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EFFECT OF DIFFERENT CONCENTRATIONS OF BIODEGRADER (*Bacillus subtilis*) ON THE SURVIVAL OF FINGERLINGS IN CRUDE OIL POLLUTED ENVIRONMENT

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ABSTRACT

The role of various concentrations of hydrocarbon degraders (*Bacillus subtilis*) in the survivability of *Clarias gariepinus* reared in hydrocarbon polluted aquaria was investigated under laboratory conditions using standard microbiological and analytical protocols. Six aquaria containing ten fingerlings each were contaminated with 100ml of crude oil and different amount (5ml, 10ml, 15ml and 20ml) of hydrocarbon degraders (*Bacillus* subtilis) in broth culture were introduced into each of the aquarium. The set-up which was adequately aerated was monitored for 21 days for survivability of the fingerslings. The results revealed changes in swimming performance and feeding pattern of the fingerlings after crude oil contamination. Reduction in swimming was observed in those fingerlings with less hydrocarbon biodegraders. Similarly, the feeding habit of those fingerlings with less hydrocarbon degraders decreased significantly. The aquarium with no biodegrader recorded 3,2 and 3 deaths on the 1st , 2nd and 3rd week respectively while the aquarium with the highest biodegraders recorded 2, 2 and 0 death on the 1st , 2nd and 3rd week respectively. The rate of death of the fingerlings revealed that increase in hydrocarbon biodegraders in the aquarium increased the survival of the fingerlings in the aquarium. Specifically, it was observed that aquarium with the highest biodegrader (20ml) recorded the lowest mortality of 40% at the end of 21 days. No mortality was recorded in aquarium with no crude oil contamination. This study revealed that in the aquarium with highest hydrocarbon degraders, the effect of crude oil was quickly neutralised thus eliminating the mortality of the exposed fingerlings on the third week of the experiment.

KEYWORD: *Bacillus subtilis*, Crude oil, *Clarias gariepinus*, biodegraders

INTRODUCTION

Petroleum contains non-aromatic, monoaromatic and an extensive suite of polycyclic aromatic hydrocarbons (PAHs), all of which can be toxic to organisms (Essien *et al.*, 2012). Environmental pollution by crude oil is a global problem as it usually leads to contamination and possible uptake and accumulation of toxic chemicals/pollutant along the food chain and harm to the flora and fauna of the affected habitat (Abraham and Essien, 2016). Aquatic environments are made up of complex interrelations between plant and animal species and their physical environment. Damage to the physical environment often lead to the death of one or more species in a food chain, which may lead to damage for other species further up the chain. The effects of oil spill on aquatic lives are caused by either the physical nature of the oil or by its chemical components (Sunmonu and Oloyede, 2007). In Nigeria, especially the Niger Delta region where we have most of the crude oil exploration installations, oil spills in the area has exposed the marine and coastal ecosystems to chronic and acute environmental damages (Campagna *et al*., 2011).

There are many techniques to remove oil contamination from the sediment and wastewater, such as chemical dispersant. However, these processes do not totally remove the oil contaminants from the environment and besides they are known to have some adverse effect on the marine habitat. The use of the process of biodegradation can transform the hydrocarbons into carbon dioxide and other compounds and is one of the promising treatments for combating oil pollution. It is cost effective, ecofriendly and cause less destruction to the environment as compared to other treatments.

In open water, fish have the ability to swim away from a spill by going deeper in the water or further out to sea, however their survival are usually threatened by the toxicity of even low concentration of crude oil in the ecosystem (Hicken.*et al.*, 2011). Studies have demonstrated increased mortality of fish as a result of oil spills (Hjermann *et al*., 2007; Fodrie *et al*., 2014). Exposure of adult fish to parts per million (ppm) concentrations of crude oil has been reported to elevate adrenaline, non-adrenaline and cortisol concentrations in plasma hematological and immune system dysfunction (Alkindi *et al.*, 1996), physical fouling, intake and accumulation of water-soluble and insoluble hydrocarbons (Gravato and Santos, 2002; Rodrigues *et al*., 2010) and severe cases of mortality. Bioremediation which mainly depend on microorganisms to degrade, transform, detoxify or breakdown the contaminants is widely employed because it is known to be cost effective and environmentally friendly technology for remedying contaminated environments (Abraham and Essien, 2016). Microorganisms degrade or transform these pollutants as they carry out their normal metabolic activities under aerobic and anaerobic conditions provided their optimum growth conditions are provided. This study aimed to explore the bioremediation potentials of microbes in increasing the survivability of fingerlings in a crude oil polluted ecosystem. Specifically, the experiment was to determine the ability of microorganisms to degrade crude oil in a polluted aquatic ecosystem and eliminate their toxic effect on fingerlings and subsequently other aquatic life.

MATERIALS AND METHOD

Sample Collection

Freshwater samples were collected from Nwaniba River in large sterile gallons and transported to the microbiology laboratory, University of Uyo, Uyo. Nwaniba River is located in Uruan Local Government Area of AkwaIbom State, Nigeria between latitude 5°2'51"N and longitude 8°2'41"E (Figure 1) while healthy fingerlings of *Clarias gariepinus* were purchased from Almond fish farm within Uyo, Akwa Ibom State.

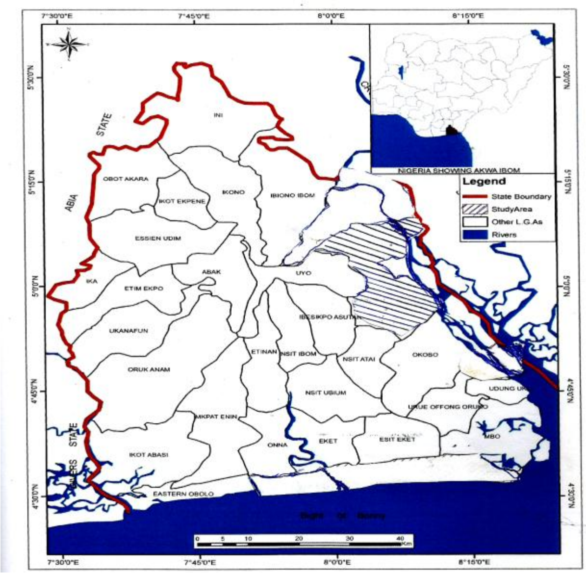

Figure 1. Map of Akwa Ibom State indicating Nwaniba River in Uruan Local Government Area.

Isolation of Crude Oil Degrading Bacteria

Crude oil degrading bacteria were isolated using an enrichment culture while the degradation potentials were determined using the hydrocarbon overlay method (Abraham and Essien, 2016). The enrichment media composed of mineral salt medium (KH_2PO4 1 g/L, Na_2HPO_4 1.3 g/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4$ 0.2 g/L, $FeSO_4{\cdot}7H_2O$ 0.05 g/L, $CaCl_2$ 0.02 g/L, $ZnSO_4{\cdot}7H_2O$ 5 mg/L, $MnCl_2{\cdot}4H_2O$ 5 mg/L, $NaMoO_4{\cdot}2H_2O$ 1 mg/L, $CuCl_2$ 0.5 mg/L), fortified with 1% crude oil. For the hydrocarbon degradation potentials, 15 g of agar-agar was added to Mineral salt medium (Same composition as enrichment medium), sterilized and allowed to set. The solidified plates were overlaid with 1% (v/v) and 2% (v/v) of sterile crude oil separately, allowed for about 15 to 30 min then the test isolates were streaked on the surface of the plate. All inoculated plates were incubated at room temperature for 5-15 days with periodic observations. Colonies that eventually developed showing area of clearing were selected and rated. The utilization was rated based on the diameter and luxurious nature of the developed colonies, i.e., '+', '++' or '+++' indicating the utilisation potentials. (Table 1). The best hydrocarbon degrader was then characterized and stored for further study.

Morphological and Biochemical Characteristics of Best Hydrocarbon Degrading Bacteria

The crude oil degrading bacterial isolates were characterized based on their cultural and morphological attributes as well as their responses to standard biochemical test as described by Collins *et al.* (2004). Twenty-four (24) hours old mono-cultures of bacteria obtained were subjected to Gram's and endospore staining and several biochemical tests such as Catalase test, Citrate Utilization test, Oxidase test, Motility test, Methyl red and Voges Proskauer test and Indole test, as well as sugar fermentation test (Cruickshank *et al*., 1975; Cowan, 1985). The results obtained were compared with characteristics described in Bergey's manual of determinative bacteriology for identification (Holt et al., 1999).

Experimental Set Up

Six aquaria (A – E; Table 1) were set up using the freshwater sample collected from the Nwaniba River. Then ten apparently health fingerlings of *C. gariepinus* were introduced into each of the aquarium, fed with Prime 53/14 (Zemach, Israel) and allowed to acclimatize for a period of seven days. On the seventh day, five of the aquaria (B – F) were contaminated with 100 ml of crude oil. Varying amount (5 ml, 10 ml, 15 ml and 20 ml) of standardized broth culture of the best hydrocarbon degrading bacteria earlier isolated were introduced into aquaria (B – E) respectively (Table 1). The hydrocarbon pollutants degrading potentials of the bacterial isolates was as depicted in Table 2.

Table 1: Summary of Experimental Set-Up

Aquarium Code	Number of fingerlings	Amount of Crude oil added (ml)	Amount of crude oil degraders added (ml)
A	10	-	-
B	10	100	5
C	10	100	10
D	10	100	15
E	10	100	20
F	10	100	-

Determination of the Effect of Hydrocarbon Degraders on Survival of Fish Sample

The effect of the crude oil and the hydrocarbon degrader on the survivability of the fish was determined by physical observations. Parameters considered during the physical observation were swimming performance, feeding performance, avoidance rate which eventually leads to the mortality of the fish samples (Table 3).

Table 2: Hydrocarbon Pollutants Degrading Potentials of the Bacterial Isolates

Isolates		W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8	W_9	W_{10}	W_{11}	W_{12}	W_{13}
Crude oil degrading rate	1%	+++	++++	+++	+	++		+++	+	++	++	+	++	+++
	2%	++	++++	++	-	+++	++	++	-	+	+	-	++	++

Key - = no growth, 1- 5 mm (weak) = +; 6 – 10 mm (moderate) = ++; 11 – 15 mm (strong) = +++; 16 – 20 mm (Strongest) = ++++

Table 3: Physical Observations of the Fingerlings Characteristics

Week	Characteristics	Aquarium A	Aquarium B	Aquarium C	Aquarium D	Aquarium E	Aquarium F
Acclimatization Period	Swimming performance	Active	Active	Active	Active	Active	Active
	Feeding performance	Active	Active	Active	Active	Active	Active
	Avoidance reaction	Non	Non	Non	Non	Non	Non
Week 1	Swimming performance	Active	Inactive	Inactive	Inactive	Inactive	Inactive
	Feeding performance	Active	No appetite	No appetite	Little appetite	Little appetite	No appetite
	Avoidance reaction	Non	Complete Avoidance	Complete Avoidance	Complete Avoidance	Complete Avoidance	Complete Avoidance
Week 2	Swimming performance	Active	Inactive	Inactive	Inactive	Inactive	Inactive
	Feeding performance	Active	Little appetite	Little appetite	Little appetite	Little appetite	No appetite
	Avoidance reaction	Non	Complete Avoidance	Complete Avoidance	Complete Avoidance	Complete Avoidance	Complete Avoidance
Week 3	Swimming performance	Steady	Steady	Steady	Steady	Inactive	Inactive
	Feeding performance	Active	Little appetite	Little appetite	Increased appetite	Increased appetite	No appetite
	Avoidance reaction	Non	Little Avoidance	Little Avoidance	Little Avoidance	Little avoidance	Complete Avoidance

RESULTS AND DISCUSSION

The result of the study revealed that the water ecosystem harbored bacterial communities with varying capability of crude oil utilization. Of the 13 bacterial isolates screened, isolate; W_2 demonstrated very strong potential to degrade the hydrocarbon. This was revealed by the remarkable increase in the biomass of the isolate when exposed to crude oil as sole carbon source. Morphological and biochemical characteristics of the best crude oil degrader revealed that the best degrader (W_2) was a specie of *Bacillus subtilis.* This high potential might be attributed to the organisms' ability to produce biosurfactants. The amphilic property of biosurfactant is believed to enhance the degradation of crude oil by reducing the surface tension between the crude oil and the test organism (Nwaogu *et al.*, 2008). In a similar study on the bacterial degradation of crude oil by gravimetric analysis, Latha and Ralaivani (2012) noted the predominance of hydrocarbon utilizing bacteria, *Bacillus cereus* and concluded that *Bacillus* sp contributes largely in the degradation of hydrocarbons and its compounds because it forms spores which can withstand adverse environmental conditions.

The effect of the crude oil and the hydrocarbon degrader on the survivability of the fish samples in the crude oil contaminated aquarium was determined by the physical observation of certain characteristics which subsequently lead to the mortality of the fish samples in the aquaria (Table 3) The mortality of the fingerlings decreased over time with increasing biodegraders' population. A change in the swimming performance and feeding pattern of fingerlings was also observed. The fingerlings which were actively swimming and feeding became inactive, sluggish and withdraw from food at the introduction of crude oil into the aquaria. According to Bagenal and Tesch (1978), the condition factor is a length-weight relationship that indicates the well-being of the fish. The lower the condition factor the poorer the growth of the fish. Exposure of fish to water-soluble fractions of crude oil may have resulted in reduced food intake and thus lower body weight. This finding simulates the work of Kicheniuk and Khan (1981), Kori-Siakpere (2000) and Akaisha et al., 2004 who noted that exposure of fish to water soluble fractions of crude oil, resulted in reduced feeding and lower body weight which are reflected in a change in the condition factor. The reduced growth performance observed in this study is also supported by the earlier work of Afolabi *et al* (1985) who also reported that water soluble fractions (WSFs) of crude oil impacted lethargic effects on fish because fish fed less aggressively, moved little and were closely found together. Similarly, Ofojekwu and Onah (2002) reported that fish are known to increase their metabolic rates and excrete aromatic hydrocarbons and consequently allocate more energy to homeostatic maintenance than storage, hence a reduction in growth rate.

Table 4: Mortality rate (%) of the fingerling in each Aquarium

	Week	Aquarium A	Aquarium B	Aquarium C	Aquarium D	Aquarium E	Aquarium F
Number of Fingerlings in each aquarium		10	10	10	10	10	10
Number of Dead Fingerlings	Acclimatization Period	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Week 1	0 (0)	3 (30)	2 (20)	2 (20)	2 (20)	3 (30)
	Week 2	0 (0)	2 (20)	2 (20)	3 (30)	2 (20)	2 (20)
	Week 3	0 (0)	2 (20)	1 (10)	1 (10)	0 (0)	3 (30)

The mortality of the fingerlings in the aquaria indicated that the increase in the biodegraders greatly influenced it (Table 4). Aquarium 'A' with no crude oil had zero mortality even to the last week of the experiment. Auqrium 'B' with 5% crude oil degraders had 70% mortality while aquarium 'E' with 20% crude oil degraders had 40% mortality in the first two weeks of the experiment and thereafter with zero mortality. It was also observed that the mortality rate increased in aquarium 'F', with no biodegraders and with 80% death of the fingerlings at the third week of the experiment, the aquarium 'E' with the highest number of biodegraders recorded no mortality at the third week. This may be due to the fact that, aquarium 'E' with the highest number of biodegraders must have, toward the third week reduced significantly the crude oil pollution level in the aquarium, thus reducing or eliminating the crude oil effect on the fingerlings. This result is in agreement with report of Onuorah *et al*. (2018) who noted that biodegraders are capable of detoxifying or complete removal of pollutants including petroleum hydrocarbon and and its associated product due to their diverse metabolic activities. In a similar work, Röling *et al.* (2004) reported that petroleum hydrocarbons phenanthrene and dibenzothiophenes were well degraded in a laboratory experiment but noted that similar degradation effects did not occur in a field experiment, which was attributed to the temperature range during the study.

CONCLUSION

This study has shown that the presence of crude oil in water bodies can affect the fish biophysics characteristic. Introduction of crude oil degrading bacterial species into such water bodies can detoxify the water by breaking the crude oil into non-toxic compounds and increase the adaptation and survivability of the fishes. The results have revealed that the activities of oil degrading (*Bacillus subtilus)* bacteria enhanced the survivability of fish samples in crude oil contaminated aquarium E as it recorded the lowest mortality the first two weeks of the experimental period with zero mortality thereafter. Regarding these results, the potentials of this bacterial strain (*B. subtilus* W_2) can be further exploited for wider application especially in the aquatic ecosystem of the Niger Delta which is prone to hydrocarbon contamination.

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