Remediation of Crude Oil Polluted River using *Nostoc* and *Oscillatoria* spp

Williams Janet Olufunmilayo & Agunkwo Mary
Department of Microbiology,
Rivers State University,
Port Harcourt, Nigeria
funmikemwilliams@ymail.com

Abstract
Two Cyanobacteria species were employed in the remediation of a crude oil polluted River. The Cyanobacteria species isolated were Oscillatoria and Nostoc species using Abattoir effluent and Aquaculture water from Eagle Island located in Port Harcourt, Rivers State. Abattoir effluent and Aquaculture water were used because they contain essential nutrients required for the growth of cyanobacteria. The concentrations of pollutant (crude oil) used were 4%, 40%, 80%. Measurement of Optical densities was done on days 0, 2, 4 and 7 after introduction of Cyanobacteria species in the different samples and a control was set up. There was consistent increase in the optical densities with increase in concentration of crude oil (pollutant) and time (days). Some physicochemical parameters (Temperature, pH, phosphate and nitrate) enhanced the growth of these organisms in the polluted environment with varying concentrations of crude oil: (Oscillatoria (O_1 4%, O_2 40%, O_3 80%), Nostoc (N_1 4%, N_2 40%, N_3 80%), Oscillatoria and Nostoc (ON_1 4%, ON_2 40%, ON_3 80%). The microbiological analysis showed that 80% mixed culture (ON_3) had higher microbial counts especially on day 7, which could be due to the presence of a mixed consortium of organisms and nutrients (Nitrogen and Phosphorus). The effectiveness of the Cyanobacteria species used in the analysis was in the following descending order: 80%ON_3> 40%ON_2> 4%ON_1> 80%O_3> 80%N_3> 40%O_2> 40%N_2> 4%O_1> 4%N_1. The Cyanobacteria species investigated in this study were highly beneficial in the remediation of the crude oil polluted River. The analysis of variance (ANOVA) showed that at 95% confident level the significant differences among the samples varied in their ability to remediate the environment. The pollutant (crude oil) was removed to an extent by all the species, either as individuals or in a mixed consortium at all concentrations during the period of analysis. Results obtained stipulate the potential of natural resources as proficient mediators for pollution control. Extension of time would result in complete remediation of this environment.

Keywords: *Nostoc spp., Oscillatoria spp., Crude oil -polluted River, Remediation.*

Introduction

Petroleum (crude oil) is a liquid fossil fuel; it is a product of decaying organic matter, such as microalgae and zooplankton. It is one of the major energy sources in the world, and is also used by the chemical industry to manufacture a large number of consumer products. However, oil drilling or transportation can cause accidents that lead to contamination of the environment (Williams and Obomelie, 2014).

Bioremediation is the employment of natural processes to remove harmful chemicals and contamination in the environment. Microalgae/cyanobacteria bioremediation is unique because it is a self-sustaining cycle. To oxidize contaminants into less-harmful metabolites, algae extract and utilize oxygen from its surrounding environment; these metabolites include

CO_2 and H_2O. For growth, algae use photosynthesis, which requires carbon dioxide (CO_2) and water (H_2O). Photosynthesis, in turn, releases oxygen that algae can employ for further contaminant oxidation, thus repeating the cycle.

Bioremediation of crude oil polluted water bodies is a method used in cleaning up crude oil polluted water bodies. Bioremediation is considered as an efficient and environmentally safe technology for inexpensive decontamination of polluted systems (Yoo *et al*., 2001).
Petroleum hydrocarbons are foremost pollutants of the aquatic environments (Williams and Obomeile, 2014).All the exploration, production, transportation and refining of oil, handling of refined product and management of oily waste activities in the petroleum industries pose serious threats to human (Kannan, 2006).The possibility of preventing oil spills and complete remediation of contaminated systems is a major environmental concern. Oil spills in marine environment are especially damaging because they cannot be contained and can spread over huge areas (Mallick, 2002).

Thus cyanobacteria have been used in waste water treatment processes and in bioremediation using microbial mats. Although cyanobacteria are known to be photosynthetic, the heterotrophic nature reported among some species puts them in the list of potential hydrocarbon degraders. For example cyanobacteria species such as *Oscillatoria salina*, *Plectonema terebrans*, *Aphanocaps sp*. and *Synechococcus sp*, have been successfully used in bioremediation of crude oil spills in different parts of the world (Raghukumar*et al*, 2001)
Cyanobacteria have shown to be highly effective as accumulators and degraders of different kinds of environmental pollutants, including pesticides and crude oil (Sokhoh *et al*., 2001).The use of cyanobacteria in the clean-up of petroleum hydrocarbon polluted sites is regarded as stimulation and augmentation process through their reported ability to release organic exudates coupled with their reported biodegradation potential (Okoh *et al*., 2001). The aim of the study was to determine the effectiveness of cyanobacteria species (*Oscillatoria salina* and *Nostoc* spp.) isolated from abattoir effluent and aquaculture in remediating crude oil polluted water bodies.

Materials and Methods

Source of Samples

Sample Collection

Abattoir effluent and aquaculture water were aseptically collected from Eagle Island abattoir and River, Port Harcourt, Rivers State. They were taken to the Microbiology laboratory, Rivers State University, Port Harcourt for analysis.

Bacteriological Analyses

Single cell technique (Burris, 2010; Williams and Youngtor, 2017) was used for the isolation of the *Cyanobacteria* in the sample. Using one millilitre (1ml) of sterile Pasteur pipette, an aliquot of the sample was placed on a clean glass slide, inserted on the light microscope, covered with cover slip and examined under x10 magnification of the light microscope and refocused at X40 magnification. The process was continued until the cyanobacteria species were properly identified by their morphological characteristics. Prior to bioremediation of the crude-oil polluted River water, plating on bacterial nutrient medium (nutrient agar, Difco, UK) was done and incubation at 30°C for one (1) week. Only axenic cultures, either uni- or multi-algal species were used.

Media Used

Nutrient Agar

This was prepared according to manufacturer's instruction of dispensing 28g of nutrient agar into 1000ml of distilled water. Mass/Volume relationship was used to compute actual required measurements. The mixture was mixed vigorously and then sterilized by autoclaving at121 ◦C at 15psi for 15minutes. Antibiotics (Chloramphenicol) and Nystatin (an anti-fungal agent) were mixed in the media and allowed to cool for about 10seconds before pouring into the petri dishes. Chloramphenicol was added to inhibit bacterial growth while Nystatin was added to inhibit fungal growth. The media was allowed to cool and solidify and dried in a hot air oven before inoculation with the river water (aqua culture).

Inoculation

One millimetre (1ml) of the sample was dispensed into 9ml of normal saline. An aliquot of the sample was transferred using a sterile pipette into the agar plate which was inoculated for one week. A pure culture of the organism was obtained by repeated sub-culturing on nutrient agar by using the streak plate method and incubated for 1 week until heavy growth was obtained. For each species, as well as for the mixed culture, nine flasks (100 ml of sterilized modified Cyanobacteria medium in 125ml, 250-ml and 500-ml conical flasks) were prepared and sterilized. Each of the nine flasks was inoculated with 5 ml of the 1-week dense individual Cyanobacteria suspensions, or with multiple species in the case of the mixed culture, incubated under the previously mentioned conditions and left to reach mid-late log phase of growth (E10 days).

Growth Monitoring

The pure cultures of *Oscillatoria salina* and *Nostoc species* were obtained and transferred by using sterile wire loop into the test tube containing normal saline until it was turbid. About 5ml of sample was taken by using a sterile pipette and emptied into the conical flasks with varying concentrations of crude-oil (4%, 40% and 80%). A mixed culture of *Oscillatoria salina* and *Nostoc spp*. was also used as well as the single cultures in the crude-oil polluted environments. Growth was monitored to determine the stimulatory or inhibitory effect of pollutants on the tested cyanobacteria species (their sensitivity or resistance) in order to define the most resistant and promising bioremediation species.

The abattoir effluent (which serves as a growth nutrient) (4,8 and 12%) was emptied into nine(9) conical flasks containing 50ml of river water polluted with crude-oil in a ratio of 4:40:80 and was labeled O-S for *Oscillatoria*, and N-S for *Nostoc*, ON_1 – ON_3 for mixed culture. Appropriate controls were also set up, containing only 4% crude polluted river (Control Oa), and 40% crude oil polluted river (Control Ob) and 80% crude oil polluted river (Control Oc). A mixed culture of these organisms was inoculated into crude oil polluted conical flasks (4,40 and 80% respectively) labelled ON_1,ON_2 and ON_3 (*Oscillatoria* and *Nostoc*). After the sample preparation, the flasks were monitored for two weeks using spectrophotometer (spectro UV Vis RS spectrophotometer UV 2500) at 600nm, and were kept close to the window for natural source of light (sunlight) to penetrate. The flasks were intermittently shaken three times daily to enhance growth by preventing sedimentation of the cyanobacteria avoiding thermal stratification which involves gas exchange between culture medium and air to ensure that cells of the population were equally exposed to light and nutrients. Optimum physical growth conditions were provided by white fluorescent lamps under light/dark regime of 18/6 hours for the duration of the experiments.

3.8 Determination of Cyanobacteria Concentration (Optical Density)

From the conical flasks containing *Oscillatoria* and *Nostoc* species as well as the mixed culture (ON1,ON2,ON3)with varying concentrations (4%, 40% and 80% respectively), optical density was determined. About 5ml of samples were used and the spectrophotometer was set at 600nm. The spectrophotometer was set at 600 nm and one (1) ml of the blank (sterile un-inoculated media) was transferred into a labelled polystyrene cuvette using sterile technique to blank the spectrophotometer. Each culture was transferred (using a sterile pipette) into labelled cuvette and the optical density reading was recorded from the spectrophotometer. The optical densities of the controls (Oa, Ob and Oc) were determined.

Physicochemical Parameters of the Samples

The physicochemical parameters were determined using standard analytical procedures (AOAC, 2000). The pH meter used was pocket-sized HANA pHep + HI 98108 with automatic temperature compensation. Total organic carbon was determined by dichromate wet oxidation method of Walkley and Black as modified by Dhyan *et al*., (1999). Nitrate content was determined using the macro Kjeldahl digestion method of Brady and Weil (1999) and available phosphorus was determined using the method reported by Olsen and Sommers (1982). Sulphate was determined using the turbid metric method. Standard methods were used for the determination of Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) (AOAC, 2000).

Total Cyanobacteria Counts

The average total Cyanobacteria population present in each sample at the beginning of the experiment (day 0) to the end (day 7) were estimated using nutrient agar. Inoculated petri dishes were incubated at inverted positions for 24hours at 37◦C, after which plates were checked, colonies counted and results obtained.

Statistical Analysis

The analysis of variance as described by Nduda and Ogolime (2000) was used to ascertain the significant difference at 95% confidence interval between the Cyanobacteria total viable counts of the different samples.

Results and Discussion

From fig.1, there was steady increase in the optical densities of the crude oil polluted water samples in all the samples;*Oscillatoria* (OS4%,OS40%, OS80%), *Nostoc* (N4%, N40%, N80%), *Oscillatoria and Nostoc*($ON_1$4%, $ON_2$40%, $ON_3$80%).

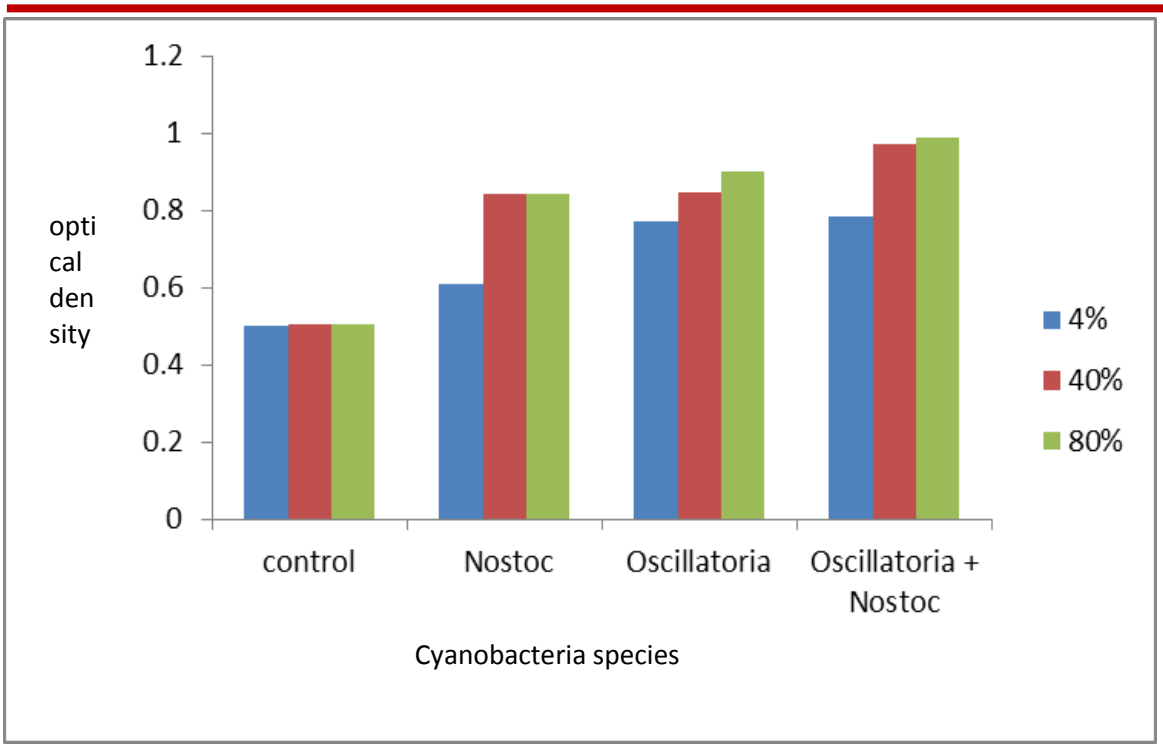

Fig1: Optical densities of the Cyanobacteria species on day 2 of experiment

The mixed culture (ON) had the highest absorbent rate due to presence of chlorophyll, nutrients (Nitrogen and Phosphorus) and other parameters (pH and Temperature).There was remediation especially because the increase in optical density grew closer to the optical density of water (1.33) (*Hale*, 1997) and one can deduce that with extension of time, remediation is likely to continue until complete remediation occurs.

Table 1: Physicochemical parameters of abattoir effluent and aquaculture water

Parameters	**Abattoir effluent**	**Aquaculture water**
Nitrate (mg/I)	5.4	5.1
Temperature (^{O}C)	26.2	29.0
Phosphate (mg/I)	0.072	0.070
Sulphate (mg/I)	1.0	1.2
pH	7.13	7.9
Total Organic Carbon	0.17	0.15
BOD	47	40
COD	275	264
DO	1.3	1.1

Table 2: Physicochemical parameters of crude polluted river water

Parameters	4%	40%	80%
Nitrate (mg/I)	5.7	5.9	6.0
Sulphate (mg/I)	1.8	2.4	2.8
Phosphate (mg/I)	0.080	0.090	0.098
Temperature (^{O}C)	28.1	28.4	29.0
pH	7.5	7.8	7.15
Total organic carbon (mg/l)	10	12	15
BOD (mg/I)	190	205	220
DO (mg/I)	1.5	2.0	2.4
COD (mg/l)	285	297	310
THC (mg/kg)	8751	8890	8971

The physicochemical parameters, pH and Temperature supported the growth of the test organisms (*Osicillatoria salina and Nostoc species*) (AOAC, 2000). The nutrients which include Phosphate (0.070–0.072mg/I), Sulphate (1.0–1.2mg/I), and Nitrate (5.1–5.6mg/I) also enhanced the growth of the organisms (AOAC, 2000). Table 2 showed increase in nutrients such as nitrates (5.7, 5.9 and 6.0mg/l in 4%, 40% and 80% crude oil polluted water respectively), sulphate (1.8, 2.4 and 2.8 in 4%, 40% and 80% crude oil polluted water respectively), and phosphate (0.080, 0.90 and 0.98 in 4%, 40% and 80% crude oil polluted water respectively).This increase occurred as a result of the introduction of pollutant (crude oil). It was observed that the higher the concentration of pollutant, the greater the increase in the concentration of nutrients. There is no doubt that decrease in dissolved oxygen was obtained due to increase in total organic carbon (TOC) content and other nutrients.

The microbiological analysis showed that there was significant difference in the total Cyanobacteria count in figs. 2a,b and c which were higher in the mixed culture ($ON_3$80%) especially on day 7, the last day of analysis. The high counts could be as a result of the presence of a consortium of organisms (ON_1, ON_2 and ON_3) or nutrients (Nitrogen and Phosphorus) that favoured the Cyanobacteria growth (Williams and Youngtor, 2017). The analysis of variance (ANOVA) of the total Cyanobacteria count in the samples showed that 80% mixed culture had the highest colonies count. The effectiveness of the cyanobacteria used in this analysis were in the following descending order: 80%ON_3> 40%ON_2> 4%ON_1, 80%O_3> 80%N_3> 40%O_2> 40%N_2> 4%O_1 4%N_1 from the analysis of variance, it showed that 80% mixed culture was more effective and capable of remediating the crude oil polluted water body (Williams and Youngtor, 2017)

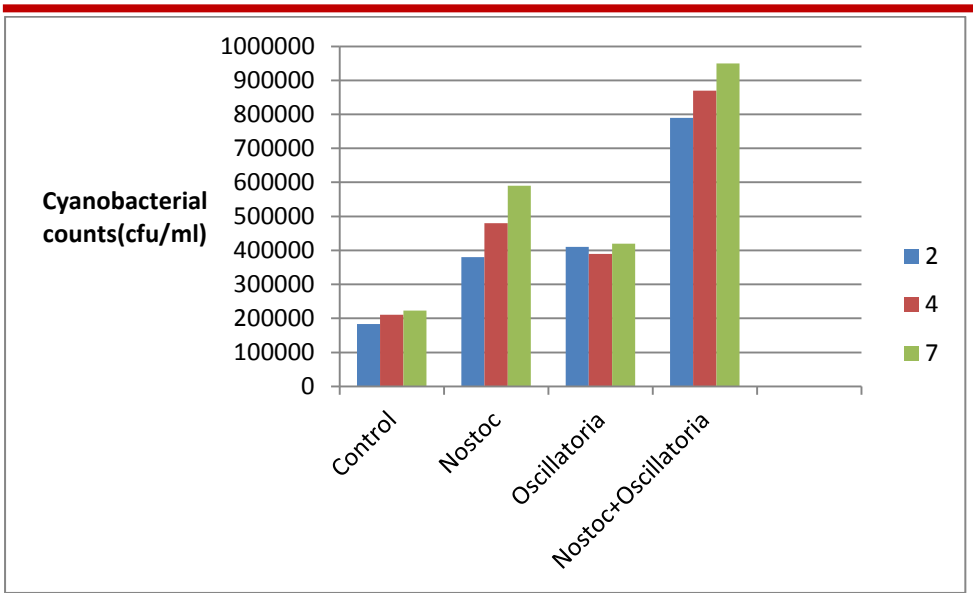

Fig. 2a: Total Cyanobacteria Counts (Cfu/ml) from day 2 to 7 from 4% crude oil concentration

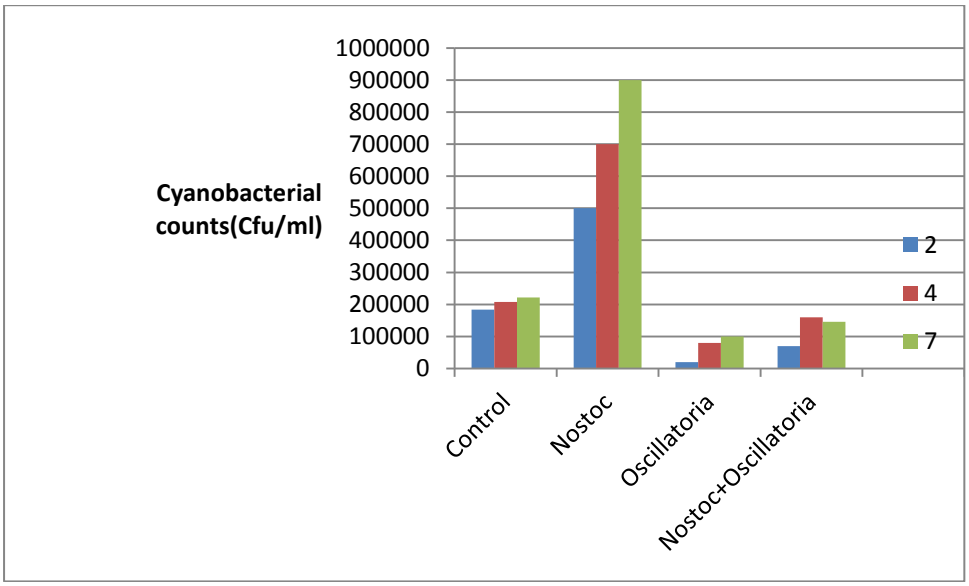

Fig. 2b: Total Cyanobacteria Counts (Cfu/ml) from day 2 to 7 from 40% crude oil concentration

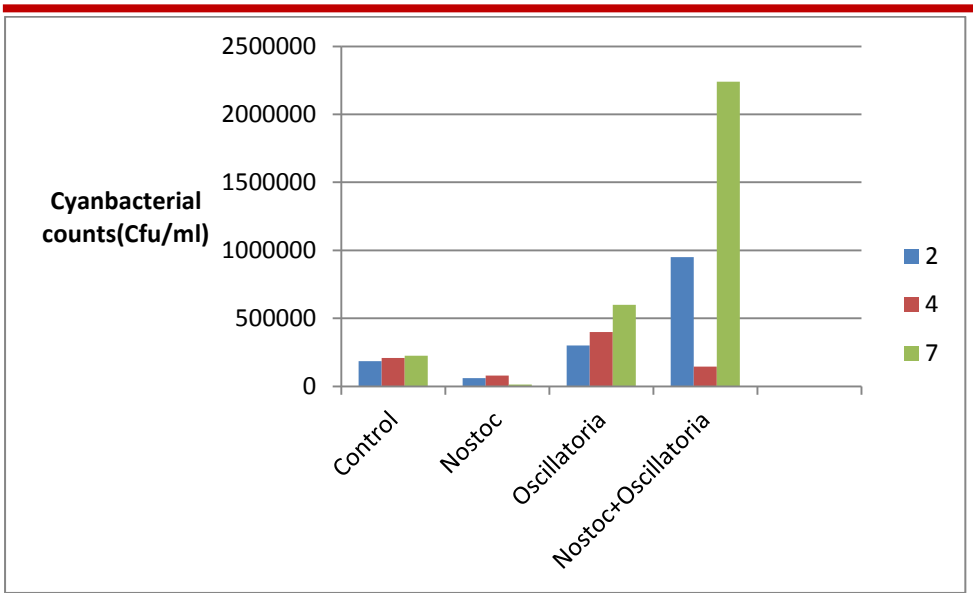

Fig. 2c: Total Cyanobacteria Counts (Cfu/ml) from day 2 to 7 from 80% crude oil concentration

Conclusion

The investigation from this study showed that Cyanobacteria species (*Oscillatoria* and*Nostoc* species) are useful bioremediation tools in crude oil polluted sites. Employment of this method in large scale bioremediation of crude oil pollution is essential. Since there is significant difference among the samples of the cyanobacteria, it means that their effectiveness varied in remediating the environment. This work is also coherent with previous studies that show abattoir effluent and aquaculture water as sources of essential nutrients and media for the cultivation of cyanobacteria.

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