil served as a sole source of carbon [17]. Colonies formed in the respective plates were counted and the mean values were recorded and expressed as cfu/ml. The mineral salt agar used for enumeration of hydrocarbon utilizing bacteria was amended with fungizone lotion while for hydrocarbon utilizing fungi the medium was amended with 250mg of tetracycline to inhibit the growth of hydrocarbon utilizing bacteria [18].

2.8.4 Composition of the mineral salt agar

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K_2HPO_4 (0.5g), $MgSO_4$.$7H_2O$ (0.3g), $NaCL_2$ (0.3g), $MnSO_4.H_2O$ (0.2g), $FeSO_4.6H_2O$ (0.02g), $NaNO_3$ (0.03g), $ZnCL_2$ (0.3g) and Agar (15g) into 1000ml of distilled water.

3. RESULTS AND DISCUSSIONS

The morphological and Biochemical characteristics of the test isolates used for the enhance biodegradation of the Degreasers (Aquabreak and Rigwash) are presented in Table1.

Table 1: Morphological and Biochemical characteristics of the test organisms

Tests	Isolate A	isolate B
Colour of colony	Creamy	Greenish
Shape of colony	Circle and raised	Round and flat
Size of colony	Tiny	Small
Cell shape	Straight long rod	Curve rod
Gram's reaction	+	-
Motility	+	+
Catalase	+	+
Oxidase	+	+
Mr	+	-
Vp	-	-
Indole	-	-
Urease	-	-
Coagulase	-	-
Citrate utilization	+	+
Ammonia oxidation	-	-
Nitrate reduction	-	+
Mannitol	+	+
Glucose	+	-
Sucrose	+	-
Probable organism	***Bacillus spp***	***Pseudomonas* sp.**

Physiochemical parameters of degreaser contaminated freshwater Ecosystem of the Freshwater contaminated with oil Aquabreak, and Rigwash and enhanced with the respective organisms were taken at constant interval of seven days for one month respectively. The total dissolved solids and P^H of the different samples were measured using a Multiple Hanna meter. The meter was calibrated and then used to measure the TDS and P^H of the samples which were put in a sterile 50 ml measuring beaker and this was done after every 7 days up to 28 days (Figures 1 and 2). The Total Dissolved Solids (mg/l) in the sample which appear in a deceasing order implies that the array of chemical contaminant is decreasing [10,16].

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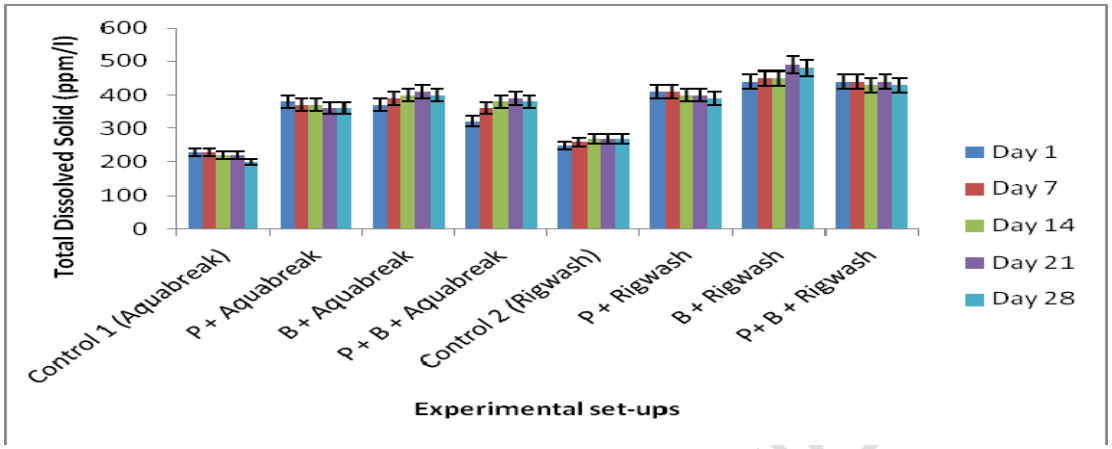

Fig 1: Changes in Total Dissolved Solids during Enhanced Biodegradation of Degreaser in freshwater

Key: **P** = *Pseudomonas* sp., **B** = *Bacillus* sp.

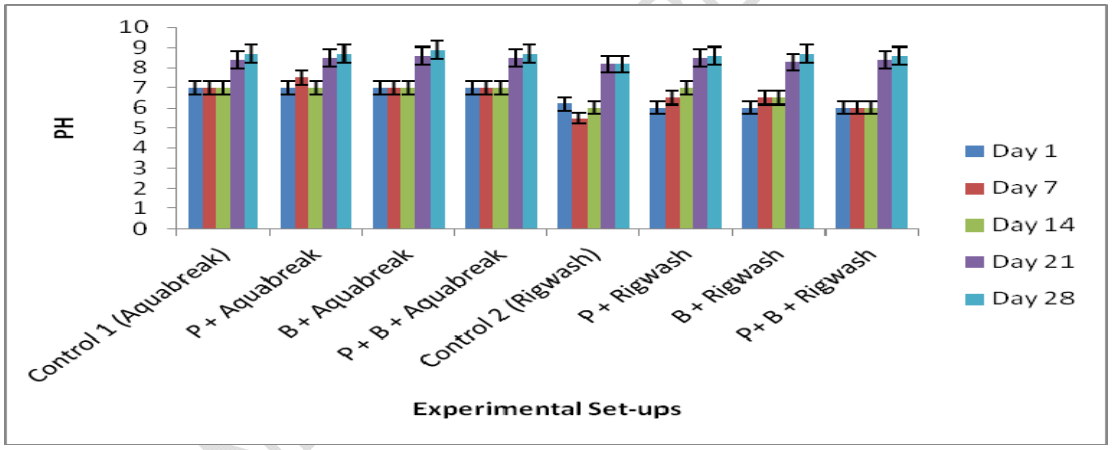

Figure 2: Changes in pH Concentration during Enhanced Biodegradation of Degreaser in freshwaters

Key: **P** = *Pseudomonas* sp., **B** = *Bacillus* sp.

The results of total hydrocarbon content (THC) in the respective freshwater contaminated with Aquabreak and Rigwash as well as the uncontaminated freshwater are presented in the Table 2. The continuesd decrease in value of THC as revealed in table indicates enhance degradation of the degreaser by the test bacteria (*Pseudomonas* and *Bacillus* species). These results are in agreement with the observation reported by Nrior *et al.*, 2017.

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Table .2 Changes in the Total Hydrocarbon Content (THC) during Enhanced Biodegradation of Degreaser in freshwater

Sample	Unit	Day 1	Day 7	Day 14	Day 21	Day 28
Control 1 (Aquabreak)	Mg/l	91.27	78.27	72.27	75.87	74.67
Pseudomonas *sp.*+ Aquabreak	Mg/l	85.47	64.27	55.87	50.67	44.27
Bacillus *sp.*+ Aquabreak	Mg/l	84.27	61.27	54.27	38.27	34.27
Pseudomonas + bacillus + Aquabreak	Mg/l	83.87	62.67	55.07	40.27	36.27
Control 2 (Rigwash)	Mg/l	198.27	197.27	195.07	194.27	192.67
Pseudomonas + Rigwash	Mg/l	197.27	176.27	157.47	154.67	151.47
Bacillus + Rigwash	Mg/l	195.87	170.67	154.27	147.87	134.47
Pseudomonas + bacillus + Rigwash	Mg/l	196.27	175.27	156.27	152.27	144.67

The result of total Microbial counts of the freshwater contaminated with Aquabreak and Rigwash, Bioaugmented with *Bacillus* and *Pseudomonas* species for enhanced degradability and unenhanced freshwater which served as control are presented in Figure 5, 6, 7 and 8. The total heterotrophic bacterial count showed that the freshwater enhanced with Bacillus species have the highest count of microbial counts followed by freshwater enhanced with both *Bacillus and Pseudomonas* species and then control (Figure 5 and 6). This shows an increase in the number of colonies as days passes. The population levels of hydrocarbon utilizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbon [17]. Fungi population showed similar trend but with a lower value ~~comparative~~ compared to Total Heterotrophic bacteria; Fungal counts (log10 cfu/ml); degreaser contaminated freshwater enhanced with Bacillus species have the highest value of log10 cfu/ml.

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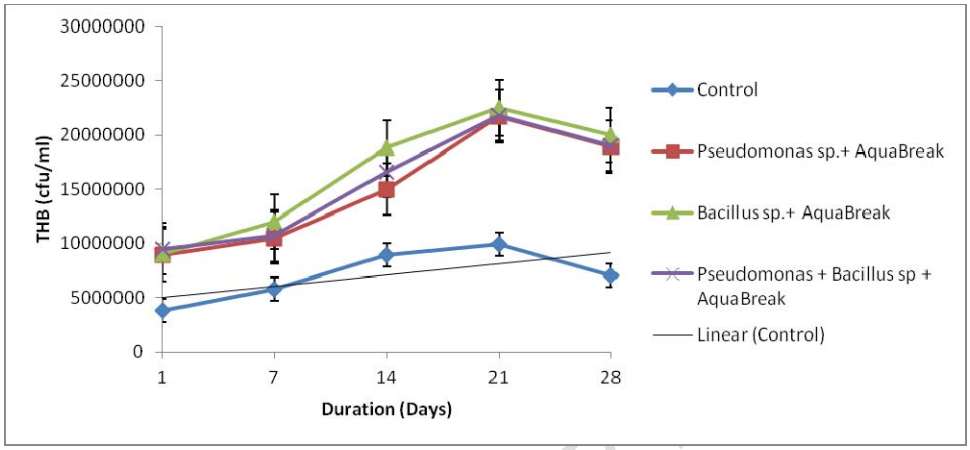

Fig.5: Changes in Total Heterotrophic Bacteria (THB) (cfu/ml) count for Aquabreak during Enhanced Biodegradation of Degreaser in freshwater

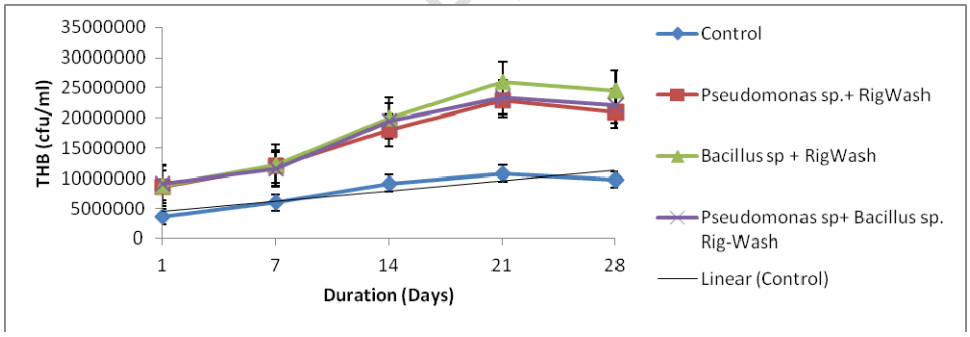

Fig.6: Changes in the Total Heterotrophic Bacterial (THB) (cfu/ml) Count for Rigwash during Enhanced Biodegradation of Degreaser in freshwater

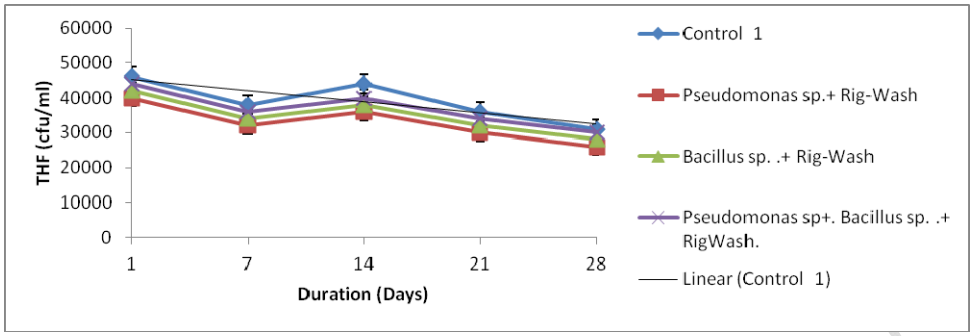

Fig.7: Changes in the Total Heterotrophic Fungi (THF) (cfu/ml) Count for Rigwash during Enhanced Biodegradation of Degreaser in freshwater

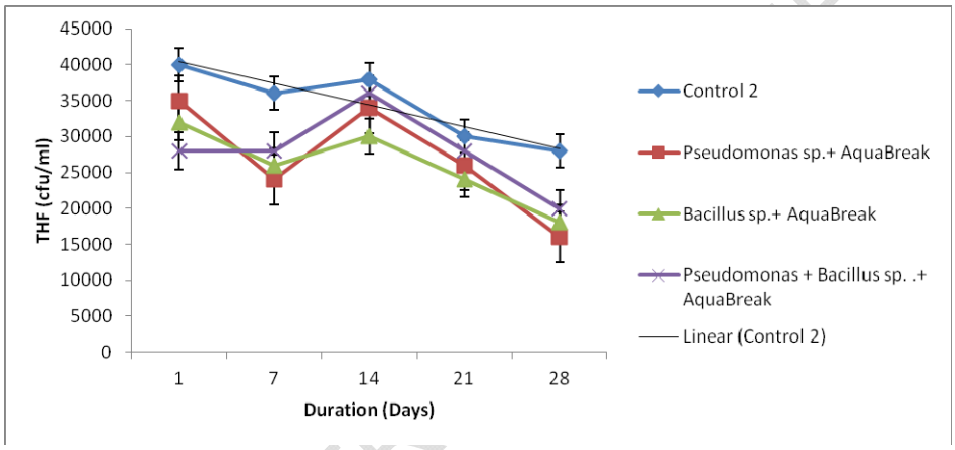

Fig.8: Changes in the Total Heterotrophic Fungi (THF) (cfu/ml) Count for Aquabreak during Enhanced Biodegradation of Degreaser in freshwater

The relative occurrence of specific genera of bacteria could be used as an index of the pollution status or biodegradation potential of an environment [18]; this fact clearly emphasizes the thought that the significance of occurrence may be due to the fact that *Pseudomonas* and *Bacillus* are more adapted to survival and biodegradation capabilities in freshwater environment. The relative occurrence of specific genera of bacteria could be used as an index of the pollution status or biodegradation potential of an environment [18]; this fact clearly emphasizes the thought that the significance of occurrence may be due to the fact that *Pseudomonas and Bacillus* are more adapted to survival and biodegradation capabilities in marine water environment. Drilling fluid utilizing bacteria isolates were *Pseudomonas, Bacillus, Micrococcus and Enterobacter*, with *Bacillus species* having the highest frequency of 42%, followed by *Pseudomonas,* with the frequency of 26%, Micrococcus and Enterobacter had 16% (Figure 9) while fungi genera were; *Aspergillus, Penicillium, Rhizopus* and *Mucor*. These groups of microorganisms are no doubt the normal flora of any situation that has to do with

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dulling fluid or mud in most familiar studies ~~while fungi genera were; Aspergillus, Penicillium, Rhizopus and Mucor. This group of microorganisms is no doubt the normal flora of any situation that has to do with dulling fluid or mud in most familiar studies.~~

The percentages of degradability potential of the respective bacterial used for the enhancement are shown in Figure 3 and 4. The figures show that *Bacillus* species has high potential to enhance degradation of both Rigwash and Aquabreak when compared to *Pseudomonas species* and consortium of both isolates. The percentage of degradability of respective set-up ranged from Control (Rigwash) (3.29%) < Pseudomonas *sp.* + Rigwash (27.56%) < Pseudomonas + Bacillus + Rigwash< (31.57 %), Bacillus *sp.* + Rigwash (37.57%) Control 2 (Aquabreak) (9.45%) < Pseudomonas *sp.* + Aquabreak (26.77%) < Pseudomonas + Bacillus + Aquabreak (31.32%) < Bacillus *sp.* + Aquabreak (32.46%)

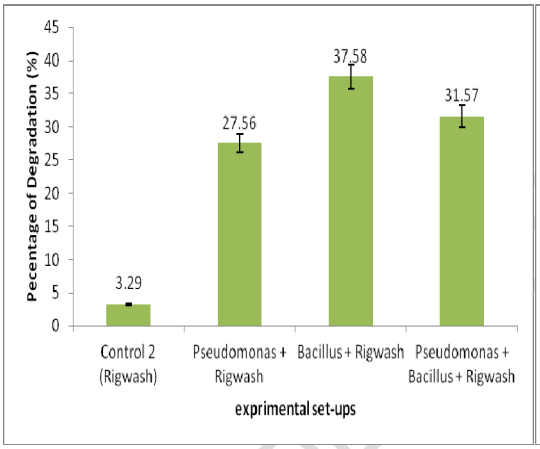

Fig 3: Degradation Percentage of Aquabreak

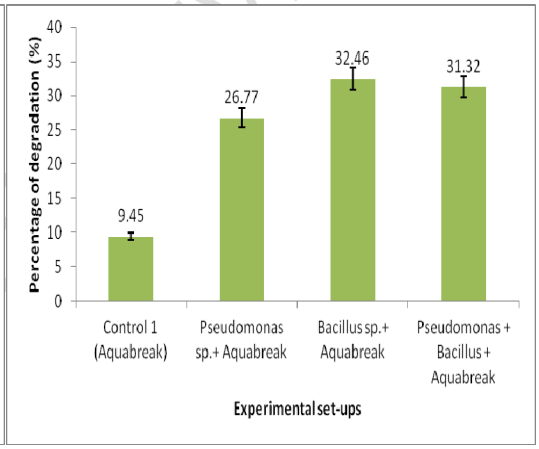

Fig 4: Degradation Percentage of Rigwash

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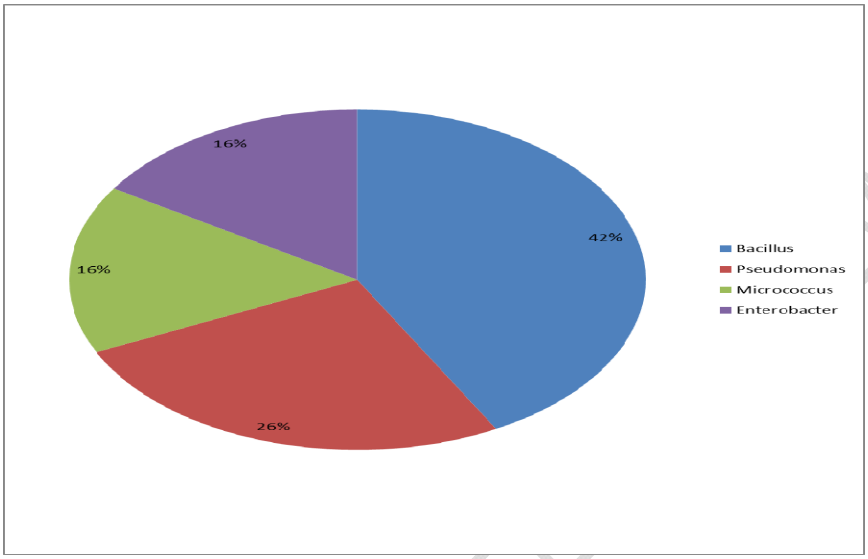

Figure 9: Percentage of Occurrence of the Bacterial Isolates From Reshwater Ecosystem

4. CONCLUSION AND RECOMMENDATION

Conclusively, the study showed that *Bacillus* species have more degradability potential than *Pseudomonas* species for both Aquabreak and Rigwash, not only that but also the result indicates that Rigwash has low biodegradation potential in fresh water Ecosystem.

Therefore it is recommended that since most oil well drilling activities in Nigeria are carried out in aquatic environments, *Bacillus* species could be used to enhance biodegradation in case of oil spill. In the course of this study it has been revealed that the two degreasers are somehow toxic to some microorganisms and therefore reduced the number of microorganisms in the sample as the days goes by. The most frequently isolated microorganisms that degrade degreaser are *Bacillus,* followed by *Pseudomonas*, *Micrococcus* and *Enterobacter*. However, it is important to note that the persistence and degradation effect in deep aquatic Ecosystems may vary from the data that were obtained in the laboratory tests due to other factors that can affect the enhanced biodegradation rate of the degreaser. Since environmental conditions in the aquatic Ecosystems vary from one location to another, it is not possible to develop a standardized procedure for biodegradability that covers all environmental conditions.

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