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Original Research Article

Hydrocarbon Biodegradation, Heavy Metal Tolerance, and Antibiotic Resistance among Bacterial Isolates from Petroleum Polluted and Pristine Soil Samples in Calabar Metropolis

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Abstract

The study was aimed at investigating hydrocarbon biodegradation, heavy metal tolerance and antibiotic resistance among bacteria isolates from petroleum polluted and pristine soil samples. The research was undertaken within a period of six months. Standard microbiological methods were used to isolate, characterize and identify bacterial isolates from the collected soil samples. Heavy metal tolerance test of the bacteria isolates was carried out using agar dilation method, while the hydrocarbon biodegradation potential of the bacteria isolates were determined using method micro-titre plate technique, with kerosene and diesel used as the hydrocarbon sources. Also the standard disc diffusion method was used to test for antibiotic susceptibility pattern of the bacteria isolates. Diesel biodegraders from the petroleum polluted soil were identified as *Serratia* species and *Enterobacter* species while kerosene and diesel biodegraders were identified as the species of *Shigella*, *Corynebacterium*, *Klebsiella*, *Yersinia*, *Aeromonas*, *Bacillus* and *Pseudomonas* while *Serratia* species and *Yersinia* species from the pristine soil showed diesel biodegradation potential and *Bacillus* species was identified as efficient diesel and kerosene biodegrader. Bacteria isolates from the petroleum polluted soil samples showed marginally higher percentage tolerability to Pb, Ni, Cr, Cd, Co and V than their pristine soil counterparts. 17 out of the 21 isolates from the petroleum polluted soil samples showed (80.95%) multiple antibiotic resistance to ciprofloxacin, doxycycline, trimethoprim, nalidixic acid, penicillin and nitrofurantoin, while isolates from the pristine sample showed resistance to only penicillin (10µg) and were susceptible to other antibiotics tested. However, in comparing the hydrocarbon biodegradation potential heavy metal tolerability and antibiotic resistance pattern, among bacteria isolates from the petroleum polluted soil samples, *Serratia* species (AMM_2), *Klebsiella* species (AMM_3), *Pseudomonas* species (AMT_4) and *Bacillus* species (AME_4) were able to biodegrade diesel and kerosene efficiently, tolerated high heavy metal concentration (3000µg/ml) of chromium, copper and lead and did not show multiple antibiotic resistance to the antibiotics they were tested against, therefore they could be useful in the bioremediation of hydrocarbon–heavy metal polluted environment, as their applicability does not pose any public health or environmental threat involving the spread of resistance genes.

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Introduction

Environmental pollution with heavy metal and petrochemical products has attracted much attention in recent decades, as cases involving their contamination of soil and water environments is on the increase (Weis and Weis, 2002; Husaini et al., 2008) and this may probably be as a result of industrial revolution (Husaini et al., 2008), as well as the presence of different types of automobile and machineries (Rajendram et al., 2002).

The presence of these heavy metals and hydrocarbon especially polycyclic aromatic hydrocarbon in the environment attracts public attention, because they are toxic, mutagenic and carcinogenic (Van Hamme et al., 2003). Prolonged exposure to high concentration of heavy metal or hydrocarbon may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased rate of cancer (Boochan et al., 2000). In addition, polycyclic aromatic hydrocarbons and heavy metals have a wide spread occurrence in various ecosystems that contributes to the persistence of these compounds in the environment (Boochan et al., 2000). With all these aforementioned negative impacts caused by the presence of heavy metals and polycyclic aromatic hydrocarbons in the environment, scientist developed conventional physical and chemical treatment methods which could help in either eliminating or suppressing the concentration of these pollutants to the nearest minimum in the environment (Oboh et al., 2006), researches has revealed the setbacks of such method involves the production of sludge which leads to disposal problems and this paved way for the development of an eco-friendly and economical option known as biological methods which involves the use of microbial cells (or its metabolites) to degrade the pollutants (Yong and Macaskie, 1998).

Hydrocarbon degrading microorganisms are widely distributed in marine, fresh water and soil ecosystems (Atlas and Cerniglia, 1995), with fungi and bacteria constituting the main components of the soil microbial biomass. Bacteria possess some attributes that 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 (Atlas and Cerniglia, 1995). 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 a ascertaining biodegradation potentials, has resulted in the development of careful practice for identification of hydrocarbon degraders (Tiku and Asikong, 2016).

In identifying useful bacteria for use in bioremediation of hydrocarbon-heavy metal polluted environment, there is a need to ascertain the antibiotics susceptibility pattern of these microorganisms in other to avoid the spread of antibiotics resistance genes in the environment. Therefore, this research work is aimed at analyzing the hydrocarbon biodegradation potentials, heavy metal tolerance and antibiotic resistance among bacteria isolates from petroleum polluted and pristine soil samples.

Materials and methods

Study site and sampling

Different auto-mechanic workshop and pristine soil environments in Calabar metropolis were sampled randomly (Table 1) (Fig. 1). The auto-mechanic workshop sampled include: Auto-mechanic workshop located in Atekong, Etta Agbor, Mbukpa and Inyang while pristine soil sampled included; Staff quarter-Unical, Cultural centre-Calabar, Botanical garden-Unical and Inyang street.

Table 1. Description of sampling locations in Calabar Metropolis-Cross River State.

Location code	Name	Latitude	Longitude	Elevation	Sample
SQU	Staff Quarters UNICAL	4°56'04.51"N	8°20'69.40"E	114.3, A12.3	Soil
CCC	Cultural Center Calabar	4°57'05.97"N	8°19'04.80"E	165.5, A22.7	Soil
AMT	Auto-Mechanic Shop-Atekong	4°58'45.24"N	8°19'59.40"E	178.3, A13.3	Soil
AME	Auto-Mechanic Shop-Ettagbor	4°57'04.43"N	8°20'04.92"E	198.1, A16.2	Soil
AMM	Auto-Mechanic shop-Mbukpa	4°56'01.81"N	8°19'14.50"E	125.5, A17.5	Soil
AMI	Auto-Mechanic shop-Inyang	4°56'02.55"N	8°19'21.40"E	135, A17.7	Soil
BGU	Botanical Garden-UNICAL	4°57'08.64"N	8°20'34.20"E	195.7, A19.6	Soil
IS	Inyang Street	4°56'02.65"N	8°19'01.95"E	125.4, A14.3	Soil

UNICAL-University of Calabar, Calabar.

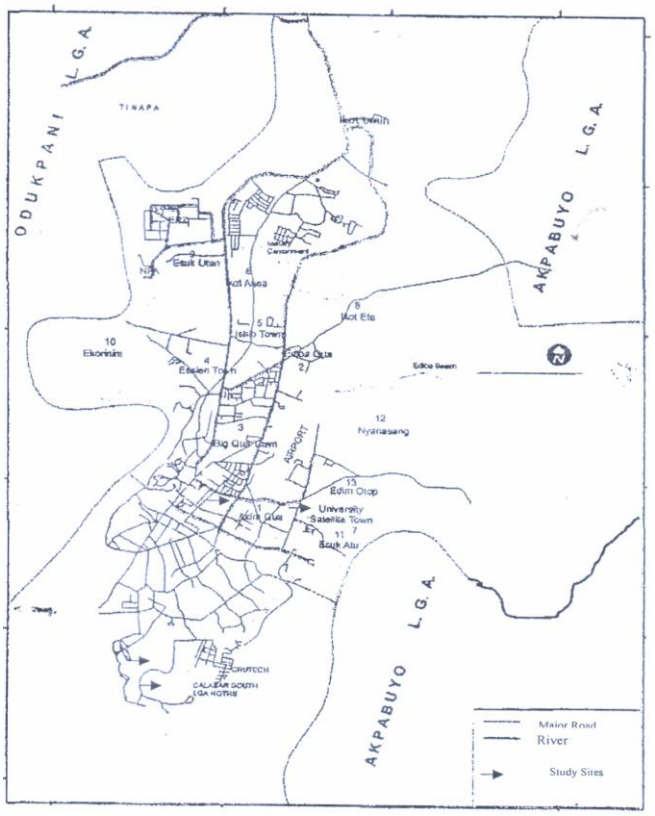

Fig. 1: Map of Calabar, showing the study area (Source: GIS Unit, University of Calabar, 2012).

Samples collection

Soil samples: Soil samples were collected without bias, 20 g each of both the auto-mechanic workshop soils and pristine soils were collected from the top, 0cm to 15cm of the soils using sterile spoon and then placed separately in an oven sterilized aluminum foil (at 16°C per hr). The soil samples were wrapped and placed in sterile 100ml universal container.

Heavy metal samples: Heavy metal salts of vanadium, chromium, nickel, lead, cobalt, cadmium and copper were obtained from Vikham Scientific Research Laboratories, located at U.J. Essuene Stadium, Calabar. The salts were used to prepare heavy metal stock solution of concentration 1000ppm.

Kerosene and diesel: Kerosene and Diesel were purchased at NNPC filling station located at Goldie Street, Calabar and were placed into five litres plastic containers.

Antibiotic discs: Commercial antibiotic discs containing the following antibiotics Trimethoprim/ Sulfamethoxazole (S X T 25µg), Nalidixic acid (NA-30µg), Ciprofloxacin (CP-5 µg), Penicillin (P-10µg), Doxycycline (D-30µg), Nitrofurantoin (F/M-300µg), all were products of Hardy diagnostics, USA and were used in accordance to the manufacturers instructions.

Media used

The media used in the study included: Nutrient agar (Hardy diagnostics, USA, Muller-Hinton agar and Tryptic soy broth (Hardy Diagnostics, USA), Bushnell Hass medium (Titan Biotech, India), Simmon Citrate medium (Accumedia, USA), Nutrient both (Hardy diagnostics, USA), Motility indole ornithine (MIO) medium (Hardy Diagnostics, USA). The media were prepared according to manufacturer instructions.

Sample preparation

This was carried out according to the method described by Eze et al. (2014). Ten grams of soil samples was aseptically weighed 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.

This was carried out according to the method described by Jamiska et al. (2011) with slight modification. 0.1ml of desired dilutions 10^{-3} – 10^{-5} were spread plated in triplicate into nutrient agar supplemented with 50µg/ml of Nystatin to inhibit the growth of fungi. Plates were incubated at 35°C and bacterial counts recorded after 24 hrs of incubation.

Purification of isolates

Following enumeration of total heterotrophic bacteria, colonies were picked at random and sub-cultured repeatedly into nutrient agar for purification. Purified isolates were stocked in nutrient agar slants for further studies.

Identification and characteristics of isolates

Purified isolates were characterized by gram morphology and biochemical test using the scheme in Bergey's manual of determinative bacteriology (Cheesbrough, 2000; Holt et al., 1994).

Hydrocarbon biodegradation potential of bacteria isolates of bacteria isolates for hydrocarbon degradation potentials using kerosene and diesel

Pure bacterial isolates from the soil samples were inculcated into 5mls of nutrient broth and incubated for 48 hrs at room temperature. 0.1ml each of the pure bacteria isolates from the nutrient broth was then inoculated into 9.9ml of Bushnell Hass broth contained in two different tubes. 700µul of sterilized kerosene and diesel were then pipette into the different test tubes. Controls containing only the kerosene and diesel were also set up.

All the test tubes were incubated for 16 days at room temperature without shaking. On 16th day, 50µl of p-iodonitrotetrazatium violet (INT) indicator was added to each tube and the tubes were further incubated for 24 hrs. Kerosene and diesel biodegradation was indicated by pink to red precipitate, while the broth turbidity was also observed. At the end of the screen test, a high degree of precipitate and broth turbidity of both kerosene and diesel observed from the biodegradation activities of the bacteria isolates were assigned +++, while the formation of moderate and low degree of precipitate and broth turbidity was assigned ++ and + respectively. However, the absence of precipitate and turbidity during the biodegradation screen test by the bacteria isolates was assigned - .

Heavy metal tolerance test

Agar dilution method as described by Lee et al. (2009) was adopted. A loopful of 12-16 hrs bacteria culture in tryptic soy broth was inoculated by streaking in duplicate on Mueller- Hinton Agar plates supplemented with increasing concentrations (20µg/ml, 100 µg/ml, 200µg/ml, 250µg/ml and 300µg/ml) of the different heavy metal used (chromium, vanadium, nickel, cobalt, copper, cadmium and lead). Plates were incubated for 24 hrs at 37°C following incubation, plates were examined visually for the presence or absence of growth was recorded as resistance while absence of growth was recorded as sensitive.

Antibiotic susceptibility testing

The standard disc diffusion method was used to test for antibiotic susceptibility pattern of the bacteria isolate. Commercial antibiotic disks (hardy diagnostics, USA) containing the following antibiotics; Trimethoprim/ sulfamethoxazole (5 x T-25µg), Nalidixic acid (Na-30µg), Ciprofloxacin (Cp-5µg), Penicillin (P-10µg), Doxycycline (D- 30µg), Ntrofurantoin (F/M-300µg) were used. The inoculums size was prepared and compared with 0.5 McFarland standard.

Results

Screening test for hydrocarbon (diesel and kerosene) biodegradation by bacteria isolates from petroleum polluted and pristine soil samples

The result of the screen test for diesel and kerosene biodegradation by bacteria isolates from petroleum polluted and pristine soil samples are presented in Tables 2 and 3 respectively. The levels of turbidity, degree of precipitate and colour of precipitate were used to assess the diesel and kerosene biodegradability of the bacterial isolates.

Out of 21 bacterial isolates, 15 isolates from the petroleum polluted soil were adjudged diesel biodegrades and were identified as *Enterobacter* species and *Serratia* species while 13 were efficient diesel and kerosene biodegraders and were also identified as the species of *Corynebacterium*, *Shigella*, *Yersinia*, *Serratia*, *Klebsiella*, *Bacillus* and *Pseudomonas* (Table 2), while out of the 14 bacterial isolates from the pristine soil sample, 3 isolates showed the capacity of degrading diesel and kerosene and were identified as *Yersinia* species and *Serratia* species. Only one isolate was efficient diesel and kerosene biodegraders and was identified as *Bacillus* species (Table 3).

Heavy metal tolerance test of bacteria isolates from petroleum polluted soil samples

Table 4 shows the result of the heavy metal tolerance test of bacteria isolates from petroleum polluted soil samples to 20µg/ml, 100µg/ml, 200µg/ml, 250µg/ml, and 300µg/ml of the different heavy metals employed. At 20µg/ml of chromium, vanadium, nickel, cobalt, cadmium, copper and lead, all the bacteria isolates from the petroleum polluted soil samples showed resistance to all the heavy metals used. At heavy metal concentration of 100µg/ml, 19 out of the 21 isolates showed resistance to chromium, 15 of the isolates showed resistance to vanadium and nickel. 11 of the isolates showed resistance to cobalt, 18 of the isolates showed resistance to cadmium, 20 of the isolates showed resistance to copper, while all the isolates showed resistance to lead.

Table 2: Screen test for diesel and kerosene biodegradation by bacteria isolates from petroleum polluted soil samples.

Isolates	Diesel				Kerosene				
	Colour of used engine oil layers	**Broth turbidity**	**Degree of precipitate**	**Colour of precipitate**	**Colour of used hydraulic oil layer**	**Broth turbidity**	**Degree of precipitate**	**Colour of precipitate**	**Probable organisms**
AMT_1	Light brown	+	+	Light brown	Colourless	+	+	Colourless	*Bacillus* species
AMT_2	Light brown	-	-	Light brown	Colourless	+	+	Colourless	*Bacillus* species
AMT_3	Light brown	-	+	Light brown	Colourless	++	+	Colourless	*Escherichia coli*
AMT_4^{**}	Pink	++	++	Pink	Light pink	+	+++	Light pink	*Pseudomonas* species
AMT_5	Light brown	+	+	Light brown	Colourless	+	+	Colourless	*Klebsiella* species
AMM_1^{*}	Pink	++	+++	Pink	Light pink	+	+	Light pink	*Aeromonas* species
AMM_2^{**}	Pink	+	+++	Pink	Light pink	+	++	Light pink	*Serratia* species
AMM_3^{**}	Pink	+	++	Pink	Light pink	+	++	Light pink	*Klebsiella* species
AMM_4^{**}	Light pink	+	++	Light pink	Pink	+	++	Pink	*Enterobacter* species
AMM_5^{*}	Light pink	++	+++	Light pink	Light pink	+	+	Light pink	*Enterobacter* species
AMM_6^{**}	Pink	++	+++	Pink	Pink	++	+++	Pink	*Aeromonas* species
AME_1^{**}	Pink	+	+++	Pink	Light pink	++	+++	Light pink	*Corynecbacterium* species
AME_2^{**}	Light pink	+	++	Light pink	Light pink	++	+++	Light pink	*Serratia* species
AME_3^{**}	Pink	++	+++	Pink	Pink	++	++	Pink	*Yersinia* species
AME_4^{**}	Light pink	+	++	Light pink	Light pink	+	++	Light pink	*Bacillus* species
AME_5^{**}	Pink	+	+++	Pink	Pink	++	++	Pink	*Serratia* species
IS_1^{**}	Pink	++	++	Pink	Light pink	++	+++	Light pink	*Shigella* species
IS_2	Light brown	-	+	Light brown	Light pink	++	++	Light pink	*Aeromonas* species
IS_3^{*}	Light pink	+	++	Light pink	Light pink	+	+	Light pink	*Serratia* species
IS_4^{**}	Pink	+	++	Pink	Pink	+	++	Pink	*Klebsiella* species
IS_5^{**}	Light pink	+	++	Light pink	Light pink	+++	+++	Light pink	*Pseudomonas* species
Control	Light brown	-	-	Light brown	Colourless	-	-	Colourless	-

Key; +++ = High, ++ = Moderate, + = Low, - = Nill, * = Efficient diesel biodegraders, ** = Efficient diesel and kerosene biodegraders.

Table 3: Screen test for diesel and kerosene biodegradation by bacteria isolates from pristine soil samples.

Diesel					**Kerosene**				
Isolates	**Colour of used engine oil layers**	**Broth turbidity**	**Degree of precipitate**	**Colour of precipitate**	**Colour of used hydraulic oil layer**	**Broth turbidity**	**Degree of precipitate**	**Colour of precipitate**	**Probable organisms**
SQU_1	Black	-	-	Black	Grey	-	-	-	*Citrobacter* species
SQU_2	Black	-	-	Black	Grey	-	-	-	*Citrobacter* species
SQU_3^*	Light Pink	+	+	Light Pink	Grey	-	-	-	*Yersinia* species
SQU_4	Black	-	-	-	Grey	-	-	-	*Serratia* species
IS_1	Black	-	-	-	Grey	-	-	-	*Yersinia* species
IS_2	Black	-	-	-	Grey	-	-	-	*Bacillus* species
IS_3^*	Light Pink	++	++	Pink	Grey	-	-	-	*Serratia* species
IS_4	Black	-	-	-	Grey	-	-	-	*Citrobacter* species
IS_5	Black	-	-	-	Grey	-	-	-	*Bacillus* species
BGU_1	Black	-	-	-	Grey	-	-	-	*Bacillus* species
BGU_2^{**}	Light pink	++	++	Pink	Light pink	++	++	Light pink	*Bacillus* species
BGU_3	Black	-	-	-	Grey	-	-	-	*Serratia* species
CC_1	Black	-	-	-	Grey	-	-	-	*Serratia* species
CC_2	Black	-	-	-	Grey	-	-	-	*Yersinia* species
Control	Black	-	-	-	Grey	-	-	-	-

Key; +++ = High, ++ = Moderate, + = Low, - = Nill, * = Efficient used diesel biodegraders, ** = Efficient diesel and kerosene biodegraders.

Table 4. Heavy metal tolerance profile of bacteria isolates from auto-mechanic workshop soils at varying concentration of different heavy metal.

Isolates	Concentration of heavy metals (µg/ml)																																		Probable organisms
	Cr					V					Ni					Co					Cd					Cu					Pb					
	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	
AMT_1	R	R	R	R	S	R	R	S	S	S	R	R	R	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	R	R	R	R	R	S	*Bacillus* species
AMT_2	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	S	R	R	R	R	R	S	*Bacillus* species
AMT_3	R	R	R	S	S	R	R	S	S	S	R	R	R	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	R	R	R	*E. coli*
AMT_4	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Pseudomonas* species
AMT_5	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	S	R	R	R	R	R	*Klebsiella* species
AMM_1	R	R	R	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	R	R	R	*Aeromonas* species
AMM_2	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Serratia* species
AMM_3	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Klebsiella* species
AMM_4	R	R	R	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	R	S	R	R	R	R	S	*Enterobacter* species
AMM_5	R	R	R	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	R	S	R	R	R	R	S	*Enterobacter* species
AMM_6	R	R	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	R	S	R	R	R	R	R	*Aeromonas* species
AME_1	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Corynebacterium* species
AME_2	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Serratia* species
AME_3	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Yersinia* species
AME_4	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	*Bacillus* species
AME_5	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	S	S	*Serratia* species
IS_1	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	R	R	R	*Shigella* species
IS_2	R	R	R	R	R	R	S	S	S	S	R	R	R	S	S	R	R	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	*Aeronomas* species
IS_3	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	R	R	R	R	S	S	*Serratia* species
IS_4	R	R	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	R	S	S	R	S	S	S	S	R	R	R	R	R	R	R	R	S	S	*Klebsiella* species
IS_5	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	S	S	S	S	R	R	R	R	R	R	R	R	S	S	*Pseudomonas* species

Key; Cr=Chromium, V=Vanadium, Ni=Nickel, Co=Cobalt, Cd=Cadmium, Cu=Copper, Pb=Lead, R=Resistance, S=sensitive.

Table 5. Heavy metal tolerance profile of bacteria isolates from pristine soil samples at varying concentrations of heavy metal.

Isolates	Concentration of heavy metals (µg/ml)																																		Probable organisms
	Cr					V					Ni					Co					Cd					Cu					Pb					
	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	20	100	200	250	300	
SQU_1	R	S	S	S	S	S	S	S	S	S	R	R	S	S	S	R	R	S	S	S	R	R	R	R	S	R	R	R	R	R	R	R	R	S	S	*Citrobacter* species
SQU_2	R	R	S	S	S	R	R	S	S	S	R	R	R	S	S	R	S	S	S	S	R	S	S	S	S	R	R	R	S	S	R	R	S	S	S	*Citrobacter* species
SQU_3	S	S	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	R	R	*Yersinia* species
SQU_4	R	R	R	R	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	*Serratia* species
IS_1	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	R	S	R	R	R	S	S	*Yersinia* species
IS_2	R	S	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	S	S	R	S	S	S	S	R	R	S	S	S	*Bacillus* species
IS_3	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	R	S	S	R	R	S	S	S	R	R	R	S	S	*Serratia* species
IS_4	R	R	R	R	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	R	R	*Citrobacter* species
IS_5	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	R	R	R	R	R	S	S	S	*Bacillus* species
BGU_1	R	R	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	S	S	S	R	R	R	R	S	R	R	S	S	S	R	R	R	R	R	*Bacillus* species
BGU_2	R	R	R	R	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	*Bacillus* species
BGU_3	R	R	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	S	S	R	R	S	S	S	*Serratia* species
CC_1	R	S	S	S	S	R	R	S	S	S	R	R	S	S	S	R	S	S	S	S	R	S	S	S	S	R	S	R	S	S	R	R	S	S	S	*Serratia* species
CC_2	R	R	R	S	S	R	R	S	S	S	R	S	S	S	S	R	S	S	S	S	R	R	S	S	S	R	R	R	R	R	R	S	S	S	S	*Yersinia* species

Key; Cr=Chromium, V=Vanadium, Ni=Nickel, Co=Cobalt, Cd=Cadmium, Cu=Copper, Pb=Lead, R=Resistance, S=sensitive

At heavy metal concentration of 200µg/ml, 13 out of the 21 isolates showed resistance to chromium, 11 of the isolates showed resistance to cobalt and nickel, 20 of the isolates were resistance to lead while all the isolates were sensitive to vanadium and cadmium at this same concentration. At heavy metal concentration of 250µg/ml, 9out of the 21 isolates showed resistance to chromium, 15 of the isolates showed resistance to copper, 16 of the isolates showed resistance to lead while all the isolates were sensitive to nickel and cobalt. At heavy metal concentration of 300µg/ml, 7out of the 21 isolates showed resistance to copper while 14 of the isolates showed resistance to lead (Fig. 2).

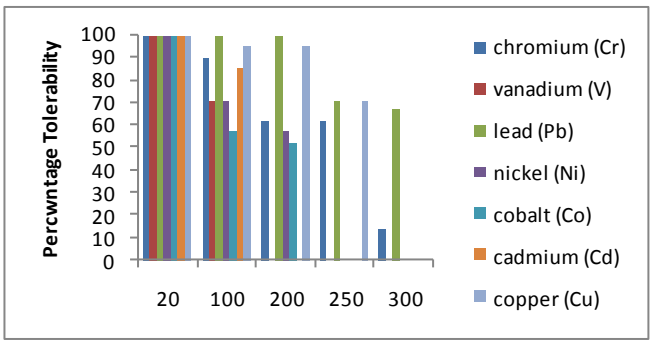

Fig. 2: Percentage tolerability of bacteria isolates from petroleum polluted soil to increasing heavy metal concentration. (Cr=Chromium, V=Vanadium, Ni=Nickel, Co=Cobalt, Cd= Cadium, Cu=Copper, Pb=Lead, Number of isolates tested = 21).

Heavy meal tolerance test of bacteria isolates from pristine soil samples

Table 5 presents the result of heavy metal tolerance test of bacteria isolates from pristine soil samples to 20µg/ml, 100µg/ml, 200µg/ml, 250µg/ml and 300µg/ml of the different heavy metals used. At heavy metal concentration of 20µg/ml, 13 out of the 14 isolates showed resistance to chromium and vanadium, while all the isolates showed resistance to nickel, cobalt, cadmium, cooper and lead. At heavy metal concentration of 100µg/ml, 9 of the isolates showed resistance to chromium, nickel and cadmium, 10 of the isolates showed resistance to vanadium, 7 of the isolate showed resistance to cobalt while 11 of the isolate showed resistance to copper and lead. At heavy metal concentration of 200µg/ml, 4 out of the 14 isolates showed resistance to chromium and cadmium, 2 of the isolates showed resistance to nickel, 7 of the isolates showed resistance to copper, 6 of the isolates showed resistance to lead, while all the isolates were sensitive to vanadium and cobalt. At heavy metal concentration of 250µg/ml, 3 out of the 14 isolates showed resistance to chromium, one of the isolates showed resistance to cadmium, 4 of the isolates showed resistance to copper and lead, while all the isolates were sensitive to nickel. At heavy metal concentration of 300µg/ml, 3 of the isolates showed resistance to cooper and lead, while all the isolates were sensitive to chromium and cadmium (Fig. 3).

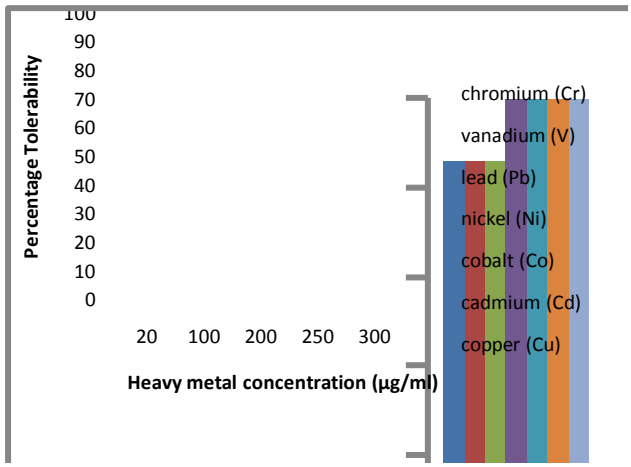

Fig. 3: percentage tolerability of bacteria isolates from pristine soil to increasing heavy metal concentration (Cr-Chromium, V=Vanadium, Ni=Nickel, Co=Cobalt, Cd=Cadmium, Cu=Copper, Pb=Lead, Number of isolates tested =14).

Antibiotic susceptibility testing of bacteria isolates from petroleum polluted soil samples

Table 6 presents the result of antibiogram of bacteria isolates from petroleum polluted soil samples. 9.5% of the bacteria isolates showed resistance to Ciprofloxacin while 90.5% showed sensitivity to Ciprofloxacin, for Doxycycline, 38.1%, 23.8% and 38.1% of the bacteria isolates showed resistance, intermediate, and sensitivity respectively to the antibiotic.

For Nalidxic acid, 19.1%, 9.5% and 71.4% of the bacteria isolates showed resistance, intermediate and sensitivity to the antibiotic, for Trimethoprim, 28.6%, 4.8% and 66.7% of the bacteria isolates showed resistance, intermediate and sensitivity to the antibiotic. For Nitrofurantoin 38.1%, 9.5%, and 52.4% of the bacteria isolate showed resistance, intermediate and sensitivity to the antibiotic. For Penicillin, 76.2% and 23.8% of the bacteria isolate showed resistance and sensitivity to the antibiotic (Fig. 4).

Table 6. Antibiogram of bacteria isolates from petroleum polluted soil samples.

Isolates	CLP (5µg)	D (30µg)	NA (30µg)	SXT (25µg)	F/M (300µg)	P (10µg)
MT_1^*	S	I	I	S	R	R
AMT_2^*	S	S	S	S	R	S
AMT_3	S	S	S	S	S	R
AMT_4	S	I	S	S	I	R
AMT_5^*	S	R	S	R	S	R
AMM_1^*	R	I	S	S	R	R
AMM_2	R	I	S	S	S	S
AMM_3	S	S	S	I	S	R
AMM_4	S	R	S	R	S	R
AMM_5^*	S	R	R	R	S	R
AMM_6^*	S	I	R	R	R	R
AME_1^*	S	S	I	S	S	S
AME_2^*	S	S	S	S	I	R
AME_4	S	S	S	S	S	R
AME_5	S	R	S	S	R	R
IS_1^*	S	R	S	S	R	S
IS_2^*	S	S	R	S	R	R
IS_3^*	S	S	S	S	S	S
IS_4	S	R	S	R	S	R
IS_5^*	S	R	R	S	R	R
%R	9.5	38.1	19.1	28.6	38.1	76.2
%I	-	23.8	9.5	4.8	9.5	-
%S	90.5	38.1	71.4	66.7	52.4	23.8

Key: S = Sensitivity, R= Resistance, I = Intermediate; * = Multiple antibiotic resistance bacteria; CIP = Ciprofloxacin, D = Doxycycline, NA = Nalidixic acid, SXT = Trimethoprim/Sulfamethoxazole, F/M = Nitrofurantoin and P= Penicillin.

Table 7. Antibiogram of bacteria isolates from pristine soil.

Isolates	CLP (5µg)	D (30µg)	NA (30µg)	SXT (25µg)	F/M (300µg)	P (10µg)
SQU_1	S	I	I	S	I	R
SQU_2	S	S	S	S	S	R
SQU_3	S	S	S	S	S	R
SQU_4	S	S	S	S	S	R
IS_1	S	S	S	S	S	R
IS_2	S	S	S	S	S	R
IS_3	S	S	S	S	S	R
IS_4^*	S	S	I	S	R	R
IS_5	S	S	S	S	I	R
BGU_5	S	S	I	S	S	R
BGU_1	S	S	I	S	S	R
BGU_2	S	S	S	S	S	R
BGU_3	S	S	S	S	S	R
CC_1	S	S	S	S	S	R
CC_2^*	S	S	S	R	S	R
%R	-	-	-	7.1	7.1	100
%I	-	-	14	-	21.5	-
%S	100	100	86	92.9	71.4	-

Key: S = Sensitivity, R= Resistance, I = Intermediate; * = Multiple antibiotic resistance bacteria; CIP = Ciprofloxacin, D = Doxycycline, NA = Nalidixic acid, SXT = Trimethoprim/Sulfamethoxazole, F/M = Nitrofurantoin and P= Penicillin.

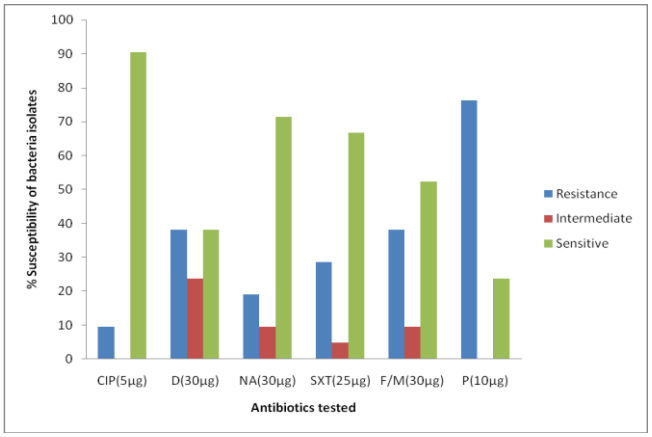

Fig. 4: Percentage susceptibility of bacteria isolates from petroleum polluted soil samples tested against. **Key:** CIP = Ciprofloxacin, D = Doxycycline, NA = Nalidixic acid, SXT = Trimethoprim/Sulfamethoxazole, F/M = Nitrofurantoin and P= Penicillin.

Antibiotic susceptibility testing of bacteria isolates from pristine soil samples

Table 7 presents the result of antibiogram of bacteria isolates from pristine soil samples. All the bacteria isolates (100%) showed sensitivity to Ciprofloxacin and Doxyclycline, 14% and 86% of the bacteria isolate showed intermediate and sensitivity to Nalidixic acid, 7.1% and 92.9% of the bacteria isolates showed resistance and sensitivity to Trimethorpim/ Sulfamethoxazole, 7.1%, 21.5% and 71.4% of the bacteria isolates showed resistance, to intermediate and sensitivity to Nitrofurantoin, while all the bacteria isolates were resistance to Penicillin (Fig. 5).

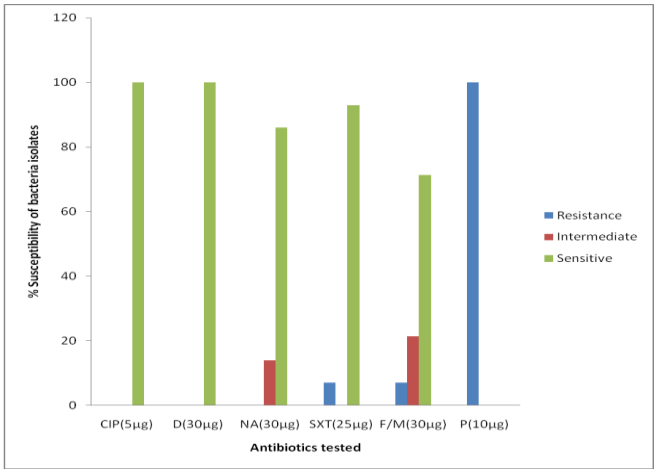

Fig. 5: Susceptibility of the bacteria isolates from pristine soil samples to antibiotics test. **Key:** CIP = Criprofloxacin, D = Doxycycline, NA = Nalidixic acid, SXT = Trimethorpim/sulfamethoxazole, F/M = nitriofurantoin and P = Penicillin.

Comparability of hydrocarbon biodegradation, heavy metal tolerance and antibiotic resistance pattern among the bacteria isolates from petroleum polluted and pristine soil samples

Table 8 presents the result of comparability of hydrocarbon biodegradation, heavy metal tolerance and antibiotic resistance pattern among bacteria isolates from petroleum polluted and pristine soil samples. It showed that *Serratia* species (AMM_2), *Klebsiella* species (AMM_3) *Pseudomonas* species (AMT_4), and *Bacillus* species (AME_4) were able to utilize both diesel and kerosene efficiently, tolerated heavy metal concentration of chromium, copper and lead at a concentration of 300µg/ml and did not show multiple resistance to the antibiotics tested against.

Discussion

Bacterial genera isolated from the petroleum polluted soil were species of *Yersinia*, *Shigella*, *Bacillus*, *Enterobacter* and *Escherichia coli*, while species of *Citrobacter*, *Yersinia*, *Serratia* and *Bacillus* were also identified from the pristine soil sample. This observation corroborates with that of Ijah and Antai (2003), who reported the presence of *Micrococcus*, *Pseudomonas*, *Bacillus*, and *Alkaligenes* species in soil polluted with Nigerian light crude oil.

Also study by Van Hamme et al. (2003), reported to have identified *Enterobacter*, *Acinetobacter*, *Micrococcus*, *Pseudomonas* and *Serratia* from soils contaminated with petroleum products. The result of the biodegradation screen test showed that a greater number of bacteria isolates with hydrocarbon (diesel and kerosene) biodegradation were from the petroleum polluted soil sample as compared to its pristine counterpart.

Fifteen out of 21 bacterial isolates (71.43%) from the petroleum polluted soil were efficient diesel biodegraders while 13 out of the 21 isolates (62%) were efficient diesel and kerosene biopdegraders, compared to the bacterial isolates from the pristine soil, in which 3 out of 14 isolates (21.3%) showed capability of biodegrading diesel while only 1 out of 14 isolates (7.14%) was efficient diesel and kerosene biodegrader. This observation agrees with findings of Mikessel et al. (1993), who reported a higher abundance of crude oil utilizing bacteria consortiums at vary site of oil seepages.

Table 8. Comparability of hydrocarbon biodegradation, heavy metal tolerance and antibiotic resistance pattern among bacteria isolates from petroleum polluted and pristine soil samples.

Isolate	Petroleum polluted soil probable organism	EDB	EKB	Heavy metal conc. (300µg/ml)			MAR	Isolate	Pristine soil Probable organism	EDB	EKB	Heavy metal conc. (300µg/ml)			MAR
				Cr	Cu	Pb						Cr	Cu	Pb	
AMT_1	*Bacillus* species	No	No	S	R	S	Yes	SQU_1	*Citrobacter* species	No	No	S	R	S	No
AMT_2	*Bacillus* species	Yes	No	S	R	S	Yes	SQU_2	*Citrobacter* species	No	No	S	S	S	No
AMT_3	*Escherichia coli*	No	No	S	S	R	Yes	SQU_3	*Yersinia* species	No	No	S	S	R	No
AMT_4^{***}	*Pseudomonas* species	Yes	Yes	R	R	R	Yes	SQU_4	*Serratia* species	No	No	S	S	R	No
AMT_5	*Klebsiella* species	No	No	S	S	R	Yes	IS_1	*Yersinia* species	No	No	S	S	R	No
AMM_1	*Aeromonas* species	Yes	No	S	S	R	Yes	IS_2	*Bacillus* species	No	No	S	S	S	No
AMM_2^{***}	*Serratia* species	Yes	Yes	R	R	R	Yes	IS_3	*Serratia* species	Yes	No	S	S	S	No
AMM_3^{***}	*Klebsiella* species	Yes	Yes	R	R	R	No	IS_4	*Citrobacter* species	No	No	S	S	R	Yes
AMM_4	*Enterobacter* species	Yes	Yes	S	S	S	Yes	IS_5	*Bacillus* species	No	S	R	S	S	No
AMM_5	*Enterobacter* species	Yes	No	S	S	S	Yes	BGU_1	*Bacillus* species	No	No	S	S	R	No
AMM_6	*Aeromonas* species	Yes	Yes	S	S	R	Yes	BGU_2	*Bacillus* species	Yes	Yes	S	S	S	No
AME_1	*Corynebacterium* species	Yes	Yes	R	R	R	Yes	BGU_3	*Serratia* species	No	No	S	S	S	No
AME_2	*Serratia* species	Yes	Yes	R	R	R	Yes	CC_1	*Serratia* species	No	No	S	S	S	No
AME_3	*Yersinia* species	Yes	Yes	R	R	R	Yes	CC_2	*Yersinia* species	No	No	S	R	S	Yes
AME_4^{****}	*Bacillus* species	Yes	Yes	R	R	R	No								
AME_5	*Serratia* species	Yes	Yes	R	R	R	Yes								
IS_1	*Shigella* species	Yes	Yes	S	S	R	Yes								
IS_2	*Aeromonas* species	No	No	R	S	S	Yes								
IS_3	*Serratia* species	Yes	No	S	R	S	Yes								
IS_4	*Klebsiella* species	Yes	Yes	S	R	S	Yes								
IS_5	*Pseudomonas* species	Yes	Yes	S	R	S	Yes								

Key: *** = Potentially useful microorganisms for bioremediation, MAR= Multiple antibiotic resistance, EDB = Efficient diesel; biodegraders, EKB = Efficient kerosene biodegraders.

This could be as a result of the presence, of a higher number of microorganisms with degrading metabolic capabilities in the contaminated soil sample (Meldina et al., 2005), as study has shown that depending on the hydrocarbon chain length, enzyme systems (such as cytochrome P450) are required to introduce oxygen into the substrate to initiate biodegradation (Van Beilen and Funhoff, 2007; Obayori et al., 2009).

Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon environments (Atlas, 1992; Amund and Nwokoye, 1993; Lal and Khanna, 1996). Hydrocarbon in the environment are biodegraded primarily by bacteria, yeast, and fungi (Broomjimans et al., 2009), with reported efficiency ranging from 50% to 80% for soil bacteria (Daugulis and McCracken, 2003), as researches have shown that mixed populations of bacteria with overall broad ezymatic capabilities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil (Jan et al., 2003), freshwater (Atlas, 1985), and marine environments (Yakimor et al., 2007).

The result of the heavy metal tolerance test of the bacteria isolates revealed that the sensitivity exhibited by bacteria isolates from both the petroleum polluted and pristine soil samples were proportional to the concentration of the heavy metals utilized. This trend was similar to report by (Rathnayake et al., 2010), who investigated the tolerance of trace metals such as Cd^{2+}, Cu^{2+} and Zn^{2+} by *Paenibacillus* species and *Bacillus thuringiensis*, isolated from a pristine soil. A greater number and percentage of bacteria isolates from the petroleum polluted soils in this study were able to tolerate the seven heavy metals (Pb, Ni, Cr, Cd, Co, Cu and V) tested compared to those from the pristine soil. This was not surprising as the marginal difference could have been due to the selective pressure from the metal content of their growth environment (petroleum polluted soil), since such sample sites are constantly contaminated with petroleum products and metal fillings (Tiku et al., 2016; Jabora et al., 2013).

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 (Misra, 1992; Nies, 1992).

The antibiogram profile of the bacteria isolates from both the petroleum polluted and pristine soil samples 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 (Kim et al., 2006). A study by Oyetibo et al. (2010), reported heavy metal resistant and antibiotic resistant among bacterial isolates, *Pseudomonas* species, *Micrococcus*, *Escherichia coli*, *Bacillus* species and *Proteus* species to Gentamycin (77.7%) Rifampicin (66.0%) and Ofloaxcin (57.3%). The resistance of the organisms to the antibiotics confirms the correlation between resistance metal ions and antibiotic. Also study by Oboh et al. (2006), also reported heavy metal resistance and antibiotics resistance bacterial species from different sources. A study by Nies (2003) 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.

In comparing hydrocarbon, heavy metal tolerability and antibiotic resistance. *Pseudomonas* species (AMT_4), *Serratia* species (AMM_2), *Klebsiella* species (AMM_3) and *Bacillus* species (AME_4), were able to utilize diesel and kerosene efficiently, tolerated high concentration (300µg/ml) of chromium, copper and lead, and did not show multiple antibiotic resistance to the antibiotics tested against, hence they could be useful in the bioremediation of hydrocarbon- heavy metal contaminated environment.

Conclusion

It is obvious from this study that petroleum polluted environments such as auto-mechanic workshops soils could serve as a source of microorganisms especially bacteria that can be useful in the bioremediation of hydrocarbon-heavy metal polluted environment. In addition, the screening of these useful microorganisms for antibiotic resistance is vital in controlling the spread of antibiotic resistant gene to other bacteria in the environment, as this could endanger both the public health and the environment at large.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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