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Antimicrobial and Metal Tolerance of Bacteria Isolated from Underground Water Sample of Aged Crude Oil Contaminated Site

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study was aimed at evaluating metal tolerant and antibiotic resistant bacteria isolated from underground water around aged crude oil polluted site. Samples were collected from different locations around aged crude oil polluted site and control sample from an uncontaminated site of Bodo community, Gokana Local Government, Rivers state, Nigeria. The samples were cultured on nutrient agar, Bushnell Hass and MacConkay agar using standard microbial technique. Antibiogram of the isolated and identified bacteria were determined by Kirby-Bauer disc diffusion method. The

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bacterial tolerance of different concentrations of the heavy metals, Chromium, Vanadium, Arsenic, Cadmium and Lead was determined. The total heterotrophic bacterial count (THBC) of the samples ranged from 1.26×10^4 CFU/ml to 3.6×10^5 CFU/ml. Count of hydrocarbon utilizing bacteria (HUB) ranged from 1.02×10^3 to 3.2×10^3 CFU/ml and the coliform count of the sample ranged from 4.2×10^3 CFU/ml to 4.0×10^3ml. The predominant bacteria identified were *Bacillus* sp, *Micrococcus* sp, and *Staphylococcus* sp, *Enterobacter* sp and *Proteus* sp. All (100%) the Gram-positive bacteria were resistant to the antibiotic, Ceftazidime, Cefprozil and Cloxacin, 92% were resistant to Gentamycin and Erythromycin while 80% were resistant to Augmentin. All (100%) of the Gram-negative bacteria isolates were resistant to cefluroxime, 66% were resistant to Augmentin and Ceftazidim, and 33% were observed to be resistant to Nitrofurantin and Gentamicin. It was observed that all the isolates were tolerant to 50 µg/ml concentration, 70 to 100% of the isolates were tolerant to 100 µg/ml concentration, 17 to 100% were tolerant to 200 µg/ml concentration while 11 to 41% were tolerant to 300 µg/ml concentration of all the heavy metals studied. From this study, it was revealed that petroleum aged contamination could be a source of heavy-metal tolerance and antibiotic resistance in bacteria.

Keywords: Metal tolerance; antibiotic resistance; multidrug resistance.

1. INTRODUCTION

"Crude oil pollution occurs when there is an introduction of crude oil into the soil and water, which interferes with the structure and texture of the soil, thereby affecting soil fertility, toxification of aquatic organisms, increase in the concentration and accumulation of heavy metal such as zinc, chromium, nickel, mercury, iron and copper" [1] and "general alteration in the natural characteristics of water bodies, which renders it unfit for man's use" [2] and "water bodies can either adapt and proliferate or become vulnerable and eliminated, when there is crude oil pollution. Those bacteria that are able to adjust to crude oil contamination by structural and physiological modification thrive due to their ability to assimilate hydrocarbon to obtain carbon and energy" [3].

"Researches have also shown that heavy metals such copper, lead, chromium etc., are released in the course oil exploration into the environment and they can be absorbed by plants via their roots which are very harmful to plants and animals. In plants, they can result in stunted growth and death while in animals; they are capable of causing genetic mutation and cancer" [1,2].

"Bacteria possess some attributes that present them as potential bioremediation agents of both hydrocarbon and metals bioremediation and some of these include; ability to withstand adverse environmental condition, low pH, low moisture content, low nutrient requirement and production of extracellular enzymes like lipase. Although they do not occur alone, but in mix consortia with heterotrophic microorganisms without degradation capabilities, thus the need to give a clear-cut distinction in ascertaining biodegradation potentials, has resulted in the development of careful practice for identification of hydrocarbon degraders" [4].

"Although some heavy metals are essential trace elements, most are toxic at high concentrations to all organisms by forming complex compounds within the cell. Microbes are known to have evolved several mechanisms to tolerate the pressure of heavy metals by efflux, complexation, or reduction of metal ions or using them as terminal electron acceptors in anaerobic respiration" [1]. "Because heavy metal and antibiotic resistance genes are often found on the same mobile genetic element, metal pollution often promotes the emergence of antibiotic resistances in exposed organisms, and as a result, there is a growing concern in natural and clinical settings" [5,6]. Bacteria which survive in such environments have developed or acquired genetic systems that counteract the effects of high metal ion concentrations overtime. Previous studies have reported the relationship between antibiotic resistance and heavy metal tolerance hence this study was to understand the potential of the indigenous microbiota to resist the inhibitory effects of the heavy metals in underground water and their antibiotic resistance potentials which might have occurred over a long period of hydrocarbon pollution.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 4 underground water samples was collected, 3 from different locations around aged

crude oil polluted site and 1 control sample from an uncontaminated site of Bodo, community, Gokana Local Government, Rivers state, Nigeria. The samples were transported aseptically in an ice-block pack to the laboratory for immediate analysis. The heavy metal salts of vanadium, chromium, arsenic, lead, copper and cadmium were obtained from a Scientific Research Laboratory, located at Choba, Port Harcourt, Rivers state. The salts were used to prepare heavy metal stock solution of concentration, 1000ppm.

2.2 Sample Culture

This was carried out according to the method described by Tiku et al. [4] with slight modification. 10ml of the water samples was aseptically measured into 90ml of sterile distilled water in a 100ml conical flask. The samples were vortexed to homogenize and allowed to stand for 10 minutes. From the initial dilution, 10-fold serial dilutions were carried out in clean sterile test tubes containing 9ml of sterile distilled water plating procedures. An aliquot (0.1ml) of desired dilutions of 10^{-3} to 10^{-5} were cultured in duplicate on sterile media (NA, Bushnell Hass and MacConkay) using spread plate culture method. The inoculated plates were incubated at 35^{o}C and bacterial counts recorded after 24hour of incubation for NA and MacConkay agar and 5-7 days for Bushnell Hass agar.

2.3 Purification / Preservation of the Isolates

Following different cultural morphological characteristic, different bacterial isolates were purified by sub-culturing on freshly prepared plates of nutrient agar plates and again, the subcultured plates were incubated for 24hours at 37^{o}C. The pure culture of the bacterial isolates were preserved on a slanted NA in bijou bottles, incubated for 24hours and the stocked slants (cultures) were refrigerated (at 4^{o}C) for further use.

2.4 Identification of Bacterial Isolates

In addition to their cultural morphology, microscopy and biochemical test such as methylred-voges proskauers (MRVP) test, sugar fermentation tests, catalase, indole, production test, test for hydrogen sulphide and gas production, citrate utilization test, urease test, etc were carried out. Base on the results of the tests by comparing with the characteristics described in Bergey's Manual of Determinative Bacteriology (1994).

2.5 Heavy Metal Tolerance Test

Agar dilution method as described by Tiku *et al*. [4] was adopted. A loopful of 12-16hr bacteria culture in tryptic soy broth was inoculated by streaking in duplicate on Mueller-Hinton Agar plates supplemented with increasing concentrations (50µg/ml, 100 µg/ml, 200µg/ml and 300µg/ml) of the different heavy metal used (chromium, vanadium, arsenic, cadmium and lead). Plates were incubated for 24hr at 37^{o}C following incubation, plates were examined visually for the presence or absence of growth. The presence of growth was recorded as resistance while absence of growth was recorded as sensitive to the metal.

2.6 Antibiogram of the Isolates

In vitro sensitivity pattern of the isolates was studied by Kirby-Bauer disc diffusion method using numbers of antibiotic discs. The inoculums were prepared by transferring colonies from the pure culture to broth (normal saline) and matched with 0.1 McFarland (containing approximately, 3.0×10^{8}cfu/ml of cells). The standardized inoculums were then applied onto Mueller Hinton (MHA) agar plate by soaking with sterile swap stick. The discs were then placed aseptically on the surface of the agar plate and the plates were incubated at 37^{o}C for 24hours for development of inhibition zone. The diameters of zone of inhibition were measured and the interpretation was made according to Clinical and Laboratory Standards Institute chart [7].

2.7 Multiple Antibiotic Resistance (MAR) Indexing of the Isolates

"The multiple Antibiotic resistance (MAR) indexing of the isolates was determined. The MAR index is defined as a/b where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the total number of antibiotics to which the isolate was exposed. Isolates with a MAR index value higher than 0.2 was considered to have originated from high-risk source of resistance" [8].

3. RESULTS AND DISCUSSION

The microbial count of the samples is shown in Fig. 1. The total heterotrophic bacterial count (THBC) ranged from 1.26×10^{4} CFU/ml to 3.6×10^{5} CFU/ml with the control sample having

the least count and the highest count was recorded in Sample B. Count of hydrocarbon utilizing bacteria (HUB) ranged from 1.02×10^3 to 3.2×10^3 CFU/ml with the control Sample having the least count and the highest count of HUB was observed in Sample A. No count of coliform was observed in sample A and the control sample however, sample B and sample C recorded coliform count of 4.2×10^3 CFU/ml and 4.0×10^3CFU/ml respectively. The total heterotrophic bacteria count of the sample recorded in this study is similar to the study of Gambo et al. [9] in which bacterial count of drinking recorded was within the range of 5.2×10^4 to 5.9×10^4 CFU/ml in the samples.

The result of heterotrophic bacterial count and hydrocarbon utilizing bacterial count of the sample were observed to be lower in the control sample in comparison to the samples from the aged-contaminated sites. This is in line with the report of Asabia et al*.* [10] in which less count of heterotrophic bacteria was observed in the control sample. Water of good quality must have a low total bacterial count fewer than 100 cfu/mL, [11] and drinking water standards demand that drinking water should have a total heterotrophic bacteria count of <1cfu/mL and should not contain coliform, therefore, the water samples analyzed in this study were below the required standard of WHO [10]. Coliform in water is an indication of faecal contamination and indicates the presence of pathogenic microorganisms. The result of this study revealed that the control sample had no presence of feacal contamination however the two samples from the aged-contaminated environment showed coliform count that exceed the NSDWQ limit of 10CFU/ml. Coliform contamination of underground water can be attributed to close proximity of the point of the underground water source to a fecal contamination source which could be a septic or sewage tank that might have percolated or seeped into the underground water with which should contain some organic matter and could be source of pathogenic bacteria [12].

The different bacteria identified were *Bacillus* sp, *Micrococcus* sp, and *Staphylococcus* sp, *Enterobacter* sp and *Proteus* sp. The identified bacteria *Bacillus* sp, *Micrococcus* sp, *Staphylococcus* sp, *Enterobacter* sp and *Proteus* sp recorded in this study is similar to those recorded in the study of Elenwo et al. [13] and Idibie et al. [12] which reported similar bacteria contaminants from borehole water samples. The bacteria, *Bacillus* sp had the highest percentage of occurrence (58%) followed by *Micrococcus* sp 23% occurrence while the least percentage occurrence was observed to be *Proteus* sp having 6% of occurrence (as shown in Fig. 2). "*Bacillus* spp are gram-positive, aerobic or facultative anaerobes and catalase positive microorganisms. They are heat-resistant spore forming microorganisms that are most often found in soil. Due to their ability to form heat-resistant spores, they are able to survive and compete with other organisms while secreting metabolites that are antagonistic to other microorganisms in form of antibiotics" [14]. The presence of *Enterobacter* in some of the samples can be attributed to the coliform counts recorded in this study [12].

The tolerance of the isolated bacterial isolates to the different concentrations of the heavy, vanadium, chromium, arsenic, cadmium and Lead is shown in Table 1. For the heavy metal, Vanadium, 100% and 76% of the isolates were tolerance to the concentration of 50 µg/ml and 100 µg/ml respectively. In the case of the heavy metal, chromium, 100%, 82%, 17%, 11% of the isolates were observed to be tolerant to the concentration of 50 µg/ml, 100 µg/ml, 200 µg/ml and 30 µg/ml respectively. For the heavy metal, Arsenic, 100%, 70%, and 17% of the identified bacterial isolates exhibited tolerance to the concentration, 50 µg/ml, 100 µg/ml and 200 µg/ml respectively while all (100%) the isolates were observed to be sensitive to the arsenic concentration of 300 µg/ml. It was also observed that 100% and 47% of the bacterial isolates were resistant to cadmium concentration of 50 µg/ml, 100 µg/ml respectively and 100% of the isolates were sensitive to concentration of 200 µg/ml and 300 µg/ml. The heavy metal, Lead produced the highest tolerance to the bacteria isolated with the population of 100% of the bacterial isolates showing complete tolerance to the concentration of 50-200 µg/ml however, 41% of the isolates showed tolerance to 300 µg/ml concentration of Lead (Pb). The heavy metal tolerance test of the bacterial isolates revealed the sensitivity exhibited by the bacterial isolates from both the underground water of aged crude oil polluted site and the control sample was in relation to the concentration of the heavy metals utilized. This trend is similar to report by Tiku et al. [4] which reported metal resistance of *Bacillus, Yersinia, Citrobacter* and *Serratia* in relation to the concentrations of Pb, Ni, Cr, Cd, V and Cu. Researches have shown that microorganisms have evolved several mechanisms to tolerate the uptake of metal ions in order to survive metal

toxicity and these mechanisms have been proven to include surface binding, reduced uptake, increased efflux intracellular sequestration, enzyme detoxication and active transport [4]. The antibiotic resistant profile of both the Gram-positive and Gram-negative bacteria is shown in Table 2 and Table 3. For the Gram-positive bacteria, 100% of the Gram-positive bacteria were resistant to the antibiotic, Ceftazidime, Cefprozil and Cloxacin, 92% of the isolates was resistant to Gentamycin and Erythromycin while 80% of the isolates was observed to be resistant to Augmentin however all the Gram-positive isolates were susceptible to ciprofloxacin. For the Gram-negative bacteria, 100% of the isolates were resistant to cefluroxime, 66% of the isolates was observed to be resistant to Augmentin and Ceftazidim while 33% of the Gram-negative bacteria was observed to be resistant to Nitrofurantin and Gentamicin. All the Gram-negative bacteria isolated were observed to be susceptive to ciprofloxacin and oflaxacin. "The antibiogram profile of the bacteria isolates from both the petroleum polluted water samples and control sample revealed varying resistance to the antibiotics tested against. This trend could be attributed to the production of enzymes which could inactivate or modify the specific antibiotics and changes in bacterial cell membrane, modification of target site and development of metabolic pathways by the bacteria" [15]. The resistance of the organisms to the antibiotics confirms the correlation between resistance metal ions and antibiotic. The study by Bai, et al. [16] also reported "heavy metal resistance and antibiotics resistance bacterial species from different sources". Other studies have speculated that "this could be as a result of the likelihood that resistant genes to both antibiotics and heavy metals could be closely located on the same plasmid in bacteria and are thus more likely to be transferred together in the environment" [4].

In this study, the bacterial genera of *Bacillus, Micrococcus Staphylococcus*, *Enterobacter* sp, and *Proteus* sp showed 100% multidrug resistance to the antibiotic tested against as the MAR index range of 0.3 to 0.8 was recorded (as shown in Table 4). This suggests that the isolates showed resistance to most of the antibiotics tested. This is similar to the study of Yitayeh et al. [7] which recorded higher multidrug resistance in isolated bacteria. This could be attributed to possession of multiple resistance genes in the bacterial genome that made them resistant to the antibiotics. Similar high-resistance patterns have been observed against these antibiotics in other studies elsewhere [17]. Multidrug resistance to antibiotics has also been attributed to persistence of heavy metals in the environment by other studies and it is a course for public health concerns [4].

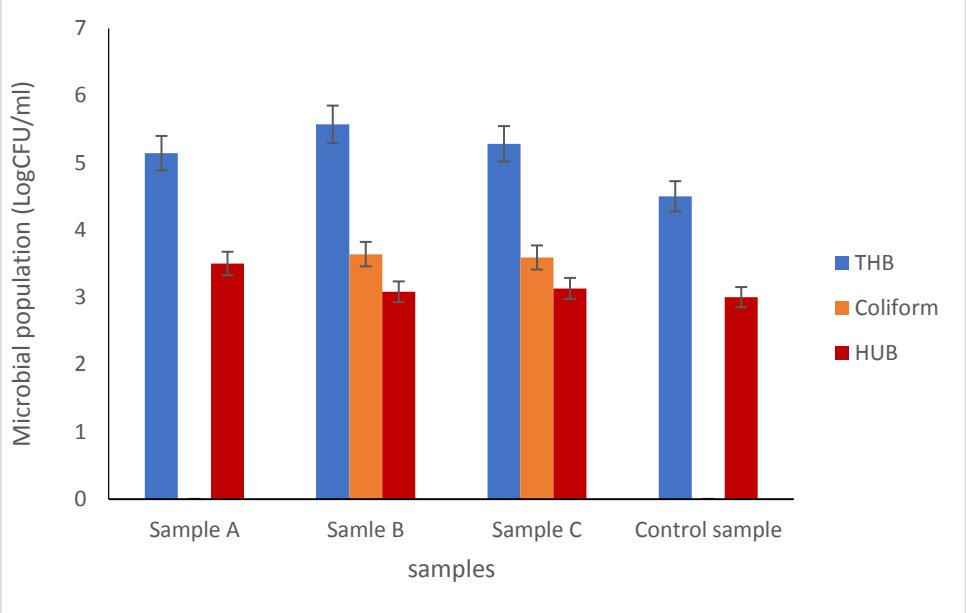

Fig. 1. Microbial population of the samples

Table 1. Heavy metal tolerance profile of bacteria isolates from the water sample at varying concentration of different heavy metal

Isolate codes	Microorganisms	Vanadium (V) (µg/ml)				Chromium (Cr) (µg/ml)				Arsenic (As) (µg/ml)				Cadmium (Cd) (µg/ml)				Lead (Pb) (µg/ml)			
		50	100	200	300	50	100	200	300	50	100	200	300	50	100	200	300	50	100	200	300
A1	*Bacillus* sp	R	R	S	S	R	R	R	S	R	R	R	S	R	R	S	S	R	R	R	R
A2	*Micrococcus* sp	R	S	S	S	R	R	S	S	R	S	S	S	R	R	S	S	R	R	R	R
A3	*Bacillus* sp	R	R	S	S	R	R	S	S	R	S	S	S	R	S	S	S	R	R	R	S
A4	*Staphylococcus* sp	R	R	S	S	R	R	S	S	R	R	R	S	R	S	S	S	R	R	R	S
B1	*Enterobacter* sp	R	R	S	S	R	S	S	S	R	R	S	S	R	R	S	S	R	R	R	S
B2	*Bacillus* sp	R	S	S	S	R	S	S	S	R	R	R	S	R	R	S	S	R	R	R	S
B3	*Bacillus* sp	R	R	S	S	R	R	S	S	R	R	S	S	R	R	S	S	R	R	R	R
B4	*Micrococcus* sp	R	R	S	S	R	R	S	S	R	S	S	S	R	R	S	S	R	R	R	R
C1	*Staphylococcus* sp	R	S	S	S	R	R	S	S	R	R	S	S	R	S	S	S	R	R	R	R
C2	*Bacillus* sp	R	R	S	S	R	R	S	S	R	S	S	S	R	S	S	S	R	R	R	S
C3	*Enterobacter* sp	R	R	S	S	R	R	R	R	R	R	S	S	R	S	S	S	R	R	R	S
C4	*Micrococcus* sp	R	R	S	S	R	R	R	R	R	R	S	S	R	S	S	S	R	R	R	R
C5	*Proteus* sp	R	R	S	S	R	R	S	S	R	R	S	S	R	S	S	S	R	R	R	R
C6	*Bacillus* sp	R	S	S	S	R	R	S	S	R	R	S	S	R	R	S	S	R	R	R	S
CT1	*Micrococcus* sp	R	R	S	S	R	R	S	S	R	R	S	S	R	S	S	S	R	R	R	S
CT2	*Bacillus* sp	R	R	S	S	R	S	S	S	R	R	S	S	R	S	S	S	R	R	R	S
CT3	*Bacillus* sp	R	R	S	S	R	R	S	S	R	S	S	S	R	R	S	S	R	R	R	S
	Percentage tolerance (%)	100	76	0	0	100	82	17	11	100	70	17	0	100	47	0	0	100	100	100	41

Table 2. Antibiogram on the gram positive bacteria isolates obtained from water samples

Isolate codes	Microorganisms	The antibiotics used							
		OFL	AUG	CAZ	GEN	CPX	CTR	ERY	CXC
A1	*Bacillus* sp	S	R	R	R	S	R	R	R
A3	*Bacillus* sp	S	R	R	R	S	R	S	R
A4	*Staphylococcus* sp	S	R	R	R	S	R	R	R
C1	*Staphylococcus* sp	S	R	R	R	S	R	R	R
C6	*Bacillus* sp	S	R	R	R	S	R	R	R
A2	*Micrococcus* sp	R	R	R	R	S	R	R	R
CT1	*Micrococcus* sp	S	S	R	R	S	R	R	R
B3	*Bacillus* sp	S	S	R	R	S	R	R	R
C4	*Micrococcus* sp	R	R	R	R	S	R	R	R
B4	*Micrococcus* sp	R	R	R	R	S	R	R	R
CT2	*Bacillus* sp	S	R	R	R	S	R	R	R
B2	*Bacillus* sp	S	R	R	R	S	R	R	R
C2	*Bacillus* sp	S	R	R	S	S	R	R	R
CT3	*Bacillus* sp	S	R	R	R	S	R	R	R
	Percentage of resistance (%)	21	85	100	92	0	100	92	100

Table 3. Antibiogram on the gram-negative bacteria isolates obtained from water samples

		The antibiotics used							
Isolate code	Microorganisms	OFL	AUG	NIT	CPR	CAZ	CRX	GEN	CXM
B1	*Enterobacter* sp	S	S	S	S	R	R	S	R
C3	*Proteus* sp	S	R	R	S	R	R	R	R
C6	*Enterobacter* sp	S	R	S	S	I	R	S	R
	Percentage of resistance (%)	0	66	33	0	66	10	33	100

Key: OFL=ofloxacin; ERY= Erythromycin, NIT= Nitrofurantoin, CXM= cefluroxime; CXC= Cloxacin, /S=sensitive, I= Intermediate; R= Resistance; AUG= Augmentin; CPX=Ciprofloxacin, CAZ= Ceftazidime; GEN= Gentamicin; CTR= Cefprozil

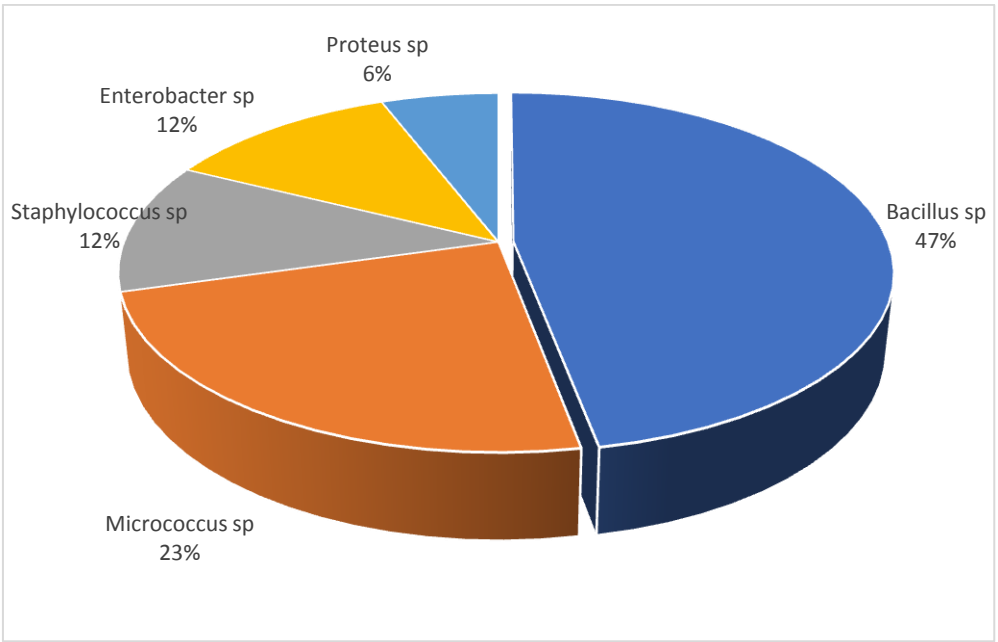

Fig. 2. Frequency of occurrence of the bacterial isolates

Table 4. Multiple Antibiotic Resistance index (MAR index) of the bacterial isolates

	MAR index							
Microorganism	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**
Bacillus sp	0(0%)	0(0%)	(0%)	0(0%)	0(0%)	0(0%)	5(63%)	3(37%)
Staphylococcus sp	0(0%)	0(0%)	0(0%)	0(0%)	(0%)	0(0%)	2(100%)	0(0%)
Micrococcus sp	0(0%)	0(0%)	(0%)	0(0%)	0(0%)	1(25%)	0(0%)	3(75%)
Enterobacter sp	0(0%)	0(0%)	2(100%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Proteus sp	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(100%)	(0%)	0(0%)

4. CONCLUSION

The results of the study showed that underground water in crude oil polluted site could be contaminated with bacteria resistant to both antibiotic and heavy metal and this could be induced by the composition of hydrocarbon overtime in the affected areas. The study revealed that hydrocarbon pollution overtime could result in correlative resistance of antibiotic and heavy metals in both pathogenic and non-pathogenic bacteria present in underground water. Underground water should undergo proper treatment before consumption to prevent bioaccumulation of heavy metal and waterborne infection in relation to the microorganism that could be present.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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