Original Research Article

Antimicrobial and Metal Tolerance of Bacteria Isolated from Underground Water sample ofAged Crude Oil Contaminated Site

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. 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 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 1.26×10^4CFU/ml to 3.6×10^5CFU/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 to4.0×10^3ml. 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 contaminationcould be a source of heavy-metal tolerance and antibiotic resistance in bacteria.

Keywords: Metal tolerance, Antibiotic resistance, Multidrug resistance

1.0 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.

2.0 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. 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.

2.3 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 concial 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. Aliquot (0.1ml) of desired dilutions, $10^{-3}$to 10^{-5} were cultured in duplicate on sterile media (NA, Bushnell Hass and MacConkay) using spread plate culture method. Plates were incubated at 35^{o}C and bacterial counts recorded after 24hour of incubation and 5-7 days for Bushnell Hass agar.

2.4 Purification / Preservation of the Isolates

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

2.4 Identification of Bacterial Isolates

For the identification of the isolates, in addition to their cultural morphology, microscopy and biochemical test such as

Comment [M2]: Add the geographical map of the study area

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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 these features, the bacterial isolates were identified. The results were compared with the characteristics described in Bergey's Manual of Determinative Bacteriology (1994).

3.5 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.

3.6 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 be 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].

3.7 Multiple Antibiotic Resistance (MAR) Indexing of the Isolates

The multiple Antibiotic resistance (MAR) indexing of the isolates was determined. The MAR index when applied to a single isolate 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]

3.0 RESULTS AND DISCUSSION

The microbial count of the samples is shown in Figure1.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 and4.0×10^{3}CFU/mlrespectively. The total heterotrophic bacteria count of the sample recorded in this study is similar to the study of Gambo et al. [9] in which bacteria count of drinking was recorded as within the range of 5.2×10^{4} to 5.9×10^{4} CFU/ml in all the

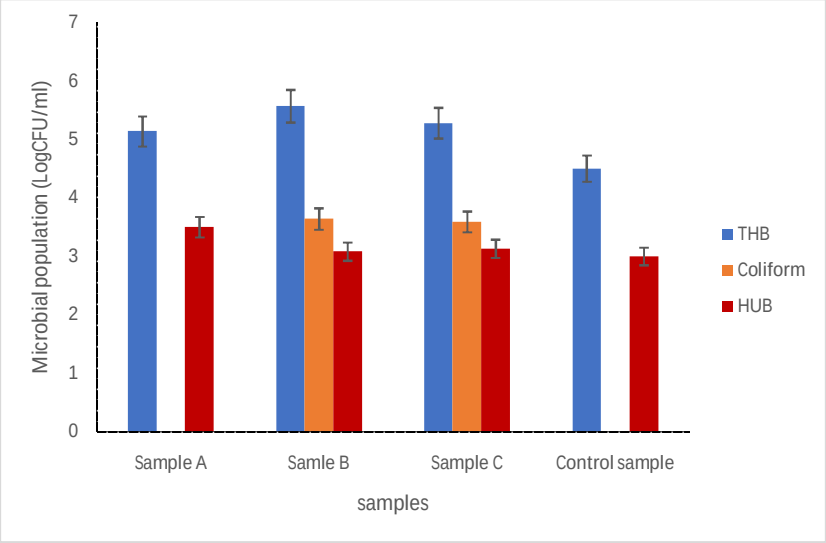

Figure 1: Microbial population of the samples

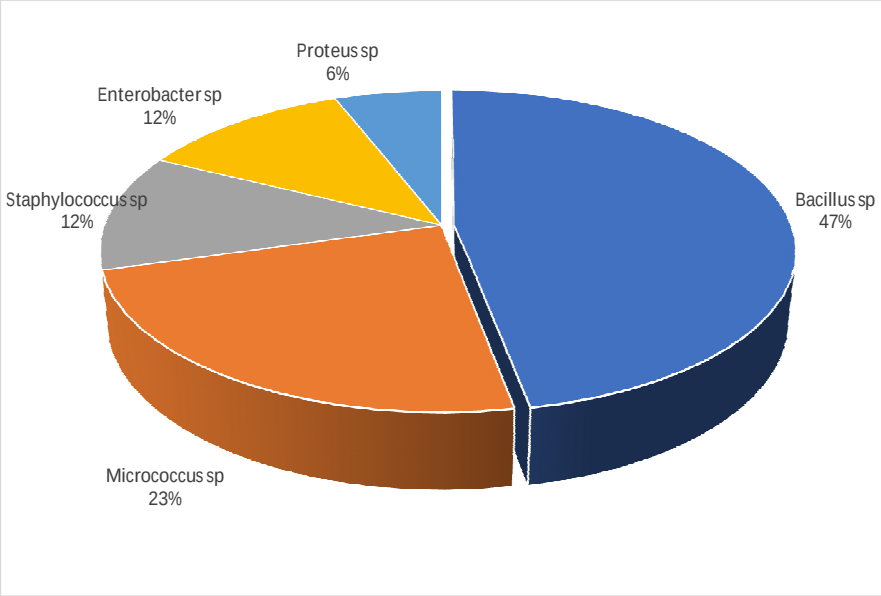

Figure 2: Frequency of occurrence of the bacterial isolates

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

Isolate code	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

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 age-contaminated sites. 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 standards demands that, drinking water should have a total heterotrophic bacteria count of <1cfu/mL and should not contain coliform. Therefore, these water samples are below the required standard of WHO [10]. Coliform in water is in an indication of faecal contamination and potent, 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 into the underground water with which should contain some organic matter [13].

The different bacteria identified were*Bacillus* sp, *Micrococcus* sp, and *Staphylococcus* sp, *Enterobacter* sp and *Proteus* sp.. The identified bacteria *Bacillus* sp, *Micrococcus* sp, and *Staphylococcus* sp, *Enterobacter* sp and *Proteus* sp recorded in this study is similar to those recorded in the study of Elenwo et al.[12] and Idibie et al. [13] 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 Figure 2. *Bacillus* sppare gram-positive, aerobic or facultativeanaerobes and catalase positive microorganisms. They areheat-resistant spore forming microorganisms that are mostoften found in soil. Due to their ability to form heat-resistantspores, they are able to survive and compete with otherorganisms while secreting metabolites that are antagonistic toother microorganisms in form of antibiotics [14]. The presence of *Enterobacter* in some of the sample can be attributed to the coliform count recorded in this study [13].

The tolerance of the isolated bacterial isolates to the different concentrations of the heavy, vanadium, chromium, arsenic, cadmium and Lead shown in Table 1. For the heavy metal, Vanadium, 100% and 76%of the isolates were tolerance to the concentration of 50 µg/ml and100 µg/ml respectively. In the case of the heavy metal, chromium, 100%, 82%, 17%, 11% of the isolated 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 respectivelywhile all (100%) the isolates were observed to be sensitive to the arsenic concentration of 300µg/ml. It was also observed that 100%, and 47%, of the bacterial isolates were resistant tocadmium concentration of 50 µg/ml, 100 µg/ml respectively and were sensitive to concentration of 200µg/ml and 300µg/ml. The heavy metal, Lead produced the highest tolerance to the bacterial 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 result of the heavy metal tolerance test of the

bacteria isolates revealed that the sensitivity exhibited by bacteria isolates from both the

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

		The antibiotics used							
Isolate code	**Microorganisms**	**OFL**	**AUG**	**NIT**	**CPR**	**CAZ**	**CRX**	**GEN**	**CXM**

Samples

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

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%)

underground water of aged crude oilpolluted site and the control sample was proportional to the concentration of the heavy metals utilized. This trend was similar to report by Tiku et al. [4] who 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 or 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 while 92% of the isolates was resistant to Gentamycin and Erythromycin while 80% of the isolates was observed to be resistant to Augmentin however, while all the Gram-positive isolates were susceptible to ciprofloxacin.From the result of the antibiotic resistance test, 100% of the Gram-negative bacteria 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 too 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 bacteria [15]. The resistance of the organisms to the antibiotics confirms the correlation between resistance metal ions and antibiotic. Also study by Bai, et al. [16], also reported heavy metal resistance and antibiotics resistance bacterial species from different sources. Other studies have speculated and have shown this to be as a result of the likelihood that resistance 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 bacteria, *Bacillus* sp, *Micrococcus* sp and *Staphylococcus* sp, *Enterobacter* sp, and *Proteus* sp showed 100% multidrug resistance to the antibiotic tested against as the MAR index 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 enable them resist the antibiotics. Similar high-resistance patterns have been observedagainst 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].

4.0 CONCLUSION

The result of the study showed that underground water has the potential for microbial contamination which could be of public interest and this could be relative of their physicochemical concentration influenced by hydrocarbon pollution that had occurred over the time. That is to say,petroleum aged contaminated environment could serve as a source of microorganisms that have the potential of heavy-metal tolerance and antibiotic resistance in correlation. Underground water should undergo proper treatment before consumption to prevent bioaccumulation of heavy metal and waterborne infection in relation to the microorganism present.

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