Toxicity Profile of Crude Oil on Degrading Bacterial Isolates

Uwem Okon Edet, Ogemdi Chinwendu Anika, Elizabeth Umoren

Abstract— In order to assess the tolerance of crude oil degrading microbes naturally present in pristine soil to crude oil toxicity, pristine soil samples were collected from the garden of the Faculty of Biological Sciences, University of Calabar, while Bonny light crude oil was collected from the retail outlet of Exxon- Mobil Nigeria Limited. Isolation and characterization of hydrocarbonoclastic microbes from pristine soil sample were all done using pour plate and vapor methods. Toxicity profiling was done using various concentrations (1%, 5%, 10%, 15% and 20% v/v) of the crude oil in mineral salt medium (MSM) inoculated with selected crude oil degraders; *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Klebsiella* species, that were designated as HUB1, HUB2, HUB3, HUB4 and control (*Escherichia coli*) grown in MSM with no crude oil. The experimental set-ups were monitored for changes in pH, temperature and bacterial counts at various time intervals (0 to 60 hours). Replicates readings were analyzed using analysis of variance for significance. Across the various concentrations, pH changed gradually from neutral to under 6 while slight fluctuations in temperature were observed across the different treatments. At concentrations of 5% and 10%, there was no tangible drop in counts but at 15 and 20%, there was a sharp decline in HUB counts, indicating that crude oil causes quantitative and qualitative changes in the composition of soil microbes. Although crude oil utilizing microorganisms have the capacity to degrade crude oil, nevertheless, HUBs have different crude oil tolerance abilities. Hence, microbes with better tolerance to crude oil effect as see with the HUB1 isolate can be employed in the process of bioremediation in the environment.

Index Terms— Autochthonous microorganisms, Bioremediation, Crude oil degrading microbes, Crude oil tolerance, hydrocarbonoclastic microbes, Toxicity profiling,

1 Introduction

Crude oil is a complex mixture of hydrocarbons (Adipah, 2019). Toxicity of petroleum and its products depends on the chemical composition and structure of hydrocarbons and studies have shown that it affects plants, animals, and can even accumulate in food chains (Ziolkowska and Wyskowski, 2010) Crude oil like other pollutants can produce varying effects on the soil microorganisms including fungi and bacteria. It can also affect soil physicochemical activity, content of soil organic matter and micronutrients in the soil (Ziolkowska and Wyskowski, 2010).

Microorganisms are very important environmentally for several reasons. They are intricately involved in various biogeochemical cycles such as nitrogen, sulfur, iron to mention just a few of them (Edet et al., 2017; Edet et al., 2018; Udotong et al., 2015). Microorganisms are able to utilize crude oil as an energy source and for support have increasingly gained acceptance worldwide because they are highly effective, readily available, cheap and safe, Overholt et al., 2015; Unimke et al., 2018). They exists as a consortia and in a delicate balance where they work synergistically to achieve the breakdown of all the complex components present therein. This balance can easily be disrupted by certain manmade pollutants that find their way into the environment (Udofia et al., 2018). It can alter the physiological and biochemical states of many organisms (Onwurah et al., 2007).

When crude oil, other petroleum based pollutants and other allochthonous compounds spill into the environment, their mobilization, transformation and utilization is ultimately the duty of autochthonous microorganisms and it affects the structure and composition of microorganisms (Colewell, 1973). It is important that an insight into the effects of petroleum hydrocarbon spill on the autochthonous microbes in any given biota and the isolation of crude oil-degraders amongst them is required for proper management of and bio-remediation of the environment (Kostka et al., 2011).

Few studies have been documented on the tolerance of indigenous microorganisms in pristine soil to the toxicity of crude oil (Mustafa et al., 2013; Babalola et al., 2016), however, the response of individual hydrocarbonoclastic microbial isolate to crude oil spillage is still poorly understood. This study attempts to fill this gap. Therefore, this research was aimed at isolating hydrocarbon utilizing bacteria and exposing them to varying concentrations of Bonny light crude oil.

2 Materials and Methods

2.1 Sample Collection

Surface and subsurface pristine soil samples were randomly sampled from eight (8) different locations in the garden of the Faculty of Biological Sciences, University of Calabar into sterile sample collection tubes using a hand held soil auger as previously described (Babalola et al., 2016). Collected samples were immediately transported to the laboratory for analysis. Bonny light crude oil sample was obtained retail outlets.

2.2 Isolation of Hydrocarbonoclastic Microorganisms

The pour plate and vapor methods of Antai et al (2014) were employed in the isolation of the hydrocarbonoclastic bacteria. Briefly, 10g of the homogenized soil and sediment sample were dissolved in sterile 90ml of distilled water and used to carry out serial dilution. Following serial dilution, freshly prepared mineral salt medium (MSM) and plate count agar were sterilized and poured into plates already containing 1 mL aliquot of the tenfold soil sample dilutions (x105) in triplicate and was allowed to gel. In addition, the crude oil and filter paper were also sterilized. Afterwards, the hot air sterilized filter papers were then soaked

with 1ml of the sterilized crude oil and carefully used to cover the lid of the agar plates and sealed with masking tape. The plates were then incubated inverted for 24 to 96hours.

2.3 Purification and Maintenance of Isolates

Distinct bacterial colonies were selected from the plates and were cultured at least thrice onto nutrient agar. The purified isolates were then stored in nutrient agar slants and stored at room temperature for further analysis.

2.4 Characterization and toxicity testing of Isolates

Isolates were subjected to microscopy and biochemical screening and this were done as previously described (Babalola et al., 2016). Toxicity test was carried out using the method described previously (Nseabasi and Antai, 2012). This was carried out on the four hydrocarbon utilizers and a control set up that received no crude oil (carbon source). Using separate conical flasks and for each of the isolate, 1, 5, 10, 15 and 20% v/v concentration of crude oil were prepared by adding appropriate volumes of MSM (4.95, 47.5, 45, 42.5 and 40ml) to the appropriate volumes of crude oil (0.5, 2.5, 5, 7.5 and 10 mL). To each of these flasks, 1ml of each of the overnight culture of each of the isolates was into the flasks containing the different crude oil concentrations. This was repeated for all the isolates. A control was set-up replacing the crude oil with glucose. The experimental set-ups were incubated at room temperature (28 ± 2oC) overnight. At an interval of 8, 16, 24, 32, 40, 48, 56, 64 and 72 h, 1ml from each conical flasks were assayed for bacterial counts (CFU mL-1) using pour plate technique.

2.5 Temperature and pH changes

Changes in pH and temperature were monitored as well as optical densities of the flasks. Temperature and pH were determined as previously described (Unimke et al., 2014) using a dual pH and temperature meter (HM Digital PH-80ph/temperature Hydro tester). Manufacturer's instructions were strictly followed in the determination process. The meter has a sensitivity of 0.01%.

2.6 Statistical Analysis

Replicates readings were analyzed using one way analysis of variance for significance.

3 RESULTS

From the morphological and the biochemical reactions of the selected crude oil degrading isolated designated as HUB1, HUB2, HUB3, and HUB4 the probable organisms are Bacillus, Micrococcus, Pseudomonas, and Klebsiella species respectively (Table 1). Across the various concentrations, pH changed gradually from neutral to under 6.00 as showed on Figure 1. Slight fluctuations in temperature in the range of 29 to 32 ℃ were observed across the different time interval (Figure 2).

From our study, at concentrations of 5% and 10% exposures of isolates to crude oil, there was no tangible drop in counts but at 15 and 20%, there was a sharp decline in HUB counts, indicating that crude oil causes quantitative and qualitative changes in the composition of soil microbes (Figure 4, 5, 6 and 7).

TABLE 1

HYDROCARBONOCLASTIC BACTERIA ISOLATES FROM THE CRUDE OIL IMPACTED SOIL SAMPLE

Sample code	Cell morphology	Gram reaction	Oxidase	Catalase	Indole	Ornithine	Motility	Lactose	Sucrose	Glucose	Gas	H_2S	Citrate	Urease	MR	VP	Probable organism
HUB_1	Rods in pairs	+	+	+	-	+	+	-	-	-	-	-	-	+	-	+	*Bacillus spp*
HUB_2	Slightly curved rods	-	+	+	-	+	+	-	-	-	-	-	-	-	+	-	*Pseudomonas spp*
HUB_3	Straight rods in pairs	-	-	+	-	+	-	+	+	+	+	-	+	-	-	+	*Klebsiella spp*
HUB_4	Spherical in pairs	+	+	+	-	+	+	-	-	-	-	-	-	+	-	+	*Micrococcus spp*

Key: HUB = Hydrocarbon utilizing bacteria, + = positive and - = negative.

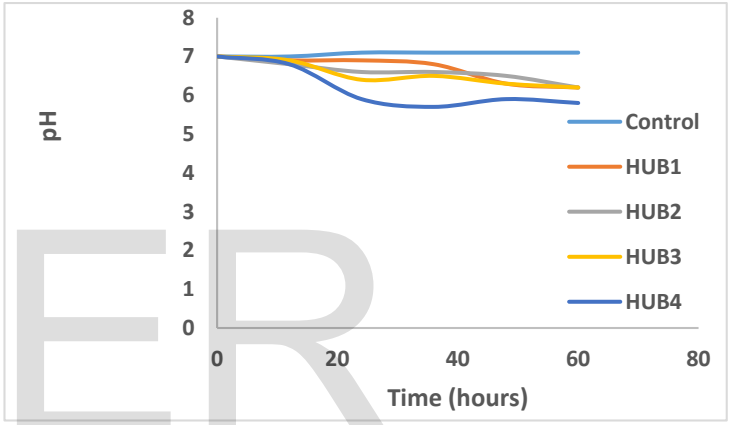

Fig 1. Changes in pH with time incubation (hours). Key: HUB = Hydrocarbon utilizing bacteria.

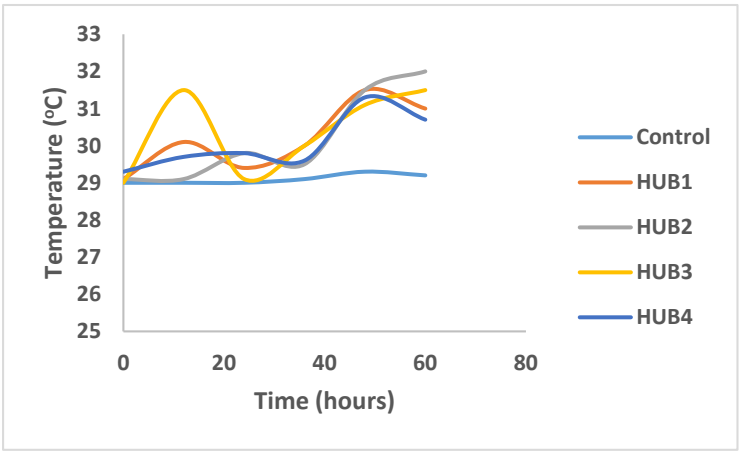

Fig 2. Changes in temperature (℃) with time of incubation (hours). Key: HUB = Hydrocarbon utilizing bacteria.

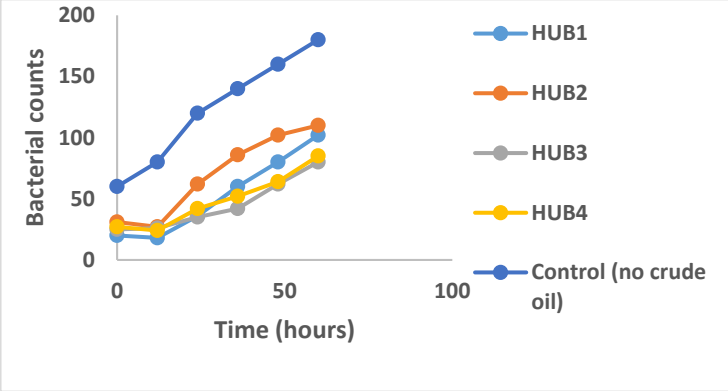

Fig. 3. HUB and control bacterial counts at 1% crude oil concentration. Control = HUB1.Key: HUB = Hydrocarbon utilizing bacteria.

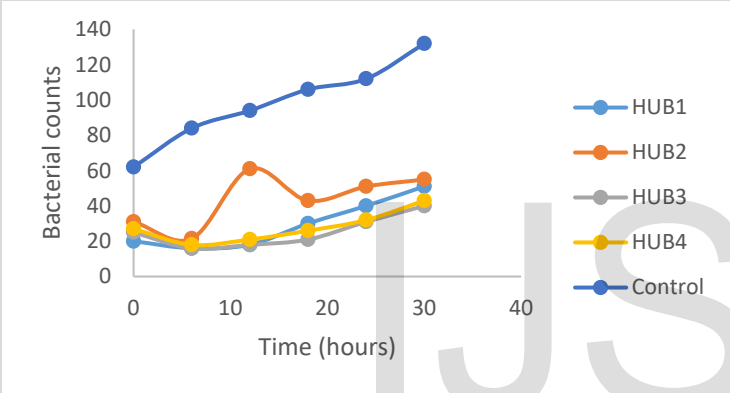

Fig. 4. HUB and control bacterial counts at 5% crude oil concentration. Control = HUB1. Key: HUB = Hydrocarbon utilizing bacteria.

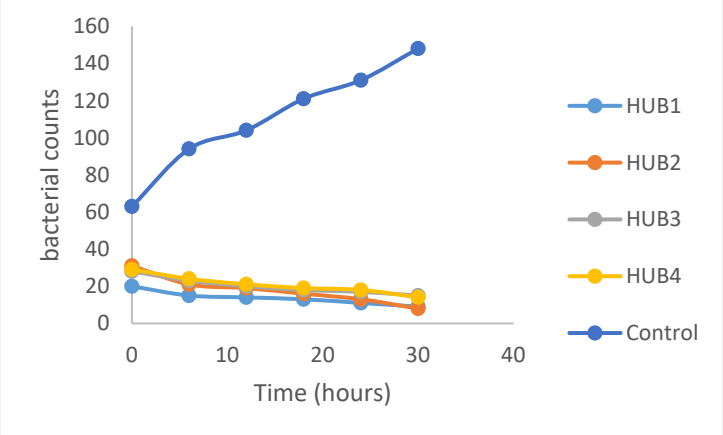

Fig. 5. HUB and control bacterial counts at 10% crude oil concentration. Key: HUB = Hydrocarbon utilizing bacteria.

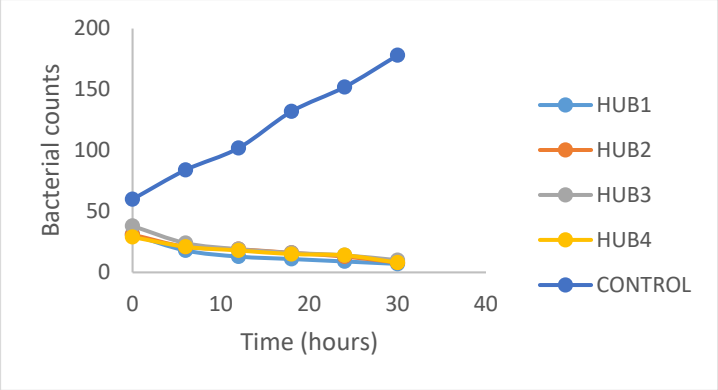

Figure 6: HUB and control bacterial counts at 15% crude oil concentration. Key: HUB = Hydrocarbon utilizing bacteria

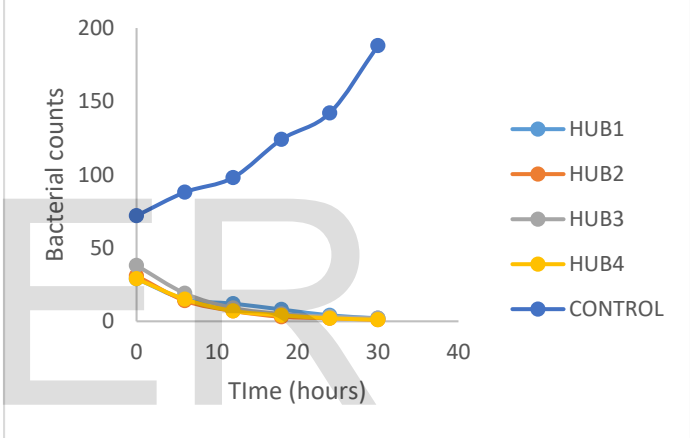

Figure 7: HUB and control bacterial counts at 20% crude oil concentration. Key: HUB = Hydrocarbon utilizing bacteria

4 Discussion

Microbes are constantly exposed to anthropogenic pollutants and they have evolved a number of mechanisms to reverse the effect of toxic pollutants and these include using them (degradation), protein repair mechanisms, and maintenance of membrane fluidity (Murínová and Dercová, 2014).Crude oil spills affects several microbiological indices such as microbial biomass carbon and microbial metabolic diversity were significantly affected by crude oil (Timmerman et al., 2003).

Contamination with petroleum substances have been shown to select some bacteria species (Saadoun et al., 2008). The bacterial isolates selected by the presence of crude oil in this study were Bacillus, Micrococcus, Pseudomonas, and Klebsiella species respectively. These isolates were also isolated in previous studies in the Niger Delta region (Nseabasi & Antai, 2012; Ekpo and Ebeagwu, 2009).

Furthermore, the presence of these contaminants inhibit catabolic activities of bacteria (Saadoun et al., 2008). In a similar study by Vyas and Dave (2007) on the effect of crude oil concentrations,

temperature and pH on growth and degradation of crude oil by marine bacteria, the pH varied between 6 and 9. The pH values of the isolates used in this study ranged from 7.0 to 5.8 and these are within range for degradation of crude oil (Vidali, 2001). From an earlier study by Zekri and Chaalal (2005) on the effect of temperature on biodegradation of crude oil, temperature during degradation varied between 35 and 75°C. In our study, the temperature range was within 32 to 29.1oC.

Fresh oil spill especially in higher concentrations have been shown to kill soil micro biota thereby reducing counts and diversity while old spill have been shown to increase diversity and counts (Saadoun et al., 2008). This has been confirmed by several authors. Hassanshahian (2014) revealed a shift in microbial community index. Ekpo and Ebeagwu (2009) also recorded a marginal decrease in microbial counts following exposure to crude oil. This was also observed by Saadoun et al (2008) who revealed petroleum contamination reduced bacterial counts and diversity and a proliferation of the genus Pseudomonas species over others in freshly contaminated soil. In our study, bacterial growth was inversely proportional to crude oil concentration. At 5% and 10% of crude oil exposures, there was no tangible drop in counts. However, at 15 and 20%, there was a sharp decline in total HUB counts, indicating that crude oil is capable of causing quantitative and qualitative changes in the composition of soil microbes. In an earlier study, 34% exposure was found to be inhibitory to growth while at 40% degradation was completely stopped (Overholt et al., 2015).

In an earlier study, hydrocarbonoclastic isolates Proteus mirabilis, Bacillus cereus, Citrobacter amalonaticus and Enterobacter sp, were subjected to various diesel oil (1, 5, 10, 15 and 20%) concentration. Their finding indicates that even amongst the hydrocarbon utilizers, higher concentration of diesel reduced the number of viable cells especially 20%. In another study, alteration of enzymes kinetics, bacterial abundance, and other physiological changes (Nseabasi & Antai (2012) were observed for isolated bacterial and fungal species. Furthermore, they showed that higher concentrations of kerosene were toxic to their isolates. Consistently, Bacillus, Pseudomonas, Serratia, and Micrococcus are less affected by the concentration used in their study as they all had 200 CFU/ml across all the concentrations. Compared to our findings, the microbial counts were lower than their reported CFU/ml. Precisely, in our study at 5, 10, 15 and 20% the HUB counts ranged between 20 -61, 29-8, 38-10 and 38-2 CFU/ml. Similar decrease in counts were also obtained for diesel with HUB isolates in another study (Nkanang et al., 2018).

5 Conclusion

Although crude oil utilizing microorganisms have the capacity to degrade crude oil, nevertheless, HUBs have different crude oil tolerance abilities. Hence, microbes with better tolerance to crude oil effect as the HUB1 isolate can be employed in the process of bioremediation in the environment.

6 References

[1] Adipah S. (2019). Introduction of Petroleum Hydrocarbons Contaminants and its Human Effects, Journal of Environmental Science and Public Health, 3(1): 1-9.

[2] Ziolkowska, T. & Wyskowski, M (2010). Toxicity of petroleum substances to microorganisms and plants. Ecological chemistry and Engineering S, 17(1), 73-82.

[3] Edet, U. O., Antai, S. P., Brooks, A. A & Asitok, A. D. (2017). An overview of culture, molecular and metagenomics techniques in description of microbial diversity. Journal of Advances in Microbiology, 7(2): 1-19.

[4] Edet, U. O., Antai, S. P., Brooks, A. A & Asitok, A. D. (2018). Isolation of hydrocarbonoclastic bacteria and plasmid analysis for alk B gene primers. South Asia Journal in Microbiology, 1(3): 1-9.

[5] Udotong, I. R., Uko, M. P., Udotong, J. I. R. (2015). Microbial diversity of a remote aviation fuel contaminated sediment of a Lentic ecosystem in Ibeno, Nigeria. Journal of Environmental and Analytical Toxicology, 5(320): 1-7.

[6] Overholt, W. A., Marks, K. P., Romero, I. C., Hollander, D. J., Snell, T. W. & Kostka, J. E. (2016). Hydrocarbon-degrading bacteria exhibit a species-specific response to dispersed oil while moderating eco-toxicity. Applied Environmental Microbiology, 82, 518-527.

[7] Unimke, A. A., Mmuoegbulam, O. A., Anika, O. C. (2018). Microbial degradation of petroleum hydrocarbons: realities, challenges and prospects. Biotechnology Journal International, 22(2): 1-10.

[8] Udofia, U. U., Edet, U. O., & Antai, S. P. (2018). Potential benefits of applying "omics" technology in cleaning up crude oil spillages in the Niger Delta region of Nigeria. Journal of Advances in Microbiology, 15(2): 1-8.

[9] Onwurah, I. N. E., Ogugua, V. N., Onyike, N. B., Ochonogor, A. E., & Otitoju, O. F. (2007). Crude oil spills in the environment, effects and some innovative clean-up biotechnologies. Int. J. Environ. Res., 1(4): 307-320.

[10] Colwell, R. R. and Walker, J. D. (1977). Ecological aspects of microbial degradation of petroleum in the marine environment. Critical Reviews Microbiology, 35, 573-580.

[11] Kostka, J. E., Prakash, O., Overholt, W. A. Green, S. J., Freyer, G, Canion, A., Delgardio, J., Norton N., Hazen, T. C. and Huettel, M. (2011). Hydrocarbon-degrading bacteria and the bacterial community response in DSGulf of Mexico beach sands impacted by the deepwater horizon oil spill. Applied Environmental Microbiology, 77: 7962-7974.

[12] Mustafa, S., Al-Douseri, A., Majki K. & Al-Saleh, E. (2013).Potential of crude oil-degrading bacteria to co-resist heavy metals in soil. Transactions on Ecology and the Environment, 173: 1-9.

[13] Babalola, T. A., Sylvester Peter Antai, S. P. and Tiku D. R. (2016). Toxicity of Crude Oil and Diesel Fuel to Some Gram Positive and Gram Negative Bacteria. Int. J. Curr. Trend. Pharmacobiol. Med. Sci. 2016, 1(3): 25-34.

[14] Antai, S. P., Unimke, A. A. and Agbor, R. B. (2014). Assessment of the heterotrophic and crude oil utilizing microorganisms of Imo river estuary of the Niger delta mangrove ecosystem. American International Journal of Biology, 2(1): 29-42.

[15] Nseabasi, N. O. and Antai, S. P. (2012). Toxic effect of kerosene contamination on the survival of bacteria and fungi species in soil from Niger delta. International Research Journal of Microbiology, 3(12): 382-387.

[16] Unimke, A. A., Antai, S. P., Agbor, R. B., Nseabasi, N. O. and Agbo, B. E. (2014). Evaluation of seasonal variation in the microbial and heavy metal concentrations of Imo River Estuary of the Niger Delta Mangrove Ecosystem. Advanced Research in Agriculture and Veterinary Science, 2014; 1(2):88–94.

[17] Murínová, S. and Dercová, K. (2014). Response Mechanisms of Bacterial Degraders to Environmental Contaminants on the Level of Cell Walls and Cytoplasmic Membrane. International Journal of Microbiology, 87: 1-16.

[18] Timmerman. M. D., Fuller, L. G. and Burton, D. L. 2003. The effects of a crude oil spill on microbiological indices of soil biological quality. Can. J. Soil Sci. 83: 173–181

[19] Saadoun, I., Mohammad, M J., Hamed, K M., Shawaqfah, M. (2008). Microbial populations of crude oil spill polluted soils at the JORDAN-IRAQ DESERT (THE BADIA REGION). Brazilian Journal of Microbiology, 39, 453-456.

[20] Ekpo, M A. & Ebeagwu, C. J. (2009). The effect of crude oil on micro-organisms and dry matter of fluted pumpkin (Telfairia occidentalis). Scientific Research and Essay .4 (8), 733-739.
[21] Vyas, T. and Dave, B. P. (2007). Effect of crude oil concentrations, temperature and pH on growth and degradation of crude oil by marine bacteria. Indian Journal of Geo-Marine Sciences, 36(1): 76-85.
[22] Vidali, M. (2001). Bioremediation: An overview. Pure and Applied Chemistry, 73(7): 163-1172.
[23] Zekri, A. Y. and Chaalal, O. (2005). Effect of temperature on biodegradation of crude oil. Energy Sources, 27:1-2, 233-244.
[24] Hassanshahian, M (2014). The effects of crude oil on marine microbial communities in sediments from the Persian Gulf and the Caspian Sea: A microcosm experiment. International Journal of Advanced Biological and Biomedical Research, 2(1), 1-17.
[25] Nkanang, A. J., Antai, S. P., Asitok, A. D. & Maurice, E. (2018). Assessment of diesel oil toxicity on some hydrocarbonoclastic bacteria isolated from IKO river estuary in the NIGER DELTA. World journal of pharmaceutical and medical research, 4(5), 48-55.

Authors' details

Uwem Okon Edet[1, 2], Ogemdi Chinwendu Anika[1 3*] & Elizabeth Umoren[4]

[1]Department of Microbiology, Faculty of Biological Sciences, University of Calabar, PMB 1115, Calabar, Cross River State, Nigeria.

[2]Obong University, Faculty of Natural and Applied Sciences, Department of Microbiology, Obong Ntak, Etim Ekpo, Akwa Ibom State, Nigeria.

[3]Department of Microbiology, Faculty of Science, University of Abuja, PMB 117, Abuja, Nigeria.

[4]PAMO University of Medical Sciences, 1 Tap Road, Elelenwo, Port Harcourt, Rivers State, Nigeria.