Original Research Article

EVALUATION OF BIOREMEDIATION POTENTIAL OF ALGAE (*Chlorella vulgaris*) IN CRUDE OIL CONTAMINATED SEDIMENT

ABSTRACT

Aim: To evaluates the bioremediation potential of algae (*chlorella vulgaris*) in crude oil contaminated sediment.
Study Design: The study employs three (3) experimental design using flat rubber, statistical analysis of the data and interpretation makes up the study design.
Place and Duration of Study: New Calabar River, Choba, Obio Akpor Local Government Area, Rivers State, Nigeria, was used for this study. New Calabar River lies between longitude 6°53.13E and latitude 4°53.52N in Choba. Bioremediation monitoring process lasted for 56 days; analyses were carried out at 14 days' interval.
Methodology: Three (3) experimental set up were employed using a using flat rubber basin, each set up contained 2500g of sediment and was contaminated with 250ml of Crude Oil except Control 1 uncontaminated sediment (Us). The set up was augmented with the *vulgaris vulgris* (CHL) except the control 1 and 2 (Us and Cs). Sediment profile like Temperature, pH, Nitrogen, Phosphorus, Potassium, electrical conductivity, moisture content, total organic carbon, soil organic matter and total hydrocarbon content (THC) before contamination was determined using standard analytical methods while parameters like Temperature, pH, Nitrogen, Phosphorus, Potassium and Total Hydrocarbon Contents (THC) were monitored throughout the experimental period. Microalgae and Hydrocarbon utilizing algae (HUA) were monitored throughout the experimental period using standard microbiological methods. Percentage Bioremediation was estimated from amount of THC reduction from day 1 (initial) of monitoring. Statistical analysis was carried out for microbiological and physicochemical parameters when treated using Statistical ANOVA to ascertain significant difference of mean values between various treatments.
Results: Results revealed the amount of hydrocarbon removed and % bioremediation efficiency after 56 days of monitoring with different treatment on the set up is given in a decreasing order as follows: (initial contamination value of 10525mg/kg) Cs+Chl (7700mg/kg; 73.15%) > control (Cs) contaminated without amendment of organisms (6345mg/kg 60.28%) > and Us uncontaminated sediment 1969.96 mg/kg. The total hydrocarbon content (THC) of the treated setup decreased from (10525mg/kg initial contamination value) at the start of bioremediation to Cs+Chl (7700mg/kg: 73.15%) at the end of bioremediation. The highest count of microalgae (log10cfu/g) for each set up during the monitoring were as follows; day 0 Us (7.65) day14 Cs (7.63) day28 Us (7.70) day42 Cs+Chl (7.60) day56 Cs (7.30). It was observed that peak count was on day28 (7.70) and a decline was on day56 (7.30). The highest Hydrocarbon Utilizing Algae (Log10cfu/g) count for each set up during the monitoring were as follows; day 0 Cs+Chl (5.23) day 14 Cs+Chl (5.30) day28 Cs (5.23) day42 Cs+Chl (4.77) > day56 Cs (5.07). Decline was observed on day 42 and peak count was on day 14.
Conclusion: Results from the study revealed that *Chlorella vulgaris* is capable of degrading hydrocarbon components. There was a faster utilization of hydrocarbon by set up with *Chlorella vulgaris* than control. Though incomplete removal of crude oil was observed in THC concentration value. This suggests that an improvement in the process is required. Such improvement could include biostimulation of the polluted sample or some chemical pretreatment of the sample. From the study, bioremediation can be said to be a viable and effective response to sediment contamination with crude oil.

Keywords: Bioremediation; Microalgae; Hydrocarbon; *Chlorella vulgaris*; crude oil contamination; Sediment; ~~Total Hydrocarbon Contents; Nitrogen; Potassium~~.

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INTRODUCTION

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The natural functioning of the environment supplies both goods and services, such as food and other products, on which man needs for the continuation of life. The environment is therefore our shared legacy and should be carefully fostered [1]. Due to human habitation and development endeavors, there has been significant environmental deterioration as a result of man's presence on earth [2]. Although these services are not designed to have a monetary value, man nonetheless depends on the ecosystem for survival since it stores a significant quantity of carbon in both plants and soils, which regulates water flow and quality as well as helps to stabilize local climates [3]. It is urgent and necessary to stop this degradation because it has endangered the sustainable development aim. The world is full of contaminated soils, but they are particularly prevalent in underdeveloped nations where environmental laws are, at best, minimal. Petroleum products are the main source of environmental pollution in the modern world since their extraction and downstream use are linked to economic growth [4]. There are numerous oil exploration and exploitation activities taking place within this incredibly valuable ecosystem. The relative cleanliness of oil contributed to its rise to prominence as the primary fuel for the globe, but the massive scale of the petroleum industry's operations eventually led to new, challenging environmental issues that we are currently experiencing [5]; [6]. Crude oil pollution is one of these environmental issues that is becoming worse every day. Since the majority of people depend on farming, fishing, and the use of water for domestic purposes, the risk to farmlands, fisheries, and potable drinking water is one of the largest worries linked with crude oil pollution in the environment [7].

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Petroleum hydrocarbons have well-established toxicity profiles for microbes, plants, animals, and people. For instance, a low concentration of crude oil or petroleum fractions (5-100 mg/l) is sufficient to kill or hinder the growth of microalgae and young marine animal forms [8]. To eliminate or degrade the dangerous components of oil sludge, advanced technology that is both affordable and efficient is essential.

Particularly in nations like Nigeria that produce oil, crude oil contamination is a recurring problem. Consequently, despite recent improvements, oil pollution will continue to be a major issue. From the point of production to the point of processing, the transportation of crude oil or its byproducts has caused spills that have had negative effects. Oil blowouts have also happened during the extraction stage, and when they do, the oil discharges itself into the ground or water while the volatile components escape into the atmosphere. Because of the threat posed by crude oil pollution, people are looking for more environmentally friendly ways to clean up petroleum-polluted environment [9]. Natural attenuation, in which microorganisms present in a particular environment break down pollutants in that environment, is the primary natural system by which petroleum hydrocarbon pollutants can be removed from the environment [10].

Bioremediation appeared to fits the requirements of the needed technology. Utilizing microorganisms to change dangerous pollutants into less toxic ones is a process known as bioremediation. Fungal bioremediation, also known as mycoremediation, has recently been the main desire for all researchers involved in the bioremediation field. Because lignin, PAHs, and some other environmental pollutants share a chemical similarity, ligninolytic fungi have been identified as the most promising candidates to degrade PAHs. Three different types of enzymes involved in the lignin breakdown are produced by white rot fungi. Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) are the enzymes [10].

For decades, bioremediation has been used to clean hydrocarbon-polluted soils because it is economical, environmentally responsible, and successful [11]; [12]; [13]; [14] In order to maximize microbial activity and the decomposition of the pollutants, bioremediation approaches for eliminating petroleum products from soil are built around methods for giving moisture, aeration, and nutrients [13]; [15]; [16]. In situ bioremediation which employ the use of indigenous microorganisms is by far the most widely used method to remove crude oil from contaminated sites amongst all the available methods [17].

Bioremediation can occur naturally and can also be stimulated by addiction of microorganisms, a process known as bioaugmentation. Bioaugmentation is the process of addition of microorganisms known to possess the enzymes to breakdown a pollutant. They act as bioremediators that facilitate the removal of

complex pollutants. Microorganisms that have been studied and known to show abilities and high capabilities of efficient degrading a wide range of environmental pollutant are usually employed in this process. They are used in this process mainly because of their diverse metabolic profile capable of transforming complex compound into harmless and less complex end products [18]. The addition of particular substances to promote native microbial assemblages (biostimulation) and/or the addition of particular microbial taxa with effective biodegradation/detoxification ability (bioaugmentation) are two common microbial-based bioremediation techniques [19].

It has been demonstrated that polycyclic aromatic hydrocarbons like naphthalene, phenanthrene, and pyrene can be effectively broken down by green microalgae from the *Selenastrum, Scenedemus*, or *Chlorella* genera [20]. and in the immobility of the metals. Exopolysaccharides play a crucial part in the methods by which microalgae remove harmful substances, lowering their bioavailability and toxicity. They can enhance the uptake of pollutants on the cell surface and/or their complexation into less accessible forms. The contaminant linked to the membrane or cell wall exopolysaccharides may either stay adherent or may be absorbed and chelated by molecules from the phytochelatin classes, depending on the microalgal taxonomy [21].

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2. MATERIALS AND METHODS

2.1 Description of the Study Area

Choba Bridge Station of New Calabar River lies between longitude 6°53.13E and latitude 4°53.52N in Choba village and close to it is an extension base of Wilbros Nigeria Limited (WNL) oil serving industry. Construction and maintenance of oil pipelines, dredging, fishing, illegal oil refineries (also known as "kpo-fire"), and wastewater irrigation activities are among the activities carried out at this station, which is located 224.25 meters from Aluu.

2.2 Sample Collection

Sediment sample was collected beneath the river at New Calabar River, Choba Bridge, Obio Akpor Local Government Area. Rivers State, Nigeria, using soil auger and was transfer into sterile container. The collected sample was immediately transported to Microbiology Laboratory, Rivers State University, Port Harcourt for microbiological and physicochemical analysis.

Water from a fish pond in Rumuagholu in the Obio Akpo Local Government Area of Rivers State, Nigeria. was used to isolate microalgae. The pond's water was green, which denotes the existence of an algal bloom. Using sterilized glass water bottles with a 200 ml capacity, water samples were taken from the pond's surface (0.0 to 0.2 m depth). A 10 L jerry can was used to help collect a significant amount of water from the pond. [22].

2.3 Media used for Analysis

The media that were used during the analysis were Mineral salt agar (MSA) and BG-11 agar. All the media were sterilized and prepared according to Manufacturer's instruction. Table 1 and 2 shows the composition compound of MSA and BG11 media respectively [23].

Table 1: Mineral Salt Agar (MSA) Composition Compound for the Analysis

Salt	Quantity (g)
K_2HPO_4	0.5
$Nacl_2$	0.3
$FeSO_4 6H_2O$	0.02
$ZnCl_2$	0.3
$MgSO_4. 7H_2O$	0.3
$NaNo_3$	0.03
$MnSO_4 . H_2O$	0.2
Distilled water	1000ml

Table 2 BG-11 Composition

Salt	Quantity (Gram/L)
Sodium nitrate	1.500
Dipotassium hydrogen phosphate	0.0314
Magnesium sulphate	0.036
Calcium chloride dehydrate	0.0367
Sodium carbonate	0.020
Disodium magnesium (EDTA)	0.01
Citric acid	0.056
Ferric ammonium citrate	0.06
Distilled water	1000ml

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2.4 Enumeration and Isolation of Microalgae for Bioremediation Treatment

The algae medium was prepared using the method described by [22]. Algae bloom visible water was fetched and filtered using filter paper**,** the filtered water was supplemented with 20 $\mu g.ml^{-1}$ Na_2CO_3 and autoclaved at 121°c at 15psi for 15minutes, and used as the culture medium for the microalgae cells. Ten milliliter (10ml) of visible algae water was transferred into two different 250ml conical flask containing 189ml of sterile algae medium, and was supplemented with 0.4ml stock solution of rifampicin to inhibit other microbial growth. The flasks were cooked with cotton wool and rubber tubing was inserted into the conical flasks, but not touching the culture in other to exchange oxygen. The flasks were placed close to the window were sunlight can get to the culture and was agitated daily for over 30 days. 0.1ml aliquot of the microalgae that popped-up after 30 days was plated in to a solidified algae medium that was supplemented with rifampicin and incubated close to the window in an upward position for 5 to 15 days.

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2.8.1 Identification of Microalgae Cells

According to macroscopy and microscopy, the microalgae cells from the culture plates were recognized, and they were compared to the observed cells with photos in a text published by [24]

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2.9 Preparation of Microalgae Suspension for Bioremediation Setup

Suspension of *Chlorella vulgaris* was prepared by transferring 10ml of the isolate (*Chlorella vulgaris)* into 250ml flask containing sterilized 200ml of BG-11media and was supplemented with 0.8g of rifampicin, the flask was covered with cotton wool and was inserted with rubber tubing and was place close to window for 14days 28days.

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Table 3 Experimental Design

Set up code	Sediment (g)	Amount of crude Oil ml	Amount of broth Chl
US	2500	-	-
CS	2500	250	-
CS+Chl	2500	250	25

Key: US= uncontaminated sediment, CS= contaminated sediment, CHL= *Chlorella vulgaris*

This bioremediation set up was monitored for microalgae count, HUA and selected physicochemical parameters from day 0 to 56 days. Such as HUA, microalgae count, total hydrocarbon content (THC), nitrogen, potassium, phosphorus temperature and pH, respectively at 14 days interval. Eighty milliliter (80ml) of sterilized water was added to the set up three times weekly and agitated for proper aeration and adequate distribution of microorganism as described by [25]; [26].

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2.10 Microalgae Analysis.

2.10.1 Enumeration of Hydrocarbon Utilizing Algae (HUA)

According to [27], the vapour phase transfer technique was used to enumerate hydrocarbon-utilizing algae (HUA). In order to prevent the growth of other organisms, the media was supplemented with rifampicin. an aliquot of 0.1 ml of the 10^{-3} dilution was spread onto solidified MSA plates using the spread plate technique. The plates were then inverted, filter paper was placed inside the inverted plate cover, and 1 ml of sterile crude oil was added as a source of carbon and energy, incubated for 5 to 7 days at 37^{0}C. The number of discrete colonies that formed were counted and given in cfu/g. This was done for US, CS and CS+CHL, respectively.

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2.10.2 Enumeration of Microalgae Count

Total microalgae count was determined by weighing 1g of sample and was serially diluted up to ten fold, using spread plate method as described by [22]. An aliquot of 0.1ml from 10^{-5} test tube was inoculated into solidified water-agar plate supplemented with rifampicin. The culture plates were incubated for 48hrs to 78hrs for count. Discrete colonies that developed were counted and expressed in cfu/g. This was done for US, CS and CS+CHL respectively.

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2.11 Physicochemical analysis of selected parameters

The Physicochemical properties such as pH, temperature, moisture content, total organic carbon, electrical conductivity, soil organic matter, nitrogen, phosphorous, potassium and total hydrocarbon content (THC) of the sediment sample was determined before experimental contamination of the sediment to establish the baseline parameters and subsequently after crude oil contamination and nutrient addition for the duration of bioremediation process for selected parameters. The following selected parameters including; pH, temperature, nitrogen, phosphorous, potassium and total hydrocarbon content (THC) were determined using the methods from [28].

The pH of the sediment sample was determined using digital pH meter, the meter was put on to stabilize for 15mins. Forty grams (40g) of soil sample was collected after calibration and weighed in duplicates after drying in oven. Slurry was prepared by adding 40 ml of distilled water to the soil and allowed to equilibrate in15min. A buffer was used to calibrate pH meter at pH 7. The pH meter was dipped into 20 ml water sample in a beaker and allowed for 5 min. The mean of the three values obtained was used and the result of the reading was recorded as pH [28].

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EC was determined on-site using a conductivity meter. The conducting cells were calibrated using recognized standards whose readings were predetermined prior to examination. The probe cells were completely cleaned with distilled water at each level of analysis, then the experiment's control was run. The EC is simultaneously measured in micro Siemen/centimeter. The total organic carbon was measured using the [29] technique.

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TOC. The material was divided into one gram (1g) and put into a clean Pyrex conical flask. 7.5 ml of strong sulfuric acid and 5 ml of potassium chromate solution were added. On an electro thermal heater, the mixture was heated to reflux for 15 minutes. The sample was diluted to 100 ml with distilled water after being allowed to cool at room temperature. Using Ferrion as an indicator, 25ml of the sample solution was titrated with 0.2 molar ferrous ammonium sulphate. A blank that contained the same oxidant (potassium chromate) and sulfuric acid as the sample was titrated. The value of the tires was recorded [30]

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Water temperature was determined using calibrated thermometer. The conducting cells were calibrated using recognized standards whose readings were predetermined prior to examination. The probe cells

were completely cleaned with distilled water at each level of analysis, then the experiment's control was run. The conducting cells were then lowered into the analytes, and a simultaneous temperature reading in degrees Celsius was taken [31].

To prevent evaporation, 200g of the moisture content sample was taken out and put in a funnel that was lined with filter paper and covered with foil. Overnight, it was left to stand. After overnight (M1), an aliquot was weighed out and baked for 24 hours at 106°C in a Gallen Kamp BS, 250, England, oven. The new weight (M2) was measured after cooling. [32].

The semi-micro Kjeldahl technique was used to determined total nitrogen. One tablet of Selenium catalyst was added, along with zero point one grams (0.1g) of the sample, and a little amount of distilled water was used to wet the mixture. Conc. H2SO4 in the amount of 5 ml was added and placed on the digesting block. Until the sample was digested, it was heated over a fume cupboard. A 50ml volumetric flask was used to prepare the digest for semi-micro distillation. Ten milliliters of the digest were added to the distillation chamber of the MARKHAM distillation apparatus after it had been turned on. Gently adding 10ml of 45% NaOH allowed the sample to be distilled into 10ml of 4% boric acid.About 50ml distillate was collected and titrated with 0.02N H2SO4 to get back a pinkish-red end point [32].

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Using distilled water, digested samples from the Kjeldah analysis were prepared up to 50ml. The spectrometer's burner chamber was aspirated with a standard potassium ion concentration in order to calibrate the apparatus and create a graph of the standard ion concentration. The employed wavelength was 760 nm. The spectrometer's aspirators tube system was flushed with water before aspirating the sample. On the spectrophotometer's screen, the potassium ion concentration in the sample was immediately shown.

One-gram air dried and sieved soil was emptied into 250ml flask and 10ml of $INK_2Cr_2O_7$ solution was pipetted into the flask and swirled gently, after which twenty milliliter (20ml) concentration of H_2SO_4 was added using automatic pipette and swirled vigorously for 30minutes, then diluted with distilled water 100ml. Ferron indicator 3 drops was made and titrated with 4Nferrons sulphate solution. Changes in color from green cast to maroon color marked the end point and tire value was taken [32].

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Methodologies were utilized to ascertain the soil's THC content ~~[33]~~. A clean beaker containing five grams (5g) of the sediment sample was filled with 10 milliliters of xylene and left with a cork lid on for 30 minutes. A sample of the extract was run through an analyzer using an infrared spectrophotometer. A calibrated curve made from the dilution of a stock solution of 1:1 Bony light crude and Bony medium was used to calculate the THC value. At 450 nm, an ultraviolet light spectrophotometer was used to measure the absorption.

The amount of pollutant remediated and the % remediation in the experiment were determined using the approach of [34].

For the amount of pollutant remediated:

Pollutant remediated amounts are equal to Initial Pollutant Concentration (day 1) minus Final Pollutant Concentration (day56)

Ba = Ic – Fc

Where:

Ba= Amount of pollutant remediated

Ic = Initial Concentration of pollutant in (day1)

Fc = Final Concentration of pollutant in plot x (day56)

For percentage remediation:

The percentage (%) remediation equals Amount of pollutant remediated divided by the Initial Concentration of pollutant (day1), multiplied by 100

% remediation = (Bc/Ic) x 100.

2.12 Statistical Analysis

Statistical Package for Social Science (SPSS) was used to analyze the data in order to do statistical analysis on the collected microbiological and physicochemical parameters. In order to determine if there was a significant difference in mean value between different treatments and the study's data, analysis of variance (ANOVA) with a P-values test of significance was conducted at a 95% level of confidence.

3. RESULTS AND DISCUSSION

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3.1 Baseline Physicochemical and Microalgae Properties of the Sediment before Application of Various Treatments for Bioremediation Analysis.

The baseline physicochemical and microalgae properties of the sediment before the application of various bioremediation treatments are shown in Table 3. Notably, major parameters such as pH, temperature, electrical conductivity, potassium, phosphorus, nitrogen, moisture content, total organic carbon (TOC), soil organic matter (SOM) and total hydrocarbon content (THC) were determined. The microalgae properties determined were Total microalgae count and Hydrocarbon Utilizing Algae (HUA). The baseline results revealed that the pH was slightly acidic with value of 6.4 for uncontaminated sediment and 6.9 for contaminated sediment, temperature was observed to be 30.8^{o}C for uncontaminated while the contaminated was 26.5 ^{o}C, electrical conductivity was 301µS/cm for uncontaminated sediment and 30µS/cm for contaminated sediment, moisture content was 12.0% and 18.6% respectively, TOC was 0.48% and 1.32% for contaminated, SOM was 0.83% and 2.27%. Nitrogen was 73.74 mg/kg for uncontaminated whereas the contaminated was 70.40 mg/kg, potassium revealed 17.75 mg/kg and 37.25 for contaminated, 5.51 mg/kg and 21.22mg/kg for uncontaminated and contaminated respectively and THC for uncontaminated was 2529.96mg/kg and contaminated revealed value of 10525.15mg/kg.

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The baseline analysis for microalgae parameters for HUA and microalgae count for uncontaminated and contaminated showed the average counts of 1.75±0.35 and 1.35±0.21 for HUA and 2.50x10^{8} for microalgae count respectively.

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Table 4: Physicochemical and Microalgae Baseline of Crude Oil Contaminated Sediment in New Calabar River

Parameters	Unit	Uncontaminated soil	Contaminated soil (50ml Crude Oil + 2500g soil)	Contaminated soil (250ml Crude Oil + 2500g soil)
Temperature	`C-	30.8	30.4	26.5
Ph	-	6.4	7.1	6.9
Electrical Conductivity (EC)	µS/cm	301	167	30
Moisture Content	%	12.0	10.2	18.6
Total Organic Carbon (TOC)	%	0.48	0.63	1.32
Soil Organic Matter (SOM)	%	0.83	1.08	2.27
Nitrogen	mg/kg	73.74	93.08	70.40
Phosphorus	mg/kg	5.51	7.39	21.22
Potassium	mg/kg	17.75	10.89	37.25
Total Hydrocarbon Content (THC)	mg/kg	2529.96	5765.95	10525.15
Microbiological	**Cfu/g**	**Average count**		**Average count**
HUA	Cfu/g	1.75±0.35(×10^{3})		1.35±0.21(×10^{3})

Cfu/ml	-	$2.50x10^{8}$

KEY: HUA= Hydrocarbon utilizing algae

Plate 1: Microalgae growth on water-agar plate

Table 5: Identification and Characterization of Microalgae Isolate

Macroscopy	Microscopy	Probable organism
Spherical shaped green photosynthetic pigment.	Cells are without flagella, thin cell wall. Cell is roughly spherical and features a cup shaped chloroplast and numerous starch grains.	*Chlorella vulgaris*

3.2 Physicochemical Parameters of the Sediment Sample during Bioremediation Monitoring.

The physicochemical changes of the sediment with various bioremediation treatments are shown in the figure 1-6 The parameters determined during bioremediation were temperature, pH, nitrogen, phosphorus, potassium (NPK) and total hydrocarbon content (THC).

Temperature value obtained is given in figure 1. The temperature values from the study were relatively same between the bioremediation set up. The highest value for each set up during the monitoring were as follows; day 0 Cs+Chl (26.9^{o}C), day14 Cs (28.6^{o}C), day28 US (28.9^{o}C), day42 Cs+Chl (27.7^{o}C), and

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day56 Cs (28.1 ^{o}C). The results of temperature and pH of the bioremediation set-up showed that temperature range as well as the pH range favored the acclimatization process for the hydrocarbon utilizing microalgae growth. It was observed that the presence of Crude oil and metabolic activities of the microorganisms tends to lower sediment pH and increase its Temperature.

pH values obtained from the set up during bioremediation is shown in figure.2 The highest value from each set up during the monitoring were as follows; day 0 Cs+Chl (7.27), day14 US (7.23), day28 US (7.08), day42 Cs+Chl (6.77), and day56 Cs+Chl (7.65). pH had a notably steady reduction during the 56 days monitoring period as metabolites were produced by the organisms during the remediation process. pH levels were shown to decrease, tending toward acidity. pH value before treatment was 6.9 during the period of monitoring, pH was found to decrease gradually and was not constant. The decrease in pH may be due to the release of organic acids in the medium. [35]., reported that optimum pH for bioremediation is between 6.0 and 8.9. He stated that changes from initial levels of pH might be as a result of release of acidic and alkaline intermediates and final products during hydrocarbon degradation which has an effect on pH. Generally, alkaline or slightly acidic soil pH enhances bioremediation, while acidic environment pose limitation to biodegradation also said that optimum pH for bioremediation of hydrocarbons is around 6-8 pH. [36]

Nitrogen value obtained after 56-days of monitoring is given in figure 3. on day 0, US (73.740 mg/kg) day14 Cs+Chl had (60.397mg/kg), day28 Cs+ (125.054mg/kg), day42 US (83.075mg/kg) day56 Cs+ had (68.399mg/kg) ~~each~~. Ranged from 39.85±14.46 to 68.94±24.46mg/kg with its mean peak value as 68.94±24.46mg/kg.

Phosphorus values recorded during bioremediation monitoring was shown in figure 4. after 56 days of monitoring as follows: day 0 recorded high value from Cs (7.390mg/kg) day14 US (0.610mg/kg), day28 Cs+Chl (0.665mg/kg) day42 Cs+Chl (0.670mg/kg) and day56 Cs+Chl (0.556mg/kg). The range value is between 0.60±0.07mg/kg-1.94±3.04mg/kg.

Potassium recorded during monitoring of bioremediation of the sediment is shown in figure 5. The highest value from each set up during the monitoring were as follows; Day0 Cs (21.220 mg/kg) day14 US (10.713mg/kg) day28 Cs (20.000mg/kg) day 42 Cs+Chl (5.250mg/kg) day 56 Cs+AChl (14.875mg/kg). With its mean peak value as 11.35±8.97mg/kg. Nitrogen, phosphorous and potassium (NPK) were shown to decrease after treatment. Nitrogen before treatment was 70.40mg/kg, phosphorous was 21mg/kg while potassium was 37.25mg/kg respectively. Nutrient availability is essential for the growth and metabolism of microorganisms which directly affects the rate of degradation of petroleum hydrocarbon. Nitrogen and phosphorus have been identified as the most growth limiting factors for organism mediated hydrocarbon degradation and potassium availability can affect bioremediation rates [37]. Hence are required by microorganisms for metabolism and other cellular functions. In the absence of one or more of these substrates, microbial growth will be limited. All biological forms must have nitrogen in order to survive. As shown in the results, set up with Cs+Chl had high rate of reduction of NPK. This could be as a result of *Chlorella* which is photosynthetic algae that has the ability of converting light energy to organic carbon. Photosynthetic organisms are independent self-supporting microorganisms. These organisms synthesize nucleic acid, amino acid, bioactive substances, sugar and organic matter by using sunlight as source of energy [38]. This explains the reduction observed in NPK.

Total hydrocarbon content (THC) value obtained during bioremediation of crude oil contaminated sediment is recorded in figure 6 and 7. The amount of hydrocarbon removed and % bioremediation efficiency after 56 days of monitoring with different treatment on the set up is given in a decreasing order as follows: (initial contamination value of 10525mg/kg) Cs+Chl (7700mg/kg; 73.15%) > control (Cs)

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contaminated without amendment of organisms (6345mg/kg 60.28%) > and Us uncontaminated sediment 1969.96 mg/kg. The total hydrocarbon content (THC) of the treated setup decreased from (10525mg/kg initial contamination value) at the start of bioremediation to Cs+Chl (7700mg/kg: 73.15%) at the end of bioremediation. The setup with higher percentage bioremediation performed better, showing the setup have positive effect on the sediment. As shown, there was a remarkable decrease in THC concentration of Cs+Chl set ups compared to the control. This can be attributed, to the fact that the microorganism in the sediment have efficient ability in utilizing, the crude as a source of carbon and energy [39]. These results could be attributed to the fact that *Chlorella vulgaris* capable of converting inorganic carbon to preformed organic molecules could have made an alternative and easier to assimilate source of carbon available for other organism to utilize. These results are in agreement with the study of [40], who studied the biodegradation of motor oil using single and mixed cultures of *Nastoc hetei* and *Synechocystis acuatilis,* and reported the highest degradation rate after 14 days using the single culture of *Nastoc hetei*. However, ~~not in line with~~ the study of [41]~~, who~~ reported faster utilization of hydrocarbon by mixed cultures than individual bacteria strains. THC concentration was still incompletely removed after the period of treatment. [42] also reported that many studies performed with soil microcosms had incomplete degradation of diesel oil.

A possible explanation to the incomplete removal of crude oil from the contaminated sediment in the present study is due to nutrient deficiency. It was shown earlier in the physicochemical properties obtained at the end of the monitoring period that nitrogen, phosphorous and potassium which has been described as growth limiting substrate were very low compared to the amount obtained before treatment. The incomplete bioremediation can also be attributed to the fact that non indigenous organism was used in the study. Different studies confirmed the efficiency of autochthonous microorganism in the decontamination of hydrocarbon polluted sites based on the fact that environmental conditions are suitable for their growth and metabolism [42].

Total counts for microalgae (log10CFU/g) is shown in fig.8 The highest count for each set up during the monitoring were as follows; day 0 Us (7.65) day14 Cs (7.63) day28 Us (7.70) day42 Cs+Chl (7.60) day56 Cs (7.30). It was observed that peak count was on day28 (7.70) and a decline in day56 (7.30).

Hydrocarbon Utilizing Algae (Log10): The highest count for each set up during the monitoring were as follows; day 0 Cs+Chl (5.23) day 14 Cs+Chl (5.30) day28 Cs (5.23) day42 Cs+Chl (4.77) > day56 Cs (5.07) figure 9 Decline was observed on day 42 and peak count was on day 14.

Hydrocarbon Utilizing Algae (HUA) which is the key player in bioremediation. The counts obtained from HUA ranged from 4.50±0.65 to 5.07±0.21 where the *Chlorella vulgaris* had higher counts compared to other treatment. The results show there was a significant difference in the treatment. Though the trend of microalgae counts and HUA counts was not consistent throughout the monitoring, there was a reduction in the counts during the monitoring. The reduction in counts could be attributed to rapid decomposition of organic matter by *Chlorella vulgaris* to produce alcohol, esters and antimicrobial substances which inhibit other pathogenic organisms [43]. Mohammed and Ijah [44] stated that the principal factors that contribute to inhibition, are low organic aids, pH, depletion of nutrients, hydrogen peroxide and low redox potential. Another possible explanation to the reduction hydrocarbon utilizing algae count in the present study is nutrient deficiency. The growth and activities of hydrocarbon microorganisms must be stimulated. This study is in agreement with the study of Swindell [45]; [46] who demonstrated that all biological forms must have nitrogen in order to survive. It is a part of the amino acids needed to make proteins. Proteins make up the majority of enzymes, hormones, and human and animal tissues. A key barrier to a microorganism's capacity to actively breakdown petroleum hydrocarbons is a shortage in nitrogen supplies.

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The lack of nutrients in crude oil-affected sediments makes bioremediation difficult. However, adding nutrients often improves soil hydrocarbon organisms, resulting in enhanced bioremediation of crude oil contaminated environment [47].

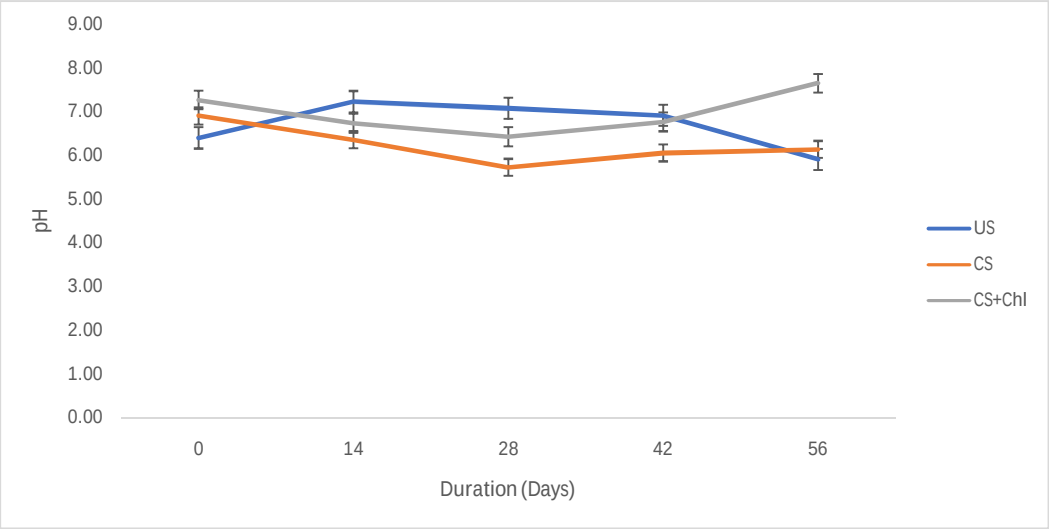

Fig. 1 Changes in pH during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

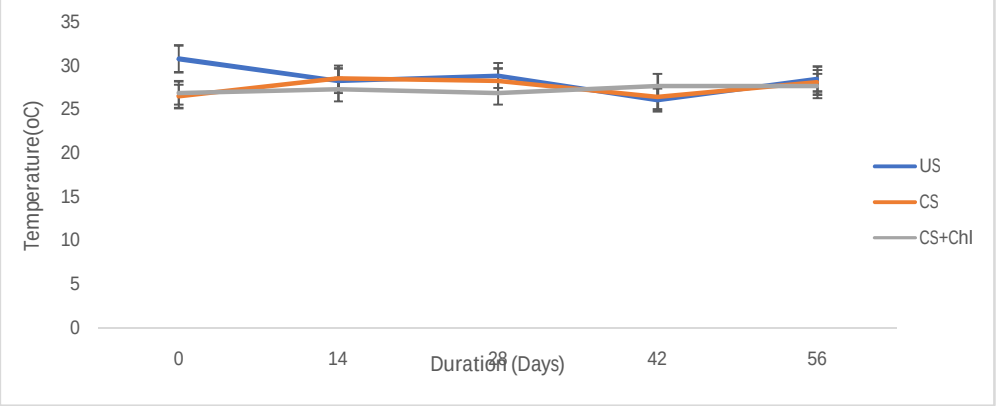

Fig. 2 Changes in Temperature during Bioremediation of Crude of Crude Oil Contaminated Sediment

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Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

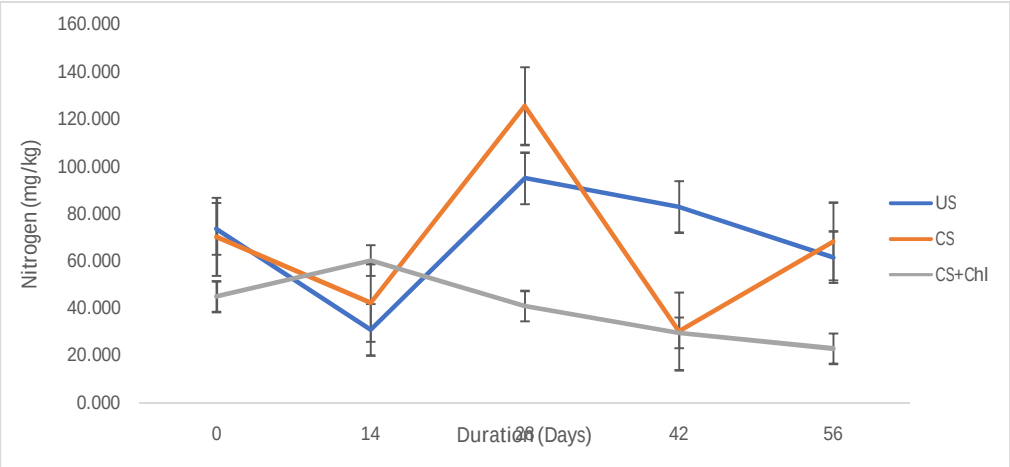

Fig. 3 Changes in Nitrogen during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

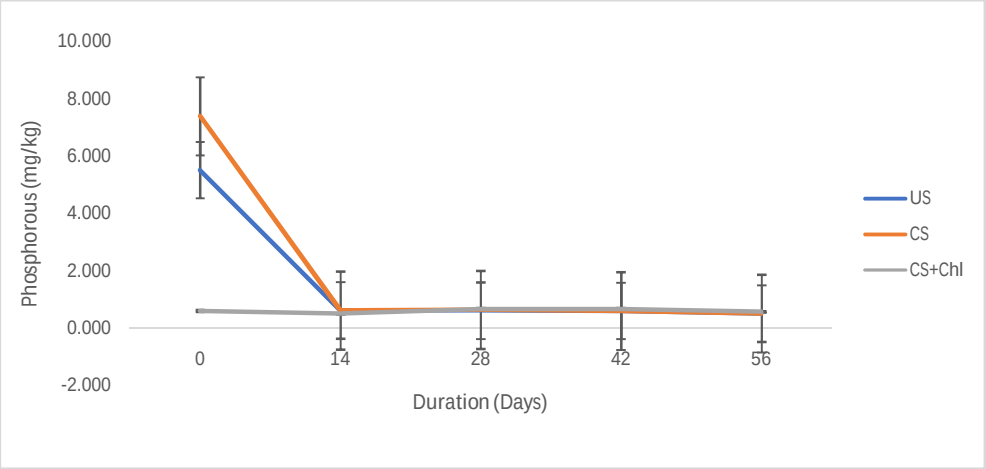

Fig. 4 Changes in phosphorous during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

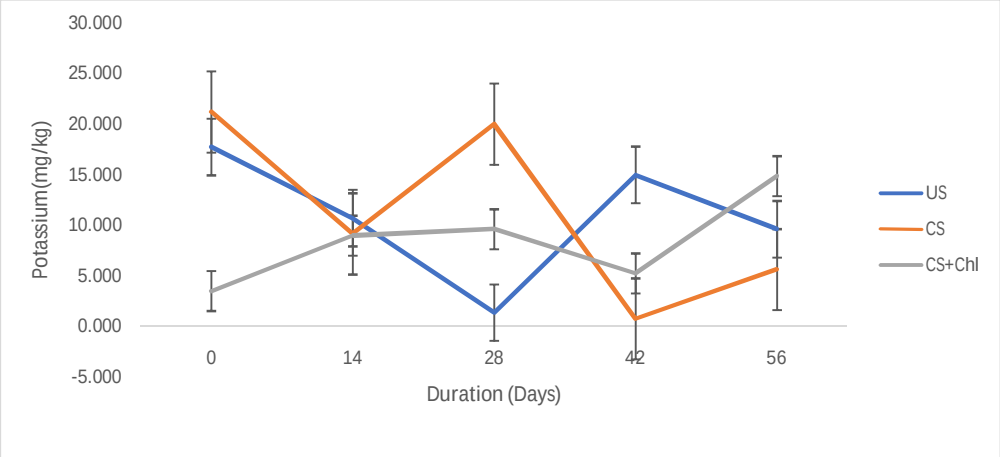

Fig. 5 Changes in potassium during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

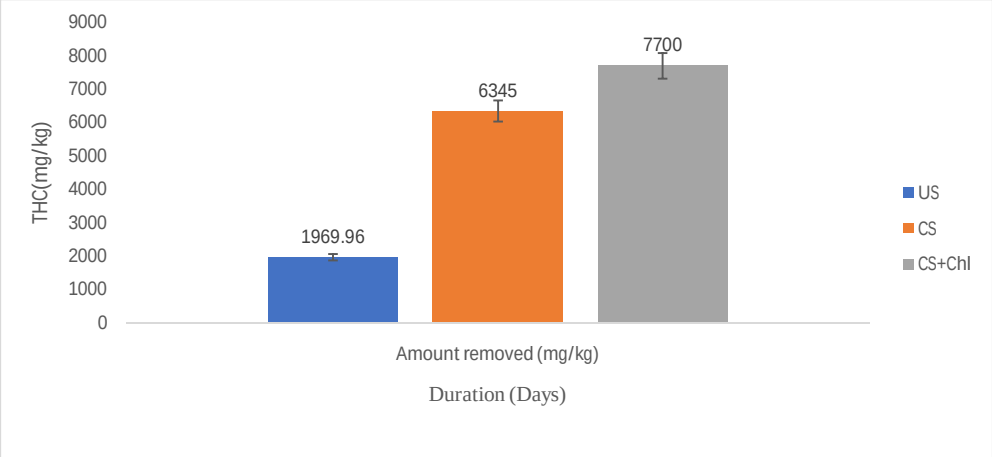

Fig. 6 Amount of THC (mg/kg) removed during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

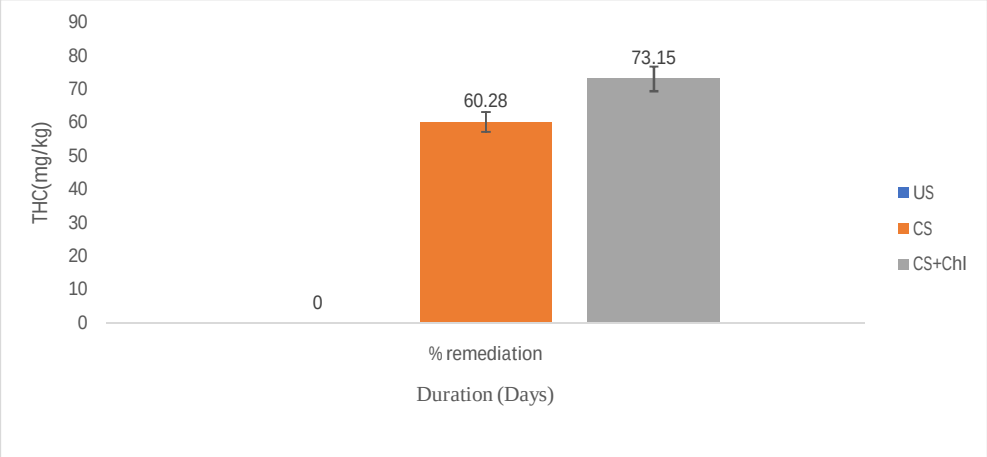

Fig. 7 THC % (mg/kg) Remediation during Bioremediation of Crude of Crude Oil Contaminated Sediment

Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

Comment [H48]: This is false this represente % not mg/Kg so please correct in the figure

Table 6 Changes in THC (mg/kg) during Bioremediation of Crude Oil Contaminated Sediment.

Treatment	0	14	28	42	56	Amount removed (mg/kg)	% remediation	Mean SD
US	2529.96	2135	560	560	560	1969.96	-	1268.992
CS	10525	7990	4470	4180	4180	6345	60.28	6269±2874.01
CS+Chl	10525	7845	4285	2825	2825	7700	73.15	5661±3406.15

Key US= Uncontaminated sediment CS= Contaminated sediment CHL= *Chlorella vulgaris*

Table 7 Mean and standard deviation of physicochemical parameters during bioremediation of crude oil contaminated sediment

Treatment	pH	Temperature	Nitrogen	Phosphorus	Potassium	THC
US	6.71 ± 0.54^{abcd}	28.52 ± 1.68^{b}	68.94 ± 24.46^{a}	1.56 ± 2.21^{a}	10.89 ± 6.25^{a}	1268.992 ± 980.82^{a}
CS	6.24 ± 0.43^{ab}	27.58 ± 1.05^{ab}	67.47 ± 36.78^{a}	1.94 ± 3.04^{a}	11.35 ± 8.97^{a}	6269 ± 2874.01^{b}
CS+Chl	6.97 ± 0.48^{cd}	27.30 ± 0.40^{ab}	39.85 ± 14.46^{a}	0.60 ± 0.07^{a}	8.45 ± 4.41^{a}	5661 ± 3406.15^{b}

*means with the same superscript down the group show no significant different ($p>0.05$). THC= Total hydrocarbon content, Us= Uncontaminated sediment, CS= Contaminated sediment, CHL=*Chlorella vulgaris*

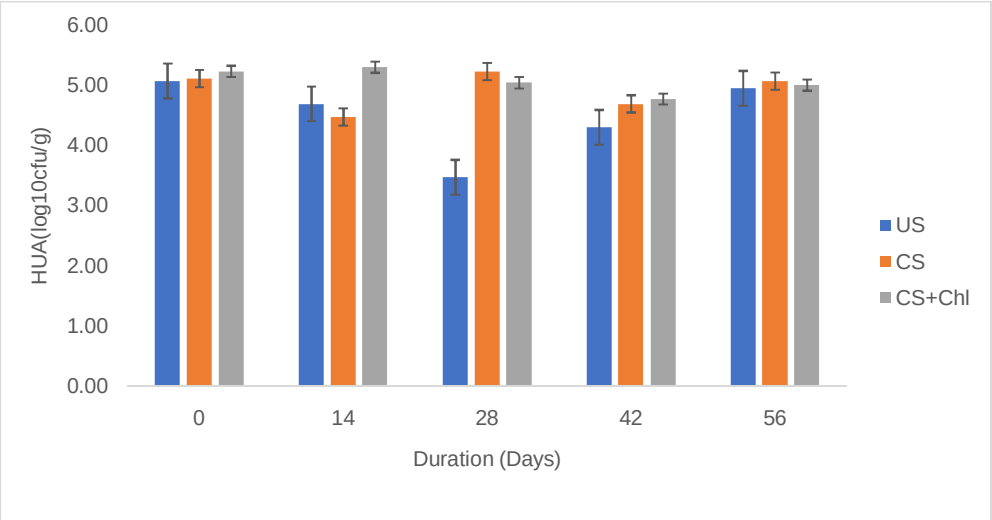

Fig. 8 Changes in total Hydrocarbon Utilizing Algae (HUA) (Log10cfu/g) during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

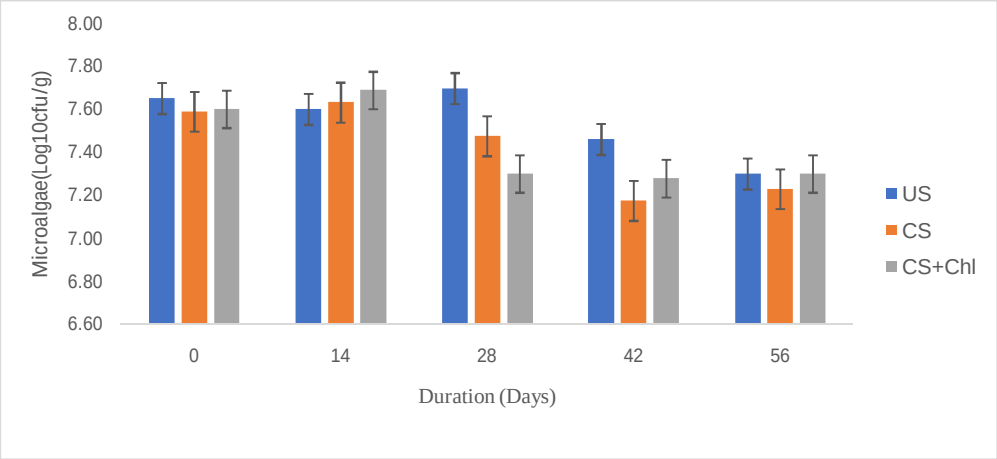

Fig. 9 Changes in Microalgae (Log10cfu/g) during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

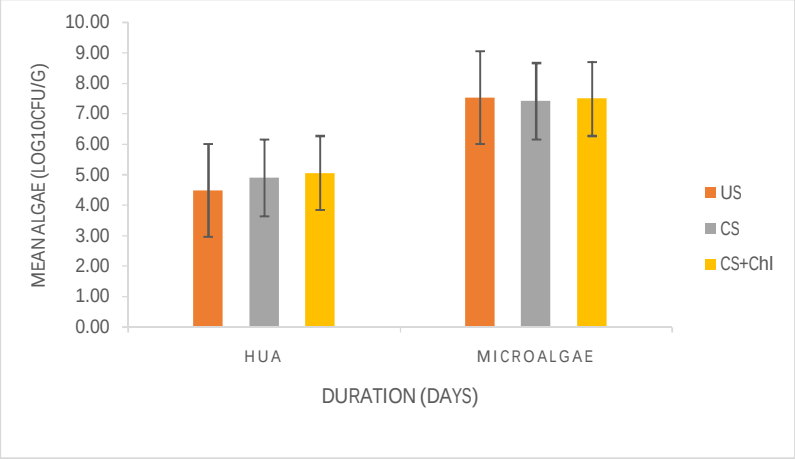

Fig. 10 mean algae (Log10cfu/g) counts during Bioremediation of Crude of Crude Oil Contaminated Sediment
Key: US= Uncontaminated sediment; CS= Contaminated sediment; CHL= *Chlorella vulgaris*

Table 8 Mean Standard Deviation Microbial (Log10 cfu/g) counts during Bioremediation of Crude Oil contaminated sediment

Treatment code	HUA	Microalgae
US	4.50±0.65[a]	7.54±0.16[a]
CS	4.91±0.32[ab]	7.42±0.21[a]
CS+Chl	5.07±0.21[bc]	7.50±0.12[a]

*means with the same superscript down the group show no significant different (p>0.05) HUA= Hydrocarbon Utilizing Algae, US= Uncontaminated sediment, CS= Contaminated sediment

CONCLUSION AND RECOMMENDATIONS

4.2: Conclusion

Results from the study revealed that *Chlorella vulgaris* is capable of degrading hydrocarbon components. There was a faster utilization of hydrocarbon by single culture than control. Though, incomplete removal of crude oil was observed in THC concentration value. This suggests that an improvement in the process is required. Such improvement could include biostimulation of the polluted sample or some chemical pretreatment of the sample.

From the study, bioremediation can be said to be a viable and effective response to sediment contamination with crude oil.

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4.2 Recommendations

Biostimulation is well known to improve the process of bioremediation by the use of effective microorganisms. This will help by providing different sources of nutrients for the degrading microorganisms.

Further studies should be carried out on bioremediation using indigenous microbial strains. Indigenous microbial strains would ordinarily acclimatize easily to the environment and should possess the enzymes required for metabolism of the target pollutant.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

References

1. Ohanmu E, Bako S, Adelanwa M. Effect of Oil Spillage on the Productivity of Pepper (*Capsicum* spp.). ~~2017~~ Lambert Academic Publishing; 2017 ~~Germany~~
2. Adebusoye SA., Ilori MO, Amund OO, Teniola OD. Olatope SO. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. *World Journal of Microbiology and Biotechnology*. 2007; *23, 8: 1149-1159.*
3. Ghazali F, Rahman R, Salleh A, Basri, M. Biodegradation of hydrocarbons in soil by microbial consortium. *International Biodeterioration and Biodegradation*, 2004; 54, 61-67
4. Okerentugba PO. Ezeronye OU. Petroleum Degrading Potentials of Single Microbial Cultures isolated from Rivers and Effluent in Nigeria. *African Journal of Biotechnology*. 2003; 2 (9), 288-292.
5. Ekanem SA, Ejue BJ, Amimi PB, Adalikwu RA. Living with oil: Towards an ethics of the environment in the Niger Delta. *Afr. Res. Rev*., 2010; 4: 17-30.
6. Eneh OC. A review on petroleum: Source, uses, processing, products and the environment. *J. Applied Sci.*, 2011; 11: 2084-2091.
7. Patrick-Iwuanyanwu KC, Onyemaenu CC, Wegwu MO, Ayalogu EO. Haematotoxic effects of diets contaminated with petroleum products (kerosene and petrol) in wistar albino rats. *Res. J. Environ. Toxicol.*, 2010;4: 134-140.
8. Rosenberg E, Ron EZ. Bioremediation of petroleum contamination. In Bioremediation: Principle and Applications. Cambridge, ~~2010~~. *UK: University Press*;
9. Chekere CB, Azubuike CC. Catechol-2,3-dioxygenase screening in putative hydrocarbon utilizing bacterial. *International Research Journal of Microbiology*. 2013;4 (1): 1-6
10. Atlas R, Bragg J. Bioremediation of marine oil spills: ~~When not –~~ the Exxon Valdez experience. *Microbial biotechnology*. 2009;2(2): 213-221.
11. Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG. Characterization of hydrocarbon degrading microbiall populations in contaminated and pristine Alpine soils. *Appl. Environ. Microbiol.* 2003;69: 3085-3092.
12. Nweke CO, Okpokwasili GC. Effects of bioremediation treatments on the bacterial populations of soil in different depths. *Nigerian Journal of Microbiology* 2004;18: 363-372.
13. Kaplan CW, Kitts CL. Bacterial succession in a petroleum land treatment unit. *Appl. Environ. Microbiol.* 2004;70: 1777-17 86.

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Comment [H58]: Rosenberg E, Ron EZ. Bioremediation of Petroleum Contamination. In Crawford RL, Crawford DL. Eds. Bioremediation: Principles and Applications. Cambridge University Press. Cambridge; 1998

Comment [H59]: When and when not – the *Exxon Valdez* experience

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14. Quatrini P, Scaglione G, De Pasquale C, Reila S, Puglia AM. Isolation of Gram-positive n-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. *J. Appl. Microbiol.* 2008;104: 251- 259.
15. Ayotamuno MJ, Kogbara RB, Ogaji SOT, Pobert SD. Bioremediation of a crude oil polluted agricultural soil polluted at Port Harcourt, Nigeria. *Appl. Energy*, 2006;83: 1249-1257.
16. Stroud JL, Paton GI, Semple KT. Microbe–aliphatic hydrocarbon interactions in soil; implications for biodegradation and bioremediation. *J. Appl. Microbiol.* 2007;102: 1239-1253
17. Ojewumi ME, Emetere ME, Babatunde DE. Okeniyi JO. In situ bioremediation of crude oil polluted soil using mathematical experimentation. *Int. J. of Chemical Engineering. ~~Airticle~~ 2017; ~~ID5184760~~.*
18. Alwan AH, Fadil SM, Khadair SH, Haloub AA. Mohammed DB. Bioremediation of the water contaminated by waste of hydrocarbon by use Ceratophyllaceae and Potamogetonaceae plants. *J. of Genetic Environmental Resource Conservation.* 2013;1: 106-110.
19. Daccò C, Girometta C, Asemoloye MD, Carpani G, Picco AM. Tosi S. Key fungal degradation patterns, enzymes and their applications for the removal of aliphatic hydrocarbons in polluted soils: *A review. Int. Biodeterior. Biodegrad*. 2020;147, 104866.
20. Ghosal D, Ghosh S, Dutta TK. Ahn, Y. ~~(2016).~~ Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): *A review. Front. Microbiol.* 7, 1369.
21. Perales-Vela HV, Peña-Castro JM, Cañizares-Villanuev RO. Heavy metal detoxification in eukaryotic microalgae. *Chemosphere* 2016; 64, 1–10.
22. Peekate LP, Chilowe EM. Use of rifampin in the development of microalgal Cultures. Basic Research Journal of Microbiology 2016;3(4) 23-29
23. Obire O, Anyanwu EC, Okigbo RN. Saprophytic and crude oil degrading fungi from cow dung and poultry droppings as bioremediating agents. *Journal of Agricultural Technology* 2008; 4(2)
24. Vuuren SJ, Taylor J, Ginkel C, Gerber A. Easy identification of the most common freshwater algae: a guide for the identification of microscopic algae in South African freshwaters. ~~ISBN 2006; 0-621-35471-6. ©~~ North-West University and Department of Water Affairs and Forestry.
25. Obire O, Nrior RR. Effect of concentrate Detergent on *Pseudomonas fluoerescens* and *Mucor racemosus*. *Current Studies in Comparative Education, Science and Technology*. 2014; 1: 189-199.
26. Chekere CB, Okpokwasili GC, Chikere BO. Bacterial diversity in a tropical crude oil polluted soil undergoing bioremediation. *African Journal of Biotechnology,* 2009; 8(11): 2535-2540.
27. Ezekoye CC, Amakoromo ER.Ibiene, AA. Bioremediation of hydrocarbon polluted Mangrove Swamp soil in Niger Delta using poultry waste. *Microbiology Resource Journal International.* 2015;19 (2): 1-14.
28. American Public Health Association. Standard methods for the examination of water and waste water. 20th ed~~n~~. *American Public Health Association, Washington;* 1998;~~1134.~~
29. Dienye HE, Woke GN. Physico-chemical Parameters of the Upper and Lower Reach of the New Calabar River Niger Delta. *J Fisheries Livest Prod.* 2015; 3:154.
30. Environmental protection agency (EPA). Conductivity. In water: Monitoring and assessment; 2012.
31. Abowei JFN. Salinity, Dissolved Oxygen, pH and Surface Water Temperature Conditions in Nkoro River, Niger Delta, Nigeria. *Advance Journal of Food Science and Technology*. 2010; 2: 36-40.
32. Ule O, Ogbonna DN, Okparanma RN, Nrior RR. Myco Enhanced bioremediation of open field crude oil contaminated soil using *Mucor racemous* and *Asoergillus niger*. *Current Journal of Applied Science and Technology*. 2021;40(1):119-141
33. Technological Partners International. Analytical manual for total petroleum hydrocarbons and polycyclic aromatic hydrocarbons analyses, Port Harcourt, Nigeria. *Technology*. 2007; 4(2): 81-89

Comment [H61]: In Situ Bioremediation of Crude Petroleum Oil Polluted Soil Using Mathematical Experimentation

Comment [H62]: 2017; 1-11

Comment [H63]: 2016

Comment [H64]: Janse Van Vuuren S, Taylor J,Gerber A, Van Ginkel C. Easy identification of the most common freshwater algae: a guide for the identification of microscopic algae in South African freshwaters. North-West University and Department of Water Affairs and Forestry; 2006

Comment [H65]: **Myco-enhanced Bioremediation in Open Field Crude Oil Contaminated Soil Using Mucor racemosus and Aspergillus niger**

Comment [H66]: verify (lost reference)

34. Nrior RR, Mene GB. Assessment of bioaugmentation efficiency of Penicillium Chrysogenum and Aspergillus Nidulans bioremediation of crude oil spill soil. Journal of Environmental Science, Toxicology and Food Technology. 2017;11(8):01-09
35. Boonchan S, Britz ML, Stanley GA. ~~(2000).~~ Degradation and Mineralization of high-molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cultures. *Applied Environmental Microbiology*. 2000;66(3): 1007-1019.
36. Mentzer E, Ebere D. Remediation of Hydrocarbon Contaminated sites. A Paper Presented at 8th Biennial International Seminar on the Petroleum Industry and Nigerian Environment, ~~November,;~~ Port Harcourt, Nigeria; 1996
37. Evans FF, Rosado AS, Sebastián GV, Casella R, Machado PL Holmström C, Kjelleberg S, Van Elsas JD, Seldin L. Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms. *FEMS Microbiol. Ecol.* 2004;49, 295–305.
38. Anibal FC, Pablo G, Lokare C. Effective microorganisms: Myth or reality? *Revista Peruana Biologia.* 2007;14(2): 315-319
39. Antai SP. Biodegradation of Bonny light crude oil by *Bacillius sp* and *Pseudomonas sp Waste Management.* 2007; 10:6164
40. Pimda W, Bunnag S. Biodegradation of used motor oil by single and mixed cultures of cyanobacteria. *African Journal of Biotechnology*. 2012; *11, 9074*
41. Sunita JV, Vivek NU. Comparative studies on bacteria consortia for hydrocarbon degradation. *International Journal of Innovative Research in Science, Engineering and Technology*. 2013;2,5378.
42. Marchal R, Penet S, Solano-Serena F, Vandecasteele JP. Gasoline and diesel oil biodegradation. *Oil and Gas Science Technology. –Rev. IFP*, 2003;58(4): 441-448.
43. Ibe CC, Uzoka CN, Ekwenye UN, Ohakwe CS, Egbuawa IO, Duru IMBiodegradation Potential of Aspergillus niger on Crude Oil Contaminated Soil. *International Journal of Scientific Research in Science and Technology*. 2017;2(4): 405-414.
44. Mohammed SSD, Ijah UJJ. Isolation and screening of lactic acid bacteria from fermented milk products for bacteriocin production. *Food Microbiology and Safty, Annals of Food Science and Technology*. 2013; 14(1): 122-128.
45. Swindell CM, Aelion CM, Pfaender FK. Influence of mineral and organic nutrients anaerobic biodegradation and the adaptation response of surface microbial communities. *Applied Environmental Microbiology*. 1988; 54(1): 212-217.
46. Van Hamme JD, Singh A, Ward OP. Recent advances in petroleum microbiology. *Microbiology and Molecular Biology Reviews*. 2003; 67(4): 503-549.
47. Beolchini F, Rocchetti L, Regoli F, Dell'Anno A. Bioremediation of marine sediments contaminated by hydrocarbons: experimental analysis and Kinetic modeling. *Journal of Hazardous Materials*. 2010; 182 (1-3): 403-407.

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