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Bioremediation potentials of Halotolerant *Oscillatoria* sp in the remediation of crude oil polluted water

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Abstract
Crude oil pollution of water bodies poses significant environmental challenges, requiring innovative approaches for effective remediation. This study explored the bioremediation potential of the halotolerant cyanobacterium *Oscillatoria* sp. in addressing crude oil contamination in water systems. Halotolerant strains of *Oscillatoria* sp. were isolated and identified from a saline environment exposed to crude oil pollution. Laboratory-scale experiments were conducted to assess the ability of *Oscillatoria* sp. to degrade crude oil compounds and restore water quality. Biodegradation efficacy was evaluated by monitoring changes in crude oil composition and water parameters, including turbidity, pH, and hydrocarbon content. Results demonstrated that halotolerant *Oscillatoria* sp. exhibited notable capabilities for degrading various crude oil constituents. Over the experimental period, a substantial reduction in hydrocarbon content by the isolate supplemented with NPK from 8.992 mg/l to 1.486mg/l was observed while reduction from 8.992 mg/l to 7.272 mg/l was observed with isolate not supplemented with NPK. Thus, *Oscillatoria* sp with NPK recorded the highest degradation rate with a percentage remediation of 83.5%. Additionally, the water quality parameters improved, indicating the cyanobacterium's potential to enhance water clarity and reduce environmental stress caused by crude oil contamination. The bioremediation potential of halotolerant *Oscillatoria* sp. offers a promising avenue for sustainable water pollution mitigation. The ability of this cyanobacterium to adapt to saline conditions while efficiently degrading crude oil compounds enhances its utility in remediating contaminated water bodies.

Keywords: Halotolerant *Oscillatoria* sp, bioremediation of crude oil

Introduction
Water is the most important natural resource and is considered to be a fundamental requirement for life. Since the pollution poses a serious threat to ecosystems and living things on earth, the alarming rate of water pollution brought on by natural and artificial sources has become a major concern. To lessen and address the current problems, researchers have used and tested a variety of detoxification procedures and processes over the years (Kumar *et al.*, 2018) [1]. Some examples of water recovery treatment conducted in several countries include advanced oxidation, adsorption, membrane bioreactor, neutralization of acid and base in the effluent as well as chemical treatment and incineration (Jayaswal *et al.*, 2018.) [2]. These traditional and physical methods, however, had some significant limitations, including delayed progress, high costs, the creation of secondary intermediates, and ineffective contamination removal from the environment. (Singh *et al*, 2017) [3]. Oil spills from underground storage tanks, pipelines, land vehicles, unintentional spills during transportation, drilling sites, and inappropriate waste disposal procedures are thought to be the origins of fresh and marine water contamination (Xu *et al.*, 2018; Hewelke *et al.*, 2018) [4, 30]. The overall volume of persistent and non-persistent hydrocarbon spillages to the environment documented in 2019 was roughly 1000 tons, according to data provided by International Tanker Owners Pollution Federation Ltd (ITOPF). Many nations now have stricter laws governing the treatment of contaminated water. For instance, Europe and a few other nations have been employing the bioremediation method to solve the problem with major successes recorded all across the world, this approach has demonstrated a significant influence (Zouboulis *et al.*, 2011) [9]. Four fractions of crude oil include saturated hydrocarbons, aromatic, resin and asphaltene. Also, the main constituents of crude oil are the complex mixture of paraffinic, aromatic hydrocarbons, as well as nitrogen, oxygen, sulfur-containing compounds and a variety of metal-containing organic and inorganic compounds. (Bachmann *et al.*, 2014; Haritash *et al.*, 2009) [5, 7].

Different eukaryotic and prokaryotic organisms may be poisoned by crude oil molecules. There are a variety of chemical, physical, and biological techniques that have been used to remove crude oil remnants from marine habitats (Bacosa *et al.,* 2013, Nwadiogbu *et al.,* 2016; Seddighi *et al.*,2015) [6, 13, 15]. Microbial bioremediation of crude oil is more effective than conventional treatments due to its affordability and lack of secondary pollution (Chandankere *et al.,* 2014; Ferradji *et al.,* 2014; Kuyukina *et al.,* 2013 and Roy *et al.,* 2014) [10, 11, 12, 14].

Crude-oil pollution tends to remain in water until appropriate remediation measures are taken. Despite many microorganisms capable of degrading petroleum components have been isolated, only few of them have been tested and reported as successful when applied for petroleum biodegradation in marine environments. Among microorganisms, only bacteria and microbial consortia have been probed as effective in the practical removal of hydrocarbons from seawater, but the cases of yeasts, algae and protozoa still are under research with highly promising potentialities (Rosales *et al.,* 2014a; Paniagua-Michel *et al.,* 2014b and Singh *et al.,* 2007) [16, 17]. The study therefore investigated the potentials of halotolerant microalgae in the bioremediation of crude oil polluted water body.

Materials and Methods

Description of the Study Area

The study area was the Bonny coal beach shore line in Bonny Local Government Area of Rivers State, Nigeria. In southern Nigeria's Rivers State, on the Bight of Bonny, is the traditional seaside town of Bonny (formerly Ibani). Additionally, it serves as the Kingdom of Bonny's capital. Today, Bonny Island is a key location for oil exports. The majority of the onshore oil extracted in Rivers State is piped to Bonny for export. It has Nigeria's largest LNG gas plant, which has six trains. The Bonny Coal Beach shore line has an area of 50 m^2 with a distance of 50 m apart and closest to the SPDC Station about 20 m away. The map of the study area is illustrated in Fig. 1

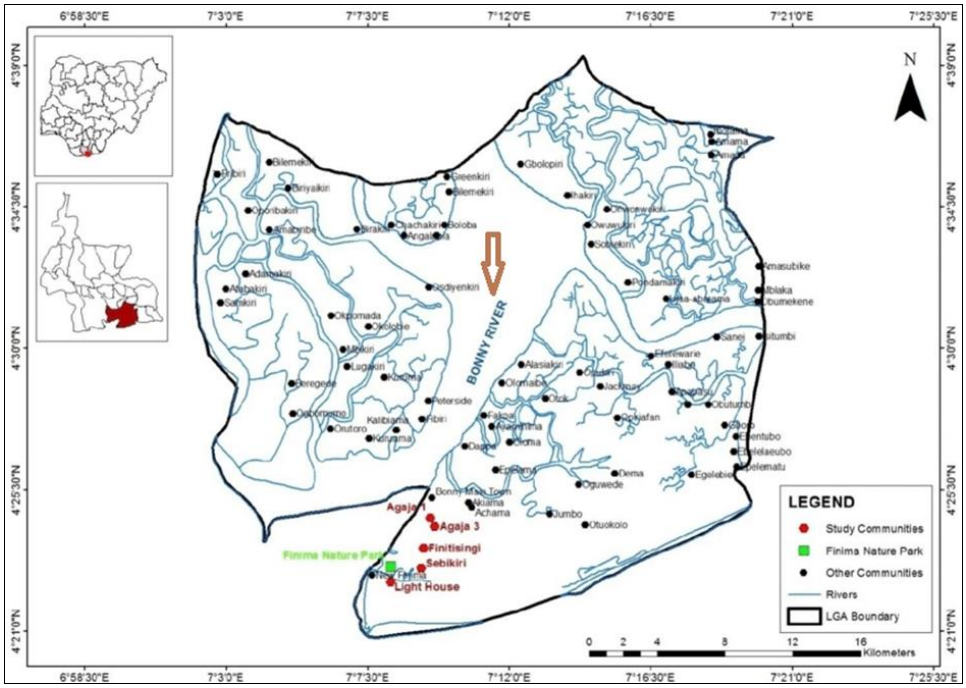


Fig 1: Map of the Bonny River

Collection of Crude-oil Polluted Water Samples

Crude oil polluted water samples were collected from Bonny coal Beach in Bonny Local Government Area of Rivers State, using two clean 5 litres plastic cans and clean amber glass bottle. Sample containers were properly covered with Teflon-lined lids in such a way as to completely protect all the water samples from any external contamination. The bottles containing the polluted water samples were properly labelled for identification and transferred to the Microbiology laboratory, Department of Microbiology, Rivers State University, in ice box, for laboratory analyses.

Isolation and Identification of Algae

The culture medium used in this study was algae water supplemented with 20 µg/ml sodium carbonate (Na_2CO_3) to which Rifampicin antibiotics was added to prevent bacterial growth and the BGII medium.

Preparation of Rifampin Algae Medium

The algae broth medium was prepared using the method of Peekate and Chilowe, (2016) [18]. In this method, the water sample showing algal bloom was filtered using a non-absorbent cotton wool. After which the filtrate was supplemented with 20 μgml^{-1} Na_2CO_3. This was later sterilized in the autoclave at 121 ^oC for 15 minutes and used as the culture medium. In other to develop bacterial free cultures, 0.8 μgml^{-1} Rifampicin antibiotics was added to the medium.

Cultivation of Microalgae Cells

Microalgae cells were cultivated by transferring 10ml fresh Bonny water (sample) to four different 250 ml conical flasks containing sterile prepared algae medium. The culture flasks were stoppered with cotton wool and curved rubber tubing obtained from clinical-drip sets were inserted through the wool, but not into the culture, to allow free exchange of air

between the internal and external air space (Peekate and Chilowe, 2016) [18]. The culture flasks were kept close to a window in the laboratory where sunlight could reach them. These flasks were agitated manually three times daily. The microalgae cultures were later purified by growing them on BGII and Rifampin agae medium. After 24-48 hours of incubation, the microalgae population in the culture flask was estimated by placing a drop of the culture on a counting chamber (Haemocytometer) and viewed under the microscope at ×10 and 40 magnifications. The cells were identified based on microscopy. This was done by placing a drop of the culture on a grease free slide. The slide was later covered with clean cover slip and viewed under the microscope using the ×10 and X40 objective lenses under the light microscope. The obtained microscopic results were referenced from atlas of microalgae cells (Vuuren *et al.*, 2006) [19].

The resulting cells were estimated using the formula

$$\text{Cells/ml} = \frac{\text{Number of cells} \times \text{dilution factor x } 10000}{\text{Number of squares counted on haemocytometer}} \text{ ..... Eqn1}$$

Where: 10^4 = constant.

Salt Tolerance Test

The microalgal isolates were screened for their ability to utilize higher concentrations of salt despite being isolated from the marine water. The salt tolerance test was carried out as described by Prescott *et al*. (2011) [20]. In this method, two concentrations of salt (10 and 40%) were prepared by dissolving 10 and 40 grams of NaCl separately into 100ml of distilled water. The brine was swirled homogeneously for easy mixing and 9 ml each were transferred into well labelled test tubes (test tube were labelled according to the salt concentrations for all the isolates tested). These were later sterilized at 12 ^oC for 15 minutes at 15 Psi. On cooling, 1ml of standardized (0.5 McFarland) cultures of the microalgae cells were transferred into separately labelled tubes. Incubation followed by keeping tubes close to light (window) for 24 hours. After incubation, microalgae population was estimated using the haemocytometer. Isolates showing higher colony forming units were selected as best halo-tolerant for bioremediation. Thus, + represents weak tolerance, ++ indicated high tolerance while the negative sign (-) showed no tolerance (Odokuma and Akponah, 2010) [27]

Screening for crude Oil Utilization

This was done in order to identify the isolates that could grow in the presence of crude oil. The Bonny light crude oil, 1mg/l, was made in 100 millilitres. Test tubes were filled with 9mL of crude oil and sterilised. The standardised microalgae cultures were transferred into the test tubes and incubated for 7 days. After incubation, 0.1 ml was withdrawn and plated using the spread plate method onto the surface of newly prepared nutrient agar plates. Incubation followed immediately by placing the plates close to the window where they could get light rays for 24 hours. Cultures that grew were counted and used for further study.

Biodegradation of Crude Oil

Prior to the biodegradation of the crude oil, the crude oil polluted water was sterilized at 121 ^oC for 15 minutes at 15 Psi. This was done to ensure the sterility of the water so that only inoculated organism can be investigated for its remediation impact. Although, one of the controls was not sterilized so as to monitor natural attenuation. Exact 1500 mL of 3% sterilized crude-oil polluted water samples was transferred into plastic containers and was labelled accordingly. Addition of the microalgal cultures followed. The experimental set-up is presented in Table 1. This was monitored for 3 months (September to November, 2022).

Percentage Biodegradation

Percentage biodegradation was calculated as follows:

Step 1: Amount of pollutant remediated equals to Initial pollutant concentration (Day 1) minus Final pollutant concentration at the end of experiment (Last day).

Step2: Percentage (%) Bioremediation equals to Amount of pollutant remediated divided by Initial pollutant concentration (Day 1) multiplied by 100.

BC =IC – FC----------Eqn. 2

Where

BC = Amount of pollutant remediated

IC = Initial concentration of pollutant (Day 0 or 1)

FC = Final concentration of pollutant at end of experiment (Last day)

$$\% \text{ Bioremediation} = \frac{\text{BC x100}}{\text{IC}} \text{ (Nrior \& Mene, 2017) ----------Eqn. 3}$$

Table 1: Experimental Set-Up of Bioremediation

SET-UP	Volume of Water	Test Isolate	Nutrient Supplement	Volume of Isolate	Final volume
Unsterilized (UPW)	1500 mL	None (control)	None	-	1500 ml
Sterilized (PW)	1500 mL	None (control)	None	-	1500 ml
PW+A	1500 mL	*O. acuminata*	None	15 mL	1, 515 ml
PW+A +NPK	1500 mL	*O. acuminata*	5.0 g	7.5 mL each	1, 515 ml

Keys: PW= sterilized water, A= microalgae; NPK= inorganic fertilizer

Physicochemistry of Water Samples

The physicochemistry of the water samples carried out included pH, electrical conductivity, salinity, total hydrocarbon content (THC), total petroleum hydrocarbon (TPH), total organic carbon, heavy metals, turbidity, biochemical oxygen demand (BOD), total dissolve solid (TDS), and total suspended solids (TSS). The method of the various parameters was done as described by (APHA, 2012). Analysis of Heavy metals (Lead (Pb), Cadmium (Cd), Chromium (Cr) and Nikel (Ni) of the water samples were done by using atomic absorption spectroscopy.

Statistical Analysis

The microbial counts were presented in log on Microsoft Excel (v16). The mean and standard deviations of microbial counts were analysed using SPSS (v 27). The means were compared using ANOVA. Means showing significant differences were separated using the Duncan Multiple range test at significant level of 0.05.

Results

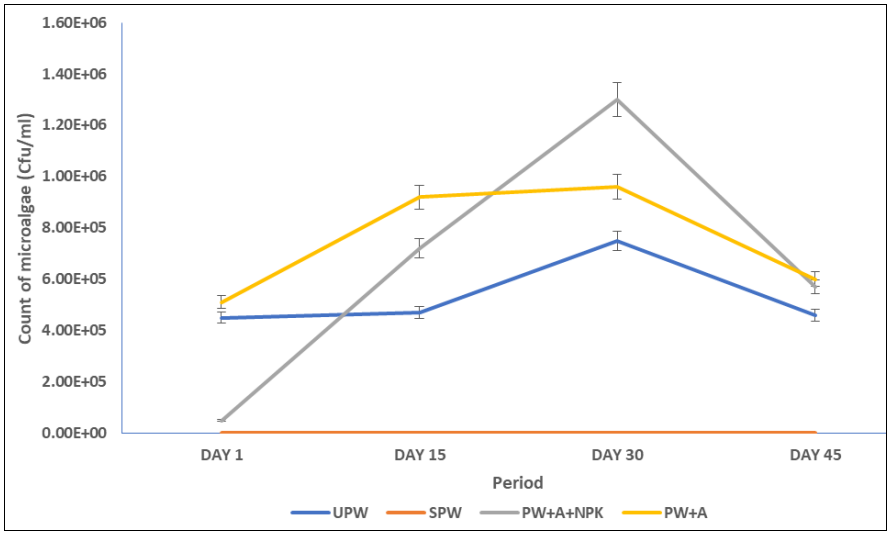
Results of the microalgae loads of the water sample was 3.5×10^5 cfu/ml. The baseline results showing the physicochemical, heavy metal parameter and TPH showed that the pH, salinity, turbidity, nitrate, phosphate, BOD, COD, total organic carbon (TOC), chromium (Cr), Iron (Fe), Lead (Pb), total petroleum hydrocarbon (TPH) and electrical conductivity (EC) were 6.4, 26.16 mg/l, 91.5 NTU, 0.6 mg/l, 20.5 mg/l, 510.13 mg/l, 5550 mg/l, 0.01 mg/l, 0.07 mg/l, 0.01 mg/l, 7.166 mg/l, 5200 mg/l and 4502 μs^{-1}, respectively.

Results of the phenotypic characteristics of the microalgae isolated from the water samples is presented in Table 2. Results showed that three microalgae isolates belonging to *Closterium* sp, *Scenedesmus* sp and *Oscillatoria* sp were isolated.

Results of the trend in the microalgal counts during the remediation period is presented in Figure 2. Results showed that the mean range of the microalgal counts in Day 1 was 0.0 to 5.1×10^5 cfu/ml. The range of the mean counts of Day 15, 30 and 45 was 0 to 9.2×105, 0.0 to 1.3×10^6 and 0 to 6.0×10^5 cfu/ml, respectively. Results further showed that the peak of microalgal growth was in Days 30 and the set-up with NPK fertilizer had higher microalgal load than the set-up without NPK fertilizer. There was no significant difference (p>0.05) in the microalgal counts supplemented with NPK and set-up without NPK. All the treatments showed decline in microalgal load in Day 45. The chromatogram of the breakdown of TPH components is presented in fig 3 to fig 7.

Table 2: Morphology and Microscopy of Microalgae in Water Sample

Isolates	Microscopy	Probable Identity
M3	Long curved / bow shaped filaments	*Closterium* sp
M2	Yellow-green small clustered round/conidia	*Scenedesmus* sp
M1	Cylindrical unbranched filaments	*Oscillatoria* sp



Keys: SPW= sterilized water, A= microalgae; NPK= inorganic fertilizer

Fig 2: Trend of the Microalga counts during bioremediation period

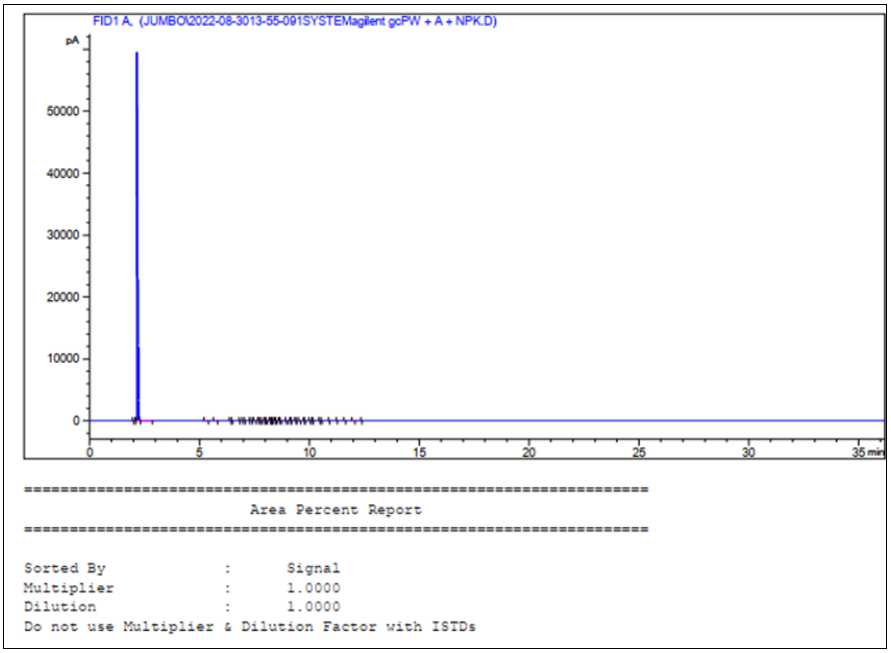


Fig 3: Total Petroleum Hydrocarbon of Contaminated water with *Oscillatoria* sp amended with NPK (Day 15)

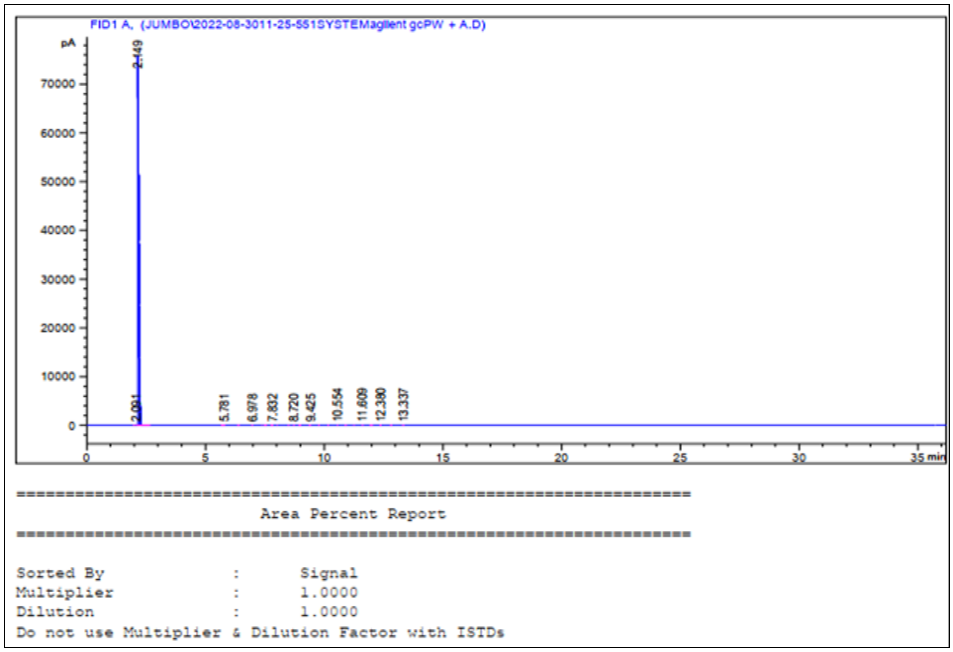


Fig 4: Total Petroleum Hydrocarbon of Contaminated water with *Oscillatoria* without NPK (Day 15)

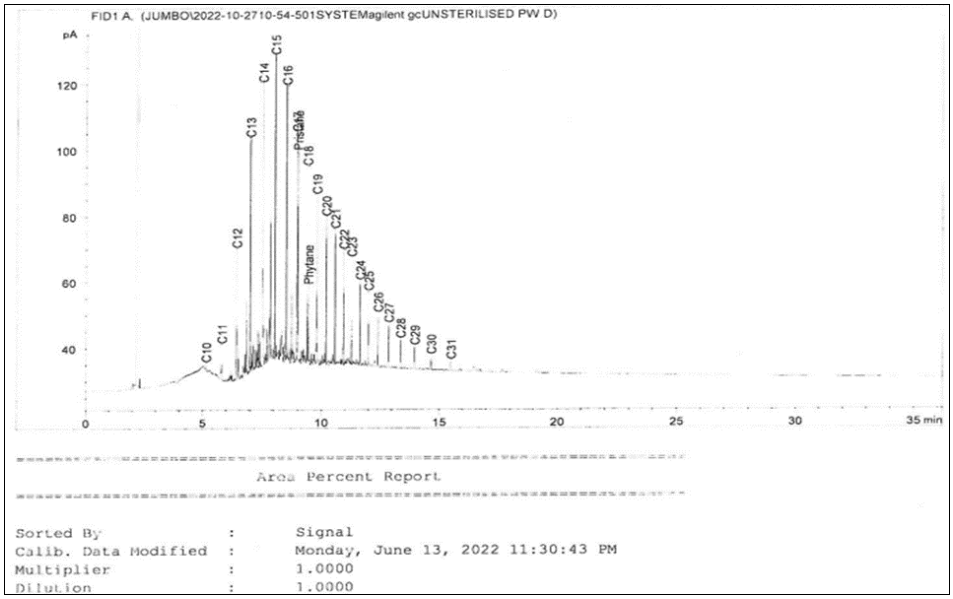


Fig 5: Total Petroleum Hydrocarbon of unsterilized Crude oil Contaminated water (Day 45)

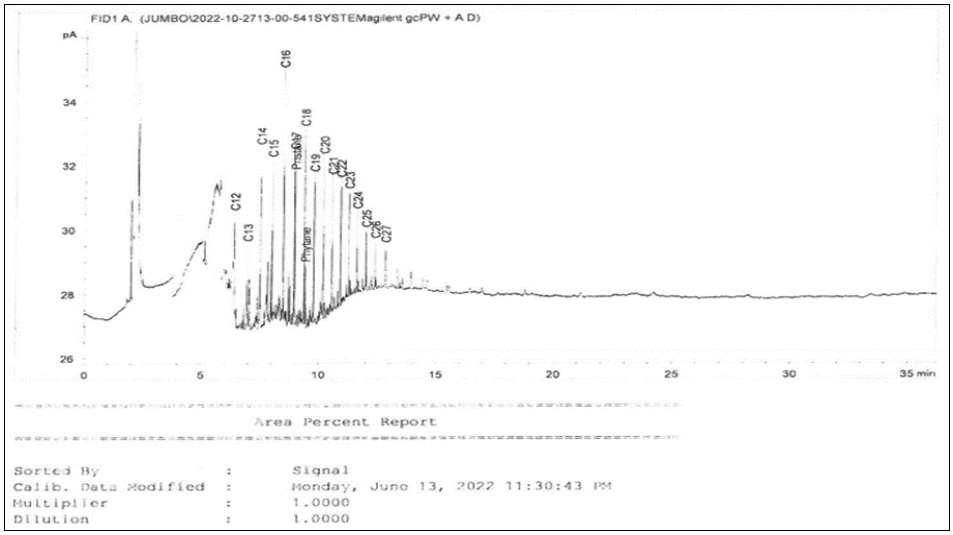


Fig 6: Total Petroleum Hydrocarbon of Contaminated water with *Oscillatoria* sp (Day 45)

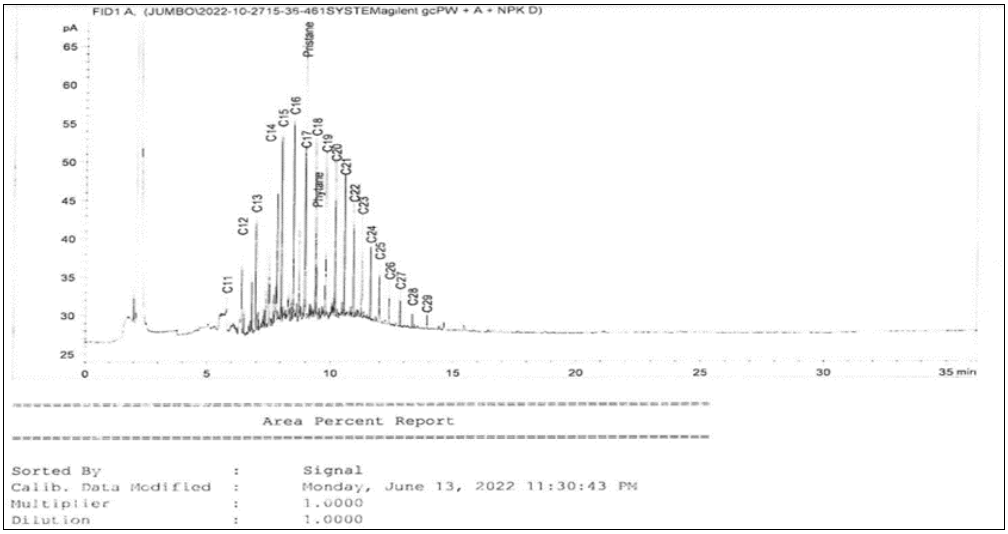


Fig 7: Total Petroleum Hydrocarbon of Contaminated water with *Oscillatoria* sp amended with NPK (Day 45)

Results of the mean physicochemical properties of the treatments is presented in Table 3. Results showed fluctuations in the paramters.

Table 3: Mean Physicochemical Parameters During Bioremediation

Treatments	pH	EC	Salinity	Turbidity	Nitrate (NO^{3-})	Phosphate (PO_4)	BOD	COD	TOC
sterilized	7.0±0.5 [b]	4199.7±10 [a]	26.3±0.2 [ab]	51.2±16.5[a]	0.66±0.24 [a]	50.9±39.40 [a]	445.2±5.0 [bc]	5862.75±57 [a]	0.01±0.00 [a]
unsterilized	6.5±0.3[b]	4449.5±35[c]	26.07±0.2[a]	43.8±40.8 [a]	0.35±0.18 [a]	14.26±6.89 [a]	494.9±12.7[c]	5444.0±121[a]	0.01±0.00 [a]
PW+A	6.5±0.2 [b]	4199.0±0.0 [b]	26.6±0.0 [b]	71.1±5.2[a]	0.36±0.06 [a]	14.26±3.73 [a]	285.5±32.9[a]	12063.25±67	0.01±0.0 [a]
PW+A +NPK	5.4±0.4[a]	5338.7±44 [d]	32.6±0.4[c]	365.2±18 [b]	0.24±0.13 [a]	115.88±157[a]	399.7±43.2[b]	928.5±194.9 [a]	0.012±0.0[a]

*****Means with similar superscript down the group share no significant difference ($p > 0.05$)
Keys: BOD: Biological Oxygen Demand; COD: chemical oxygen demand, TOC: total organic carbon; PW: pasteurized water; A: Microalgae, NPK: inorganic fertilizer, EC: electrical conductivity, SPW= sterilized water

Discussion

The importance of microalgae in the treatment of water as well as in bioremediation have been documented (Olufunmilayo and Agunkwo, 2018) [28]. Shafi (2020) [29] posited that due to the presence of useful components such as pigments/carotenoids, polyunsaturated fatty acids, vitamins and antioxidants as well as their physiological and biochemical characteristics microalgae have a wide range of applications. More so, Albert and Anyanwu (2012) have reported the use of the microalgae *Oscillatoria* in the bioremediation of hydrocarbon polluted water body and this agreed with the present study. Thus, the presence of microalgae in crude oil polluted environment could mean that they have adapted structures that aid in the utilization of crude oil components. Generally, the microalgae (*Oscillatoria* sp) used for the bioremediation gradually increased from the initial population in day 1 to higher population in day 15 and 30 with a sharp decline in day 45 during the period of bioremediation and this corroborates with the degradation rate of the crude oil component.

The highest microalgae growth was observed in Day 30 and this could be due to the presence of hydrocarbon decomposed and organic nutrients (nitrate and phosphate), microbial activity and as a result, petroleum degradation was in a maximum rate. Similar findings were reached by Sang-Haw *et al*. (2007) [22], who came to the conclusion that during the first 30 days of the 105-day testing period, the population of microorganisms that broke down hydrocarbons rose quickly. They suggested that this discovery could be used as a gauge for whether bioremediation of oil-pollution was feasible. However, over time, as a result of oil-resistant components with high chains and in the presence of fewer nutrients, bacterial growth and oil degradation declined (Schaefer and Juliane, 2007) [23]. This corroborates the present study which also showed a decline in the microalgae population in Day 45. Thus, since the set-up in the present study was a closed system in which intermittent addition of nutrients were not carried out even though the set-up was continually agitated, the depletion of nutrient could have impacted on the degradation rate of the crude oil. Furthermore, Ramsay *et al.* (2000) [24] found that continuous ventilation and fertiliser addition had a significant impact on the proliferation of hydrocarbons degrading microorganisms when they studied the effects of bioremediation on the microbial population in oil deposits.

The fluctuations observed in the pH especially the increased pH in the set-ups across the period of monitoring could be attributed to the amount of hydrogen ion deposited as the remediation continued. The pH (Acid and alkaline) plays major role on nutrient availability to plants, type of organisms found in the environment and solubility of metal. Statistically there was significant difference ($p \leq 0.05$) between the pH values from the respective set-ups and the length of bioremediation. The observed decrease in the pH of the treated samples could mean that the crude oil increased the acidity of the samples and this would be a reflection of the high bacterial and microalgae population observed during the bioremediation process. This corroborates a previous study that reported that decrease in pH of polluted samples is indicative that petroleum

pollutants make habitats more acidic, which could lead to changes in biodiversity. More so, the phosphate and nitrate of the sterilized sample (set-up) was not depleted and was still very much high after the period of monitoring whereas the phosphate and nitrate of the samples not amended with NPK depleted faster and were less than the values recorded for those treated with NPK. The high phosphate and nitrate observed in the sterilized sample could be attributed to the lack of microorganisms to utilize the nutrients for growth while the depletion in the nutrients observed in the treatments with *Oscillatoria* sp could be attributed to the use of these nutrients to increase in population which is reflected in the high counts observed during the period of bioremediation. Similar study have reported depletion of phosphate and nitrate concentration during the periods of bioremediation (Albert and Anyanwu, 2012) [25].

The degradation of total petroleum hydrocarbon (TPH) by the microalgae in the water samples as observed showed significant difference ($p< 0.05$) with the control from the statistical point of view. Thus, crude oil degradation in set-up without NPK fertiliser was less while set-up with NPK fertiliser had higher crude oil degradation rate. This corroborates the study of Chorom *et al.*, (2010) [26] who also made similar observation that oil degradation was lower in the samples without treatment but faster in the samples treated with 2 tons/ha of fertiliser than it was in the samples treated with 1 tons/ha of fertiliser. There was a significant ($p<0.05$) reduction in the TPH content from the initial stage of the remediation process to the final stage. The microalgae; *Oscillatoria* sp which was supplemented with NPK reduced the TPH values from 8.992 mg/l to 1.486mg/l while *Oscillatoria* sp without NPK reduced the TPH values from 8.992 mg/l to 7.272mg/l. Thus, *Oscillatoria* sp with NPK recorded the highest degradation rate with a percentage remediation of 83.5%. Similar findings were reported by Sang- Hwan *et al.* (2007) [22] who found that while only 18% of the hydrocarbon was removed from the non-fertilized treatment, the initial amount of oil-polluted soil (9320344 mg/kg) was reduced to 42-51% in the fertilised treatment.

Conclusion

In conclusion, this study highlights the significance of halotolerant *Oscillatoria* sp. in the bioremediation of crude oil-polluted water. The microalgal' dual ability to tolerate salinity and degrade hydrocarbons showcases its potential as a bioresource for sustainable water treatment strategies. Leveraging the bioremediation capacities of halotolerant *Oscillatoria* sp. contributes to addressing the global challenge of water pollution and underscores the importance of harnessing natural organisms for environmental restoration.

Conflict of Interest

Not available

Financial Support

Not available

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