BIO-CHEMICAL TRENDS IN ANAEROBIC BIODEGRADATION OF RAW AND TREATED HYDROCARBON POLLUTED WATER USING *ASPERGILLUS niger* AND *PSEUDOMONAS aeruginosa.*

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ABSTRACT:

The anaerobic bioremediation of raw and treated hydrocarbon polluted water samples using *Aspergillus niger* and *Pseudomonas aeruginosa* has been investigated. The rate of bioremediation of the two polluted water samples was nearly the same as it took 65 days for effective bioremediation to occur with both microbes. The overall bioremediation effects, however, of *Pseudomonas aeruginosa* (bacteria) on the Biological Oxygen Demand (BOD) and the Total Hydrocarbon Content (THC) were higher than that of *Aspergillus niger* (fungi). Furthermore, the treated crude polluted water (TCPW) sample was easier to biodegrade than the raw crude polluted water (RCPW) samples. The bioremediation of the TCPW samples gave 99.5% for BOD and 98.9% for THC using bacteria while for fungi; it gave 96.1% BOD and 95.4% for THC. For the RCPW samples bioremediation with bacteria gave 96.6% for BOD and 96.0% for THC while bioremediation using fungi gave87.1% for BOD and 86.5% for THC.

Index terms: Anaerobic, Bio-remediation, Pseudomonas aeruginosa, Aspergillus niger and polluted water

I. INTRODUCTION

Until recently, practical applications of in situ bioremediation have focused mostly on aerobic microorganisms which gain energy by oxidizing organic compounds to carbon dioxide with oxygen serving as the electron acceptor. However, this approach has had limited success, because oxygen, an absolute requirement for aerobes is scarce in almost all contaminated environments. The scarcity of oxygen in many contaminated sub surface environments has raised interest in the bioremediation potential of anaerobes. Anaerobic bacteria are present in soil and are a part of the normal flora of humans and other animals as well as the insects being investigated. This microbial life in the absence of oxygen is beginning to show significant potential for solving one of the important present day problems of environmental pollution and degradation. Normally the hydrocarbon contaminated soils or water lack oxygen. Bio-stimulants (urea, ammonia based fertilizers) are added sometimes and these can potentially exert an oxygen demand due to biological ammonia oxidation. Also mass transfer of oxygen may not be sufficient to replenish oxygen consumed by microbial metabolism. Under such conditions, anaerobic hydrocarbon degradation may be of relevance. [1-2, 3-4]

In quantitative terms, crude oil is one of the most important organic pollutants in marine environments and the current global rate of natural seepage of crude oil at 600,000 tonnes per year, with a range of 200,000 to 2,000,000 tonnes per year. [5] Methods for restoring oil-polluted sites vary from complete removal of the affected soil to doing nothing at all and "letting nature take its course".[11] Natural re-vegetation of an area affected by light crude oil spillage has occurred without any special treatment.[6-8] At low levels of contamination of crude oil, cultivation of soil without nutrient amendment is possible.[9] Physical methods such as incineration may destroy indigenous organisms, including oil-degrading microbes, and increase the toxicity of the petroleum residue. Sinking the oil with heavy hydrophobic agents such as ground chalk merely removes the oil to anaerobic sediments or deep ocean floor, where long persistence of the oil pollutant is bound to occur. Large quantities of oil accumulating on the bottom foul the ocean floor and also tend to coalesce and rise again as large droplets. Mechanical removal of stranded oil from sand dunes or salt marshes is far more damaging than leaving it alone: Not only is the ecological balance disturbed, but the aesthetic effect may also be irreparable.[10] Chemical methods for removing or dispersing spilled oil from the environment were condemned because of their side-effects on the ecosystem and their toxicity, which is sometimes more pronounced than that of the oil itself.[11] Chemical dispersants may inhibit microbial activity by damaging cell membranes or essential enzymes, or by altering the surface tension of the water in which microbes live. Furthermore, dispersed oil is never recovered from the environment, and its ultimate fate remains unknown. The key players in bioremediation are microorganisms that live virtually everywhere **[1-2]** They are ideally suited to the task of contaminant destruction because they possess enzymes that allow them to use environmental contaminants as food and because they are so small that they are able to contact contaminants easily. Indigenous populations of microbial bacteria can be stimulated through the addition of nutrients or other materials. Exogenous microbial populations can be introduced in the contaminated environment. **[6, 8, 1-2]** A few examples of micro-organisms that can biodegrade petroleum and aromatic rings are pseudomonas, proteus, bacillus, aspergillus etc. [12] Under ideal conditions, bacteria can reproduce rapidly, producing a new generation every 20 to 30 minutes. Thousands of different species of bacteria exist everywhere in our world, and most of them carry on bacterial digestion in some way. However, some of them are found only in a specific environment, require specialized types of food, or have very unique niches. Bacteria can be further separated into aerobic types, which require oxygen to live, and anaerobic, which can live without oxygen. **[1-3]** Facultative types can thrive under either aerobic or anaerobic conditions. Certain bacteria belonging to the *Bacillus* and *Pseudomonas* species have these desirable characteristics. They consume organic waste thousands of times faster than the types of bacteria that are naturally present in the waste. They grow and reproduce easily, are non-pathogenic, and do not produce foul odors or gas **[1-3]**. While fungi have the ability to mineralize, release and store various elements and ions and accumulate toxic materials**[1-3]** They have also shown the removal of metals and degradation and mineralization of phenols and other phenolic compound, petroleum hydrocarbons, polycyclic aromatic hydrocarbons, poly chlorinated biphenyls, chlorinated insecticides and pesticides and other substances in various matrices. Hence this comparative studies of the activities of *Pseudomonas aereginosa* (bacteria) *and Aspergillus niger* (fungi) in anaerobic bio-degradation of hydro-carbon polluted water.

SCOPE AND LIMITATIONS

The research was carried out in a laboratory setting where bioremediation process was simulated. Samples of hydrocarbon polluted water could not be obtained from polluted oil fields but were instead prepared in the laboratory as described in the methodology. These samples as prepared in the laboratory are simplified versions usually different in terms of complexities, compositions, concentrations, temperature and pH conditions, etc from real time situations. Oil spillages can occur both on land, flowing and stagnant water bodies with biodiversities. Millions of micro-organisms are associated with polluted sites that make it difficult to capture such real time conditions in any laboratory set-up.

II. MATERIALS AND METHODS

The raw and treated crude oil samples (escravoes light) used for this study were sourced from Shell Petroleum Development Company (SPDC) in Warri, Delta state Nigeria, October 2010. The fungi (*Aspergillus niger*) and bacteria (*Pseudomonas aeroginosa*) used for this study were cultured in the Microbiology Department of Covenant University using Nutrient Agar (NA) and Potato Dextrose Agar (PDA) respectively as feed

Sample Preparation: The crude oil polluted water samples were made by adding 300ml of the two crude oil samples respectively to 3000ml of water in the ratio of 1: 10. Three different samples were prepared for each type of crude and stored in three different black plastic containers until required. Before the experiment was started the crude oil polluted water samples were made to stand for 1 week to allow the indigenous microbes to grow and accustom to the medium. Then, 0.2M sodium nitrate was prepared by dissolving 56.1g of sodium nitrate in 3300ml (3.3L) litres of crude oil polluted water. The *Aspergillus niger* and the *Pseudomonas aeruginosa* were respectively inoculated into four containers, two each for the raw and treated crude oil polluted water and the last two kegs were left as control.

III. Analyses Description

1. Biological Oxygen Demand

Reagents used: Winkler's solution A, Winkler's solution B and Starch solution

Procedure

Two 250 ml reagent bottles were first filled up completely with the crude polluted water sample and stoppered tightly. To one of the bottles, 1.5 ml each of Winkler's Solution A and B were added, and precipitant was formed. The precipitant was dissolved with 2 ml of concentrated sulphuric acid to form a golden brown solution. 50 ml of the resulting solution was poured into 250 ml conical flask and 3 drops of starch indicator were added and titrated against 0.2 M Sodium thiosulphate ($Na_2S_2O_3$) with initial blue black coloration and the volume of 0.2 M ($Na_2S_2O_3$) solution used was recorded and after titrating, it turned colorless detecting the endpoint. The second bottle was covered with black cellophane bag to prevent the penetration of light and then left at room temperature ($29^0C - 30^0C$) for 5 days. At the end of 5 days, the above procedure was repeated for the contents of the second bottle and the volume of 0.1N $Na_2S_2O_3$ used was recorded. This was done for the two raw crude polluted water samples, the two treated crude polluted water samples containing the two microbes respectively and the two control samples.

The BOD of the sample was calculated as follows:

$BOD_5 = DO_0 - DO_5$

Where;

DO_0 = Dissolved oxygen concentration at zero time

DO_5 = Dissolved oxygen concentration after 5 days incubation period
The above procedures were repeated for other samples

2. Total Hydrocarbon Content

The oil content of the polluted water was determined by shaking 5 g of a crude oil-water sample with 10 ml of toluene. The two phases formed (water phase and crude oil-toluene phase) were separated by using a 250ml separating funnel. A sample of the crude oil-toluene phase is poured in a glass cuvette and the absorbance was checked using a spectrophotometer at wavelength 420 nm. A standard curve of the absorbance of different known concentrations of crude oil in the toluene phase was first drawn. After taking readings from the spectrophotometer, oil concentrations in the polluted water sample were then calculated after reading the concentration of the oil in the extract from the standard curve. With reference to the standard curve, the hydrocarbon content of the oil was calculated by interpolating values on the concentration-absorbance curve.

IV. RESULTS AND DISCUSSION OF RESULTS

Table 3.1: Biological Oxygen Demand for Anaerobic Bioremediation Treated Crude Polluted Water.

	TREATED CRUDE		
Days	**fungi**	**bacteria**	**Control**
0	1638.7	1638.7	1638.7
5	1589.3	1501.6	1611.3
10	1237.7	1077.6	1553.2
15	1052.1	848.7	1409.3
20	928.3	719.5	1007.3
25	811.7	497.3	933.8
30	732.6	266.1	816.9
35	518.2	107.9	748.7
40	382.6	83.9	653
45	278.1	67.7	570
50	166.5	39.3	421.6
55	105.3	25.8	389.1
60	88.6	17.2	215.8
65	67.1	8.9	136.5

Table 3.2: Biological Oxygen Demand for Anaerobic Bioremediation Raw Crude Polluted Water.

	RAW CRUDE		
Days	**fungi**	**bacteria**	**Control**
0	1882.7	1882.7	1882.7
5	1809.3	1762.7	1817.6
10	1743.3	1699.4	1787.6
15	1684.7	1512.3	1725.8
20	1609.1	1311.7	1652.2
25	1469.8	1101	1593.8
30	1300.7	986.2	1413.9
35	1117.6	821.7	1292.8
40	973.8	687.3	1017.6
45	809.3	435.9	923.7
50	692.6	299.7	793.1
55	413.9	173.9	614.4
60	315.8	92.5	501.7
65	243.6	64.7	470.2

Table 3.3: Total Hydrocarbon Content for Anaerobic bioremediation of Treated Crude Polluted Water.

	TREATED CRUDE		
Days	**fungi**	**bacteria**	**Control**
0	8634.8	8634.8	8634.8
5	8558.8	8034.8	8453.4
10	8318.6	6111.1	8387.3
15	8223	3814.1	8169.2
20	6833.3	3666.6	8088.2
25	3809.6	3474.4	8002.5
30	3610.7	3145.9	7187.5
35	3264.4	2311.3	5339.1
40	2695.1	1634.9	3655.4
45	1333.8	988.8	3420.8
50	963.2	625.2	2883.9
55	887.1	416.4	1413
60	732.2	243.6	819.7
65	394.8	93	706.4

Table 3.4: Total Hydrocarbon Content for Anaerobic bioremediation of Raw Crude Polluted Water.

	RAW CRUDE		
Days	**Fungi**	**bacteria**	**control**
0	9218.3	9218.3	9218.3
5	8959.4	8322.4	8796.8
10	8504.1	8004.5	8711.4
15	7326	6995.3	8516.3
20	6837.9	6092.4	8138.2
25	5517.3	5148	7343.5
30	5048.8	3836.2	7295.4
35	3642.6	3022.7	7146.6
40	3249.8	2673.8	6862.7
45	2843.7	1706.2	4424.1
50	2560.3	1549.1	3894.1
55	2259.5	1277.3	2982.7
60	1973.2	895.43	2565.7
65	1248.6	372.19	1836.2

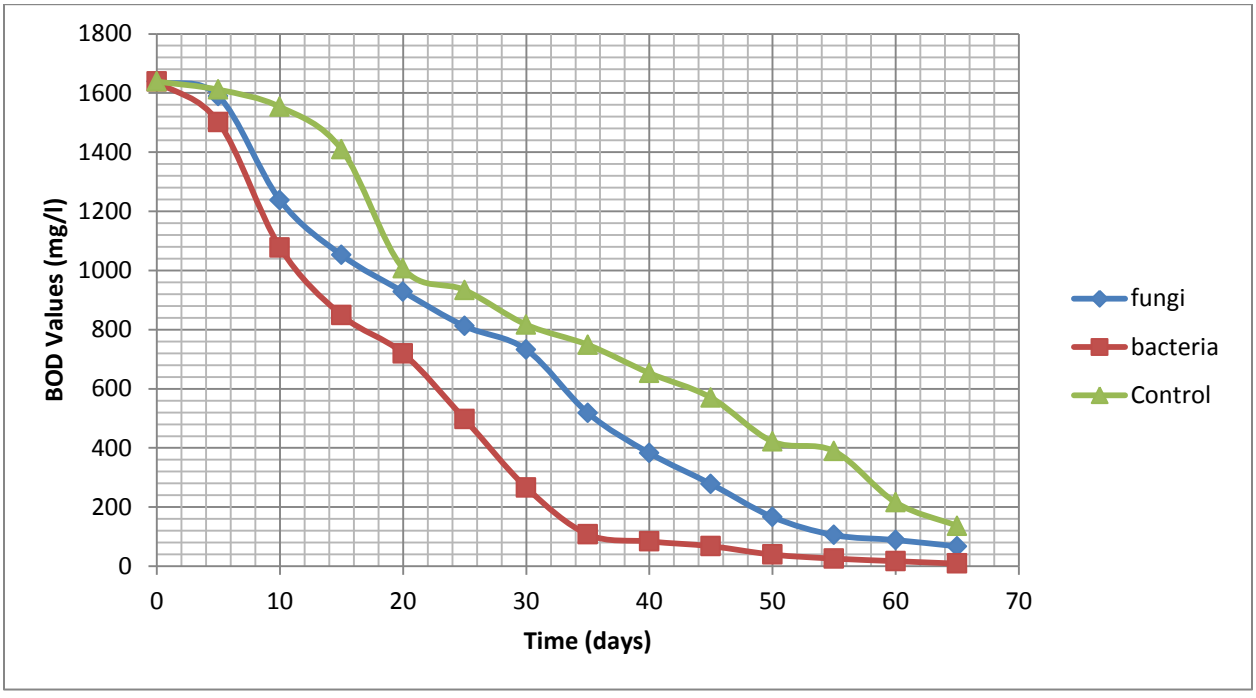

Fig. 3.1 Biological Oxygen Demand for Anaerobic Biodegradation of Treated Crude Polluted Water Sample

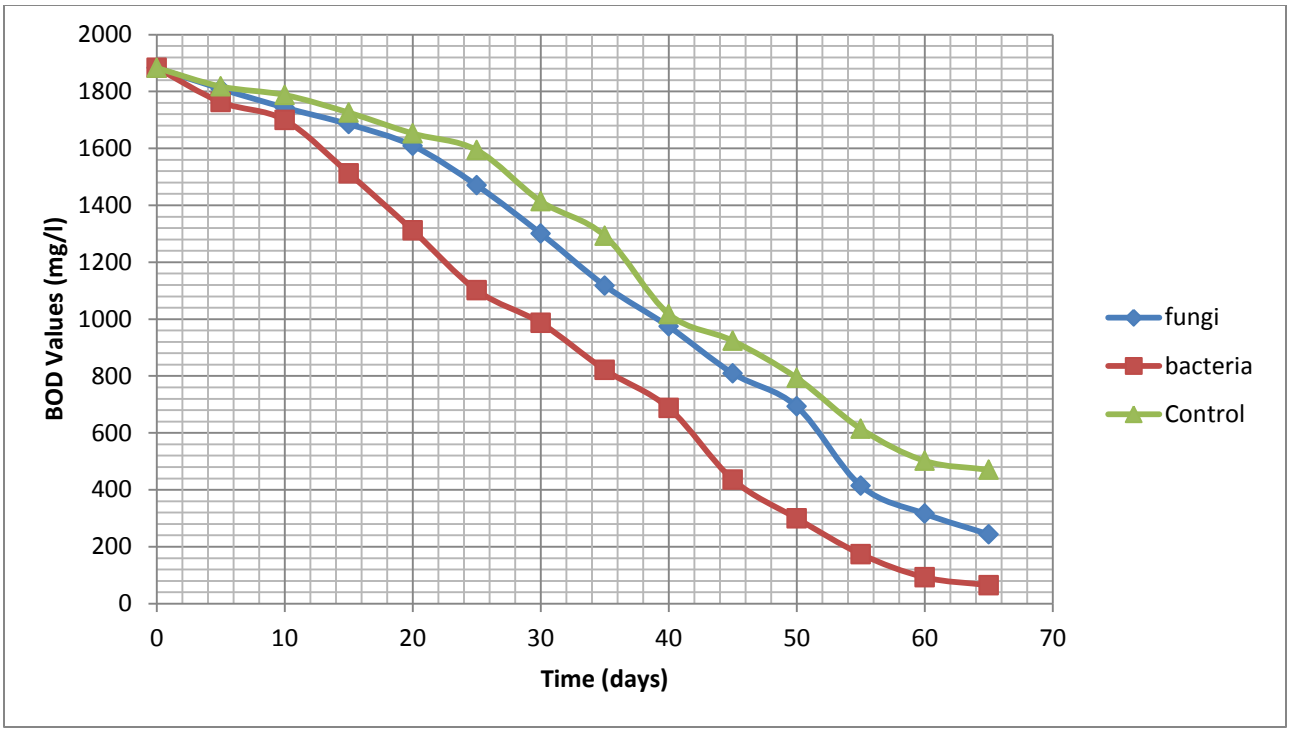

Fig. 3.2: Biological Oxygen Demand for Anaerobic Biodegradation of Raw Crude Polluted Water Sample.

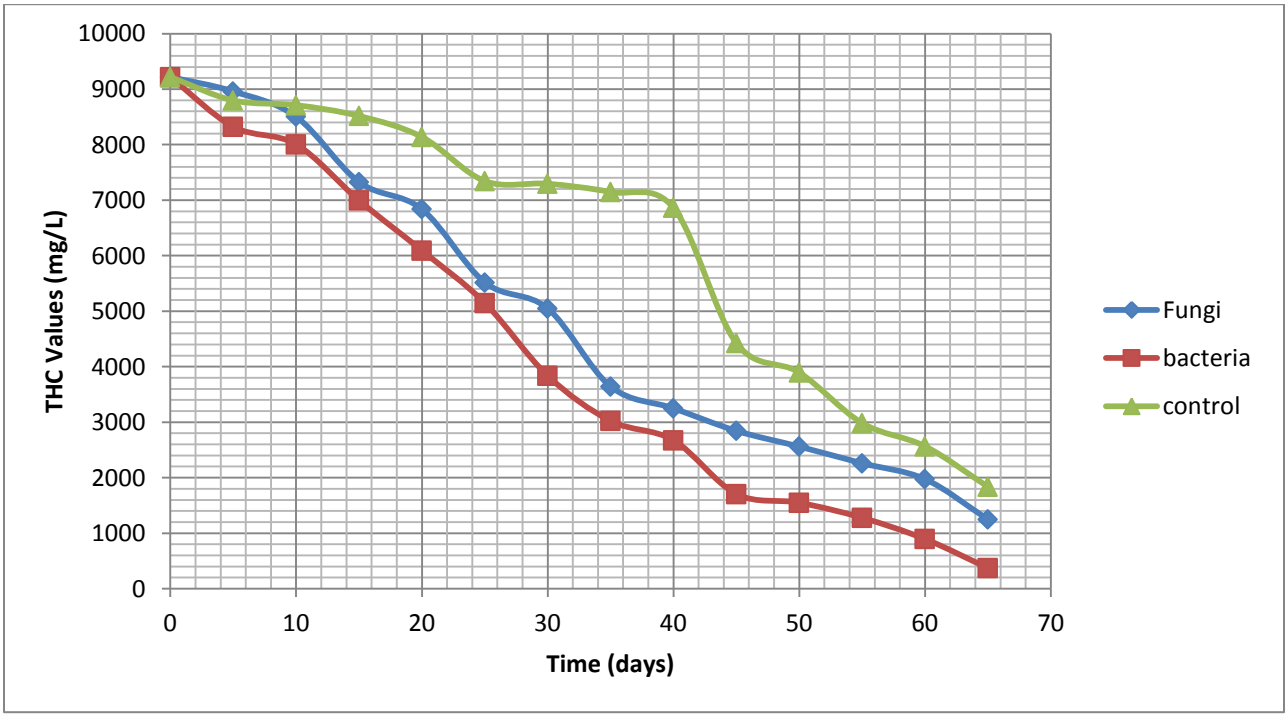

Fig. 3.3 **Total Hydrocarbon Content for Anaerobic Bioremediation of Raw Crude Polluted Water Sample.**

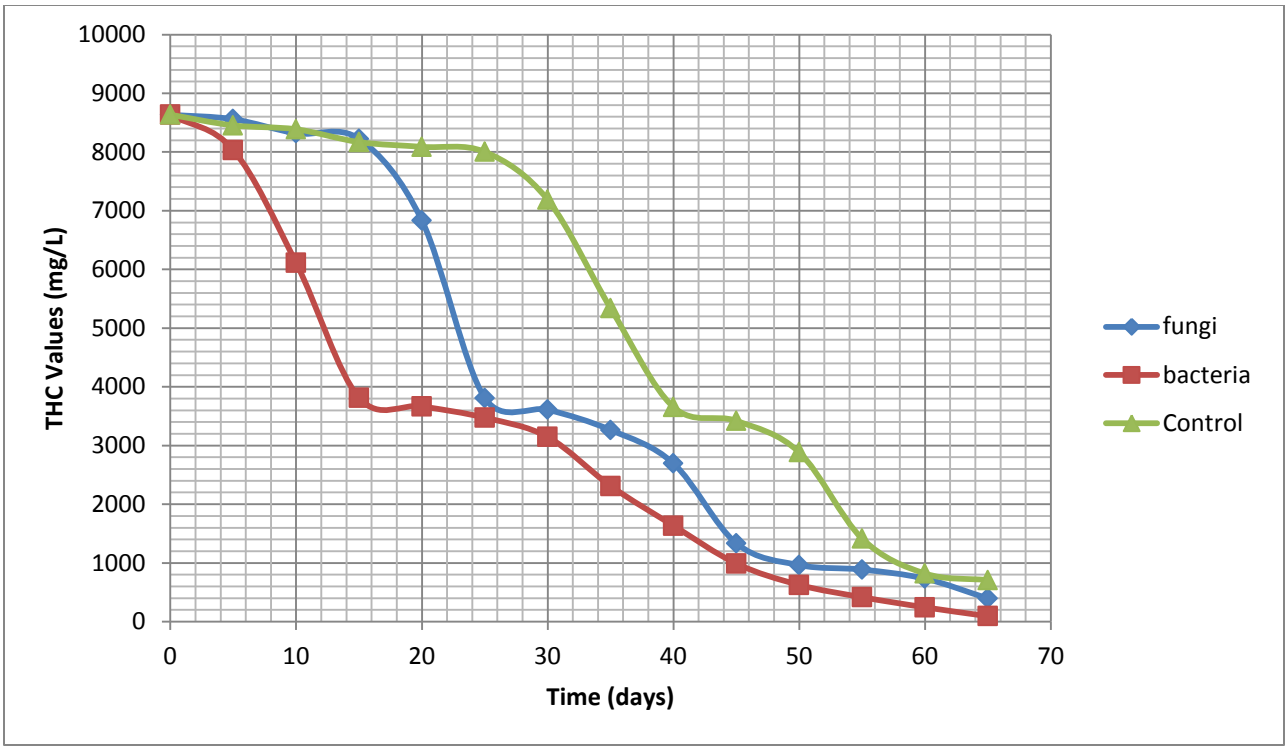

Fig. 3.4 **Total Hydrocarbon Content for Anaerobic Bioremediation of Treated Crude Polluted Water Sample**

Figs.3.1 to 3.4, depict anaerobic bioremediation of both raw and treated crudes using two different micro-organisms, *Pseudomonas aeroginosa* (bacteria) and *Aspergillus niger* (fumgi). The results showed generally that it took a maximum of sixty five (65) days for significant Bio-remediation to occur. The *Pseudomonas aeriginosa* (bacteria sample) was seen to biodegrade the hydrocarbon in both the raw and treated crudes faster than the *Aspergillus niger* (fungi sample) and the control sample. Generally too, the treated crude was easier to biodegrade than the raw crude with the two microbes. For the treated crude polluted water samples, the BOD values of the sample with bacteria fell from 1638.7mg/l to 8.9mg/l (99.5%), the sample with fungi fell from 1638.7mg/l to 64.7mg/l (96.1%) and the control sample recorded 91.7% bioremediation. For the raw crude, the BOD values of sample with bacteria fell from 1882.7mg/l to 64.7mg/l (96.6%), the sample with fungi fell from 1882.7mg/l to 243.6mg/l (87.1%) while the control sample had only 75% bioremediation. Similar trends were also observed for the THC values of both the treated and raw crude oil polluted water samples with the two microbes. For the treated crude polluted water samples, the THC values of the sample with bacteria fell from 8634.8mg/l to 93mg/l (98.9%), the sample with fungi fell from 8634.8mg/l to 394.8mg/l (95.4%) and the control sample recorded 91.8% bioremediation. For the raw crude, the THC values of sample with bacteria fell from 9218.3mg/l to 372.2mg/l (96.0%), the sample with fungi fell from 9218.3mg/l to 1248.6mg/l (86.5%) while the control sample had only 80% bioremediation. The set up was controlled to eliminate air and the nutrient- sodium nitrate used was not sufficient by itself for increased rate of biodegradation of the polluted water sample. Hence the longer bioremediation time of more than 60 days. The lower curves in the all the figures indicate that the bacteria have faster growth and biodegradation rates than fungi. Tables 3.1 to 3.4 are also indicative of this,

where both BOD and THC values for each period of measurements are much lower for samples with bacteria than those of fungi. This does confirm the fact that *Pseudomonas aeruginosa* thrives well under anaerobic conditions than *Aspergillus niger*. Figs3.1 to3.4 and Tables 3.1 to 3.4 much lower values of both BOD and THC were recorded (higher bioremediation rate) for the treated crude polluted water than for the raw crude polluted water sample. This is so because the raw crude oil polluted sample contains not only hydrocarbons which serve as energy and food for the microorganisms but also other elements like nickel, copper, vanadium and iron which are not favorable to the growth of the microorganisms. The presence of these other elements slow down the activity of the microorganism hence the growth rate is reduced and their demand for oxygen is reduced. This leads to the slow fall in the BODs of the raw crude oil polluted water. The treated crude oil polluted water which contains basically hydrogen and carbon has a higher demand for oxygen and thus their BOD and THC values fall faster. As the THC values reduce the hydrocarbon content of the polluted sample also reduces. This is as a result of the release of enzymes by the microorganisms which feed on the hydrocarbons and thereby converting them to less toxic substances such as CO_2 and H_2O.

V. CONCLUSION

From the results gotten from this research work, we can conclude that:

Pseudomonas aeruginosa is a better microbe for anaerobic bioremediation than *Apergillus nigger*. The *Pseudomonas aeruginosa* biodegrades the hydrocarbon in both the raw and treated crude polluted water samples faster than the *Aspergillus niger*. It is easier to biodegrade treated crude oil polluted water than raw crude oil polluted water. The presence of heavy metals in the raw crude polluted water samples slows down the activity of the microorganisms used hence the growth rate is reduced and their demand for oxygen is reduced. Anaerobic bioremediation as carried out in this research work was successful and hence could be applied in practical terms to combat environmental pollution challenges since hydrocarbon polluted sites are usually oxygen lean. Its development, therefore, shall in no small way help to alleviate challenges arising from oil spillages. More research work should however, be focused on:

(i) finding more effective anaerobic micro-organisms that could bioremediate hydrocarbon polluted sites at relatively shorter intervals of times than those considered here.

(ii) extensive research on the combined effect of bioremediation conditions (temperature, bacteria load, nutrients, pH etc) for optimal bioremediation should be done.

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