ORIGINAL RESEARCH ARTICLE

ASSESSMENT OF BACTERIAL COMMUNITIES IN A HEAVY METAL CONTAMINATED UNDERGROUND WATER IN IKWO SALT MINING SITE

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Abstract

Heavy metal contamination of underground water due to natural and anthropogenic sources is a global environmental concern. Microbial remediation of a hydrocarbon-contaminated site can be accomplished with the help of a diverse group of microorganisms. The objectives of this project were to determine the bacterial diversity in a heavy metal contaminated region of Enyigba, Ebonyi state. Four samples were collected from the study area (Royal Mining Salt, Enyigba) and a control from Alex Ekwueme Federal University Ndufu-Alike, Ebonyi state in triplicates. The probable tolerant genera were identified using Enterotube which was based on biochemical reactions. The physico-chemical parameters were analyzed and the different concentrations of heavy metals (Zinc, lead, Cadmium, Chromium and Arsenic) were determined using AAS (Atomic Absorption Spectrometer) and its corresponding effects on bacterial communities were determined using the statistical package IBM SPSS Version 22.0. The dominant genera isolated were *Pseudomonas spp, Acinetobacter spp, Vibrio spp,* and *Enterobacter spp* in descending order of abundance. The most tolerant genera were subsequently utilized by examining their potential to degrade hydrocarbon. This was achieved by checking the abilities of the isolates to produce biosurfactant and calculating the emulsification index. *Pseudomonas spp* (86%) was found to have the highest index, followed by *Acinetobacter spp* (67%). The presence of Sulphate reducing bacteria (SRB) and Acid producing bacteria (APB) were confirmed using API RP-38 and ZPRA-5 test broth respectively. Their presence was attributed to the high concentration of sulphate. This study summarizes the potentials of microbes in hydrocarbon degradation.

Biological; Microbiology: Key Words: *Heavy metals, Bacteria, Hydrocarbon, Biosurfactant*

1.0 INTRODUCTION

Water, soils and sediments contamination with heavy metals is an unfortunate by-product of industrialization, and has become a global ecological issue. The release of high volume of heavy metals as a result of agricultural and industrial activities has led to the deterioration of the soil and water, threatened the depending fauna and flora and also human health through the food chain (Ansari and Malik, 2007). Contamination of underground water by heavy metals is often associated with mining activities and the corresponding processing of ores. Dissolved form of heavy metals enters the surface water and in association with substances washed off the ground, they migrate over long distances (Frankowski *et al*., 2009). According to Theodore (1997), heavy metals refer to Lead (Pb), Mercury (Hg), Iron (Fe), Copper (Cu), Manganese (Mn), Cadmium (Cd), Arsenic (As), Nickel (Ni), Aluminum (Al), Silver (Ag), and Barium (Ba). Metals are known to be associated with crude oil in pyrrolic structures known as porphyrins. The ability of some microorganisms to tolerate heavy metals and the ability of some to promote transformations that render them less toxic, make organisms that live in heavy metal contaminated sites potentially useful in bioremediation. Bioremediation is of paramount importance to Oil and Gas sector as it plays a pertinent role in oil spill cleanup and degradation of harmful components of crude oil. As noted by Claude E. ZoBell in 1946, many microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and energy. Microbial biodegradation of pollutants has increased in current years as mankind attempts to find viable means to clean up contaminated environments (Diaz, 2008). Biodegradation of hydrocarbons by natural populations of microorganisms represent one of the primary mechanism by which petroleum and other hydrocarbon pollutants are eliminated from the environment. Microbial remediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms. These microorganisms can degrade a wide range of target constituents present in oily sludge (Eriksson *et al*., 1999; Barathi and Vasudevan, 2001).

Zengler et al., (1999) recorded that bacteria can degrade petroleum under both aerobic and anaerobic conditions. Many hydrocarbon-degrading microorganisms produce extracellular emulsifying agents. In some cases, growth on hydrocarbon induces emulsifier production (Hisatsuka *et al*., 1971). Hence, microbial remediation of oil polluted sites has helped beyond measures in restoring sanity to the biosphere and ecosystem at large.

The purpose of this study was to isolate and characterize the cultivable bacterial populations present in the contaminated study area. The search for bacteria populations adapted to metal-stressful conditions may be a good approach to find species or strains having the capacity to tolerate or accumulate metals and with potential applications for bioremediation strategies. For this reason, this study focused on the isolation and identification of microorganisms with the ability to eliminate these pollutants.

2.0 MATERIALS AND METHODS

2.1 Study Area

The sample was collected from a heavy metal polluted site located in Enyigba LGA, Ebonyi state. The area is bounded by Latitude 6º 10/ N - 6º 13/ N and Longitude 8º 07/ E - 8º 10/ E and covers a surface area of 33.06km^2. It is about 14 kilometers south of Abakaliki, the capital city of Ebonyi State, in the southeastern part of Nigeria. The pit was about 200 feet and the sample was collected with the help of the site operators. A control sample was collected from a machine drilled well at Alex Ekwueme Federal University Ndufu Alike Ikwo, Ebonyi State.

2.2 Sample Collection

Four water samples were collected from the study area in triplicates. A control sample was also collected from Alex Ekwueme Federal University Ndufu-Alike Ikwo, Ebonyi State using 500ml plastic containers, filled to the brim and tightly corked. The containers were rinsed three times with the water sample before collection. Water samples for bacterial analysis were then transported to the Research Laboratory, Alex Ekwueme Federal University Ndufu-Alike, Ikwo at 4 ºC and were assayed within 24 hours of sampling.

2.3 Sterilization and Aspetic Techniques

All glass wares used such as the conical flasks, test tubes, beakers, glass slides, McCartney bottles and petri dishes were thoroughly washed using detergents, rinsed in clean water and allowed to dry properly. The inoculating loops were sterilized by flaming over a spirit lamp until red hot and then allowed to cool before use. All growth media and diluents used were sterilized in an autoclave at a temperature of 121°C for 15 minutes and pressure of 15ppi. The work bench was cleaned using 70% alcohol before and after each analysis.

2.4 Water Analysis.

The pH of the water samples were measured using a surface-testing Sentix® Sur pH electrode. All assays were conducted in triplicates. Metal analysis were performed by a certified analytical company (Home Water Research Laboratory, Abakaliki, Ebonyi State) using standard atomic absorption spectrometry techniques described by Pratt and was measured calorimetrically (Hesse, 1971).

2.5 Microbiology Methods

2.5.1 Use of SRB Test Kit

Gas detection tubes were used to sample gas production. API RP-38 and ZPRA-5 broth media were used to analyze the presence of SRB (Sulphate reducing bacteria) and APB (Acid producing bacteria) respectively in samples. This was done by injecting 1ml of the water sample immediately after sample collection using sterile syringes. It was left for 7 days and color changes were recorded.

2.5.2 Preparation of Water Samples

For the isolation and enumeration of the culturable bacterial population, ten-fold serial dilutions of the samples were made. Diluted samples were plated onto general purpose media (Nutrient Agar) at 37°C, for 24 hours and the colonies were monitored after 24 hours.

Colony forming units (CFU) per milliliter (ml) was calculated using the formula:
Mean Count = (Dilution used × number of colony/amount plated [ml])

2.5.3 Media Preparation

The media for the isolation were selected for their ability to support the growth of a wide variety of the microbial populations and for their selective isolation of certain microbial species.

Nutrient media was prepared according to the manufacturer's instruction by dissolving 14g of nutrient agar into 500 ml of distilled water which was then sterilized by autoclaving at temperature of 121°C for 15 minutes. The medium was then dispensed into plates (labelled W1-10^{-3}, W1-10^{-4}, W1-10^{-5}, W2-10^{-3}, W2-10^{-4}, W2-$10^{-5,}$ W3-10^{-3}, W3-10^{-4}, W3-10^{-5}, W4-10^{-3}, W4-10^{-4}, W4-10^{-5}, W5-10^{-3}, W5-10^{-4}, W5-10^{-5}) and then allowed to cool and solidify. 0.1ml from the dilutions ($10^{-3,}$ $10^{-4,}$ and 10^{-5}) was plated onto the sterile nutrient medium. The plates were inverted and incubated at 37°C for 24 hours.

Minimal salt Agar (MSA) was prepared according to Mills *et al* (1978) with the following composition [in (g/l)]: NaCl (10), MgSO4.7H2O (0.42), KCl (0.29), KH2PO4 (0.83), Na2HPO4 (1.25), NaNO3 (0.42) in 1000ml of distilled water. It was autoclaved at a temperature of 121°C for 15 minutes and the sterile medium was poured into Petri dishes (labelled W1-10^{0}, W1-10^{-1}, W2-10^{0}, W2-10^{-1}, W3-10^{0}, W3-$10^{-1,}$ W4-10^{0}, W4-10^{-1}, W5-10^{0}, and W5-10^{-1}) and allowed to gel. 0.1ml from the stock samples and the dilutions (10^{-1}) were then plated onto the sterile mineral salt medium (Mills *et al* 1978). A sterile filter paper was placed on each of the petri dishes' cover and 3 drops of crude oil was dropped on each of the filter paper using pipette. The petri dishes were covered, wrapped properly with masking tapes to prevent penetration of any other carbon source and incubated in an inverted position at 37°C for 4 days.

2.5.4 Bacterial Isolation and Storage of the Bacterial Strains

Bacterial isolates were enumerated daily in the different cultivation media. From the 4th day, single colonies were selected based on their different morphologies and colors. All strains were then streaked using sterile loop to NA in petri dishes. All isolated strains were purified by subculturing and pure cultures collected using 10µl sterile loops and were stored in Mccartney bottles containing nutrient agar.

2.6 Characterization of the Bacterial Isolates Recovered Enterotube Test

BBL Enterotube manufactured by Becton Dickinson Laboratories, D-69129, Heidelberg, Germany was used. Oxidase test were run on the pure isolates to differentiate oxidase positive from oxidase negative microorganisms. Oxidase positive organisms were inoculated into enterotube 1 and oxidase negative into enterotube 11. It was incubated for 24hours at 37°C and the results recorded.

2.7 Screening for the Ability of the Isolates to Produce Biosurfactants

After 7 days of incubation, about 10mls of the samples were taken to calculate the emulsification index of the bacterial isolates. The emulsification test was carried out as described by Balogun and Fagada (2010). 3ml of crude oil was added to 3ml of culture supernatant and vortex for 2 minutes. It was then allowed to stand for 24 hours. The emulsification index (E_{24}) was determined using the formula:

Emulsification index (E_{24}) = $\frac{\text{Height of emulsification layer (cm)}}{\text{Total height of the liquid column (cm)}} \times 100$

2.8 Data Analysis

The data obtained from this research was analyzed using the statistical package IBM SPSS version 22.0.

3.0 RESULTS

3.1 Physico-chemical Analysis

The average temperature, pH, and sulphate level of the samples used in this study are shown in Table 3.1. The values were relatively similar because the samples were collected from the same site except for the control which was gotten from a non-contaminated site. The average value of the analyzed heavy metals showed relatively high concentrations when compared to the control as represented in table 3.2. Zinc was shown to have the highest concentration in comparison with other heavy metals in the analyzed sample.

Table 3.1: Analysis of the Physico-Chemical Parameters.

LOCATION	TEMPERATURE (^{0}C)	pH (@ 25°C)	SULPHATE (mg/l)
W1	29.00 ± 0.46^{b}	5.93 ± 0.03^{c}	67.87 ± 0.07^{a}
W2	26.67 ± 0.09^{d}	6.69 ± 0.01^{a}	57.93 ± 0.07^{c}
W3	27.00 ± 0.058^{d}	6.76 ± 0.01^{a}	67.67 ± 0.17^{b}
W4	29.93 ± 0.06^{a}	6.20 ± 0.00^{b}	68.00 ± 0.00^{a}
CONTROL	27.87 ± 0.07^{c}	6.03 ± 0.09^{c}	1.03 ± 0.03^{d}

Values are Mean ± standard error of triplicate determination. Means in the same column with different alphabets as superscripts are significantly different ($P<0.05$).

Keys:

W1 = Well 1

W2 = Well 2
W3 = Well 3
W4 = Well 4

Table 3.2: Heavy Metal Analysis
The concentrations of heavy metals present in Royal Salt mining, Enyigba

LOCATION	ZINC (ppm)	LEAD (ppm)	CHROMIUM (ppm)	ARSENIC (ppm)	CADMIUM (ppm)
W1	0.05 ± 0.00^{d}	0.02 ± 0.00^{c}	0.00 ± 0.00^{d}	0.04 ± 0.00^{b}	0.02 ± 0.00^{c}
W2	0.09 ± 0.00^{c}	0.03 ± 0.00^{c}	0.01 ± 0.00^{c}	0.01 ± 0.00^{c}	0.04 ± 0.00^{b}
W3	0.23 ± 0.00^{b}	0.03 ± 0.00^{b}	0.01 ± 0.00^{b}	0.05 ± 0.00^{a}	0.04 ± 0.00^{b}
W4	0.29 ± 0.29^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}	0.01 ± 0.00^{c}	0.05 ± 0.00^{a}
CONTROL	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}

Values are Mean ± Standard error of triplicate determinations. Means in the same column with different superscripts are significantly different ($P<0.005$).

3.3 Sulphate Reducing Bacteria (SRB) and Acid Producing Bacteria (APB) Test

The API RP-38 test broth tested positive for W1, W2, W3, and W4, signifying the presence of SRB in the study area. Also, the ZPRA-5 test broth for APB tested positive, signifying the presence of APB in the study area. However, the control sample tested negative for both APB and SRB.

Table 3.3: Color Changes Obtained in the Test Broth.

SAMPLES	API RP-38 BROTH MEDIA (SRB)	ZPRA-5 BROTH MEDIA (APB)	RESULTS
W1	Black	Orange	+
W2	Black	Orange	+
W3	Black	Orange	+
W4	Black	Orange	+
CONTROL 1	Colorless	Colorless	-

KEY: + = positive
- = negative

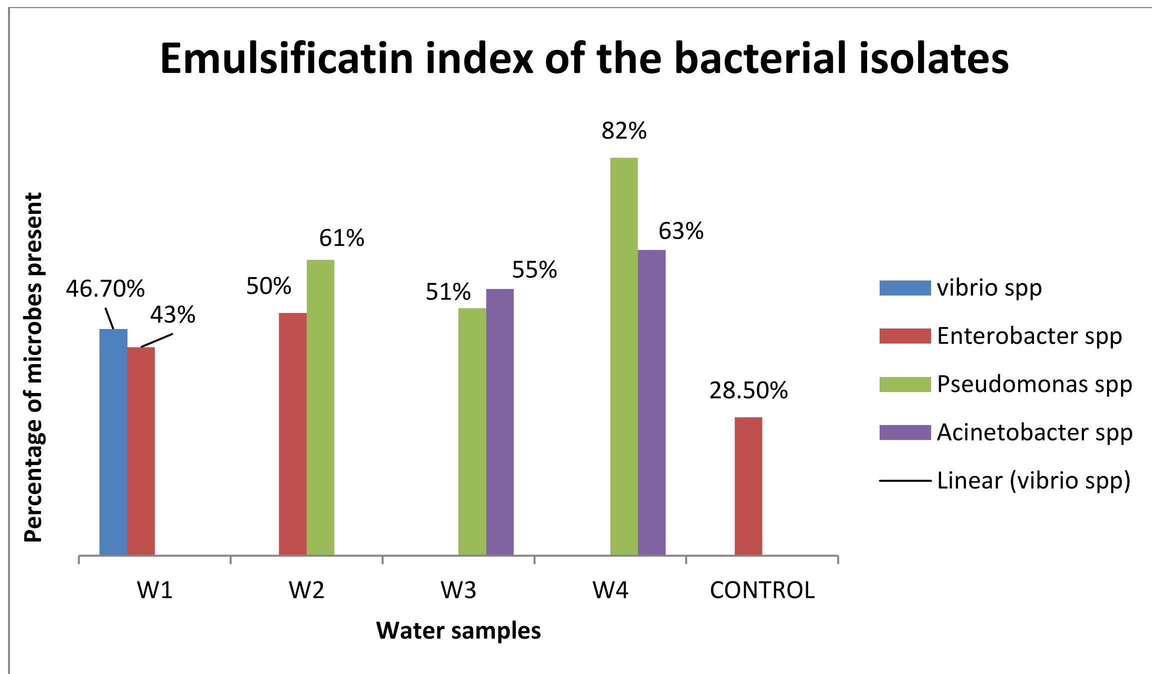
3.4 Microbial Count on Nutrient Agar

The mean aerobic count of the microbes in the fifth dilution of the samples was done and this was shown to be lower than the control. The average count for W1, W2, and W3 were relatively equal, having an average microbial count of 2.0×10^6 CFU/ml. W4 had a lesser load of 1.0×10^6. The control however had the highest count of 2.3×10^7.

3.5 Emulsification index (E_{24})

Pseudomonas spp had the highest emulsification index, while *Enterobacter spp* had the least value as shown in figure 3.2.

Figure 3.1: Biosurfactant potential of the isolates represented as Emulsification Index



3.6 Identification of the Bacteria Isolates Using Enterotubes

Four different species of bacteria was identified in the samples. *vibrio spp*., and *Enterobacter spp*., was the dominant specie in W1. W2 contained *pseudomonas spp*., and *Enterobacter spp*. W3 and W4 contained *Acinetobacter spp., and pseudomonas spp.* The control sample was found to contain *Enterobacter spp.*

Table 3.5: Characterization of microorganisms using enterotube

CODE	CAT	OXI	VP	UREA	GLU	ARA	H2S	LAC	D-rib	INO	D-xyl	SOB	PROBABLE ORGANISM
W1 (1)	-	+	+	-	+	+	+	-	-	-	-	-	*Vibrio* spp
(2)	+	-	-	-	+	-	+	-	-	-	-	+	*Enterobacter* spp
(3)	-	+	+	-	+	+	+	-	-	-	-	-	*Vibrio* spp
W2 (1)	+	+	-	-	+	-	-	-	+	-	+	-	*Pseudomonas* spp
(2)	+	-	-	-	+	-	+	+	-	-	-	+	*Enterobacter* spp
(3)	+	+	-	-	+	-	-	-	+	-	+	-	*Pseudomonas* spp
W3 (1)	+	+	-	-	+	-	-	-	+	-	+	-	*Pseudomonas* spp
(2)	+	+	-	-	+	-	-	-	+	-	+	-	*Pseudomonas* spp
(3)	+	-	-	-	+	-	-	-	-	-	-	-	*Acinetobacter* spp
W4 (1)	+	+	-	-	+	-	-	-	+	-	+	-	*Pseudomonas* spp
(2)	+	-	-	-	+	-	-	-	-	-	-	-	*Acinetobacter* spp
(3)	+	+	-	-	+	-	+	-	+	-	+	-	*Pseudomonas* spp
CONT (1)	+	-	-	-	+	-	+	+	-	-	-	+	*Enterobacter* spp
(2)	+	-	-	-	+	-	+	+	-	-	-	+	*Enterobacter* spp

Key: + = present
- = absent

4.0 DISCUSSION

This study offers a description of underground water bacterial composition in the mining area of Enyigba, Ebonyi state.

The physico-chemical analysis carried out in underground water from Royal Salt mining, Enyigba showed that the dominant temperature ranges from 26.7- 29.0^{0}C, pH 5.9-6.8, and the sulphate level, 57.9-68.0mg/l owing to the discharge of sulphates from mines. However, the control sample from Alex Ekwueme Federal University Ndufu Alike Ikwo, Ebonyi State has low sulphate level (1.03mg/l).

Figure 1 shows the color changes obtained in the SRB test kit signifying the presence of Sulphate Reducing Bacteria (SRB) and Acid Producing Bacteria (APB) which are black and orange respectively. However, the control showed no color change signifying the absence of SRB and APB.

From the identification tests carried out using enterotubes, *Pseudomonas* spp, *Acinetobacter* spp, *Vibrio* spp and *Enterobacter* spp were suspected as shown in Table 5. This has shown that the diversity is narrow in terms of genus suggesting that toxicity in this area has influenced the bacterial community. Interestingly, members of the *Pseudomonas*, and *Acinetobacter* groups are typically resistant to metals, (Cu, Ni, Cd, and Zn) (Dhalkephalkar and Chopade, 1994; Brim *et al*., 1999; Boswell *et al*., 2001; Abou-Shanab *et al*., 2007; Ansari and Malik, 2007), suggesting that the abundance of toxic heavy metals in the contaminated region of Enyigba may have selected for these microorganisms. On "well 4", the least number of bacteria were recovered, which could be attributed to stress. This is in accordance to Giller *et al*., (1998) findings.

From the emulsification test (E_{24}) carried out, *Pseudomonas* spp was found to have the highest emulsification index owing to its high production of biosurfactant followed by *Acinetobacter* spp, and, *Enterobacter* spp, then, *Vibrio* spp as shown in figure 2 above. The microbial community analysis in conjunction with potential rates of microbial activity suggests that these groups have a high potential for bioremediation and should be explored further. This is further shown in the work of Zettler *et al*., 2002; Baker and Banfield, (2003), where prokaryotes and eukaryotes were found in heavy metal contaminated sites and their ability to tolerate heavy metals and for some to promote transformations that render them less toxic, makes these organisms potentially useful in bioremediation.

Microbial population and diversity can be altered by heavy metals. Heavy metals are likely to affect the microbial populations in an environment by reducing abundance and species diversity and selecting for a resistant population (Gadd, and Griffith, 1990). This is reflected in the results obtained upon culturing in nutrient agar. W4 with the highest concentration of heavy metals (Zn=0.29, Pb=0.04, Cr=0.03, As=0.01) had the least number of microorganisms (1.0×10^6) as opposed to the control with least heavy metal concentration (Zn=0.00, Pb= 0.00, Cr= 0.00, As= 0.00). This corresponds to Tsai *et al*., 2005 studies which have shown that heavy metals can significantly alter the microbial populations and diversity.

Using minimal salt agar with hydrocarbon as the only source of carbon, the bacterial isolates from the contaminated site showed a confluent growth while that from the control region showed minimal growth because of the inability of the indigenous bacteria to utilize hydrocarbon due to its complexity. The dominant gram-negative bacteria belonged to the class *Proteobacteria* with *Pseudomonas* (50%) as the representative

genera. Also, the genera, *Acinetobacter* (16.7%) and *Vibrio* (16.7%) were isolated. The control showed the least growth on minimal salt and the genus *Enterobacter* spp. was isolated, displaying that the water in the control region is contaminated with *Enterobacter spp.* The metal resistance systems inherent or ubiquitous in microorganisms (Trevors, Oddie and Belliveau, 1985) may explain the readiness of some bacterial groups to survive the addition of metal contaminants over time.

Sulphates are usually released during mining. This explains why Sulphate Reducing Bacteria (SRB) and Acid Producing Bacteria (APB) were found in the study area owing to the presence of high concentration of sulphate. These were identified based on the color changes produced in the test kits. They were not isolated in the course of this work because of limitations in apparatus and equipment. SRB has being associated with metal immobilization and corrosion. Hence, further studies should be channeled towards exploiting its potential.

5.0 CONCLUSION

A range of culturable bacteria, mostly belonging to *Proteobacteria* was obtained from a heavy metal-contaminated site in Ebonyi state of Nigeria. These showed to be resistant to high levels of Cd, Zn, Pb, Cr and As. They also exhibited traits of been used for remediation of oil polluted sites, highlighting the potential for finding new species metabolically active in heavy metal contaminated environments.

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