Multiple Metal Tolerance of Bacteria Isolated from Selected Rivers of Cavite, Philippines

Jamaica V. Quiñones-Espineli[1], Yolanda A. Ilagan[2]

[1]Instructor and [2]Professor, respectively, Department of Biological Sciences, College of Arts and Sciences, Cavite State University, Indang, Cavite

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Corresponding author:

ABSTRACT

Metals, in trace amounts, play a role in almost all the metabolic processes, growth and development of microorganisms and in excess can be harmful to them. Increasing concentrations of metals beyond bacteria's tolerance levels force them to develop mechanisms to survive in metal-rich environments. In this study, 190 bacteria which belonged to the genera Aeromonas (49 isolates), Bacillus (21 isolates), Citrobacter (1 isolate), Corynebacterium (22 isolates), E.coli (7 isolates), Enterobacter (5 isolates), Klebsiella (1 isolate), Microccoccus (3 isolates), Morganella (2 isolates), Proteus (3 isolates), Providencia (1 isolate), Pseudomonas (32 isolates), Serratia (16 isolates), Shigella (2 isolates), Staphylococcus (15 isolates), and Yersinia (9 isolates) were isolated from nine rivers of Cavite, Philippines, namely: Ikloy River in Indang, Lit-litan River in Alfonso, Kaong River in Silang, Maragondon River in Ternate, Pulunan and Puting Tubig Rivers in Trece Martires City, Ylang-Ylang River in Gen. Trias, Cañas River in Tanza, and Dasmariñas River in Dasmariñas. The bacterial isolates were tested for their tolerance against lead, copper, mercury, manganese, iron, and zinc at 100, 150, 200, 250, 400, 800, and 1600 ppm concentrations. A decrease in the number of tolerant isolates was observed as the concentration of copper, mercury, and zinc increased. No isolate was able to tolerate 1600 ppm of copper and mercury. Serratia was observed to be the most tolerant genus to lead, copper, zinc, manganese and iron while the genus Bacillus was observed to be the least tolerant to almost all the metals used in this study except to mercury. Four metal tolerance patterns were exhibited

by the bacterial isolates with penta-metal tolerance as the main pattern.

Introduction

Water covers over 70% of the Earth's surface and is a very important resource for people and the environment (Guide Network, 2017). Ninety-seven percent is salt water, 2% is glacier ice, and less than 1% is the amount of freshwater that we can actually use (American Water Works Association, 2002). This little percentage of freshwater that serves most of life's needs is facing a major problem—pollution.

Many of our water resources lack basic protection, making them susceptible to pollution leading to water contamination. Some of the major water pollutants include microbes, nutrients, heavy metals, organic chemicals, oil and sediments (UNESCO-World Water Assessment Programme, 2009). Heat, which raises the temperature of the receiving water, can also be a pollutant (United Nations Department of Economic and Social Affairs, 2014). Heavy metals in ecosystems have received extensive attention because they are toxic, non-biodegradable, and easy to accumulate and magnify in organisms (Zhuang & Gao, 2014). Concentrations of heavy metals in aquatic ecosystems have increased considerably due to the inputs of industrial waste, sewage runoff and agricultural discharges (Prica *et al.*, 2008; Yang *et al.*, 2012). Microorganisms have developed several mechanisms to tolerate high concentrations of heavy metals. Resistance to toxic metals of bacteria probably reflects the degree of environmental contamination with these substances and may be directly related to exposure of bacteria to them (Suriya, Bharathiraja & Rajasekaran, 2013).

Bacterial heavy metal tolerance has implications. Some implications are beneficial like the use of bacteria to clean up metal-contaminated areas. However, other implications are not as beneficial like the correlation between metal tolerance and antibiotic resistance. Studies revealed that when bacteria are exposed to high levels of contamination, they can develop high tolerance with these contaminants and at the same time, they can be more resistant to antibiotics (Spain, 2003).

This study aimed to isolate, identify and analyze heterotrophic metal tolerant bacteria in selected rivers of Cavite, Philippines. Likewise, the physico-chemical characteristics and bacterial population of the rivers were also determined.

Methodology

Materials and Media

This experimental research used plating media that included Cetrimide Agar (CA), Tryptone Glucose Yeast Agar (TGYA), Mc Conkey Agar (MCA), and Brain Heart Infusion Agar (BHIA). These media were sterilized using a pressure cooker at 15 lbs/inch^2 for 15 minutes. All glasswares were cleaned thoroughly and sterilized in the oven at 160-180 ^{o}C for one to two hours before use.

Water Sample Collection

Water samples were collected from selected rivers in upland and lowland Cavite, namely: Litlitan River in Alfonso; Dasmariñas River in Dasmariñas City; Ylang-Ylang River in General Trias; Ikloy River in Indang; Kaong River in Silang; Cañas River in Tanza; Maragondon River in Ternate; and Puting River and Pulunan River in Trece Martires City. Three sterilized bottles were filled with each of the water samples. These samples were collected 20 meters apart against the flow of the river. The bottles were placed in a cooler with ice in order to stop the multiplication of microorganisms before processing.

Determination of the Physico-Chemical Characteristics of the Water Samples

The water samples were analyzed for different parameters in the laboratory of the Department of Biological Sciences, Cavite State University. Different physico-chemical parameters like pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), and total dissolved solids (TDS) were analyzed using CyberScan 6000 Series Meters (Thermo Fisher Scientific).

Bacterial Isolation

Serial dilution of the water samples was performed. One-tenth ml of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were plated on selective media CA and MCA and general purpose media TGYA and BHIA with three replicates each. Inoculated plates were incubated for 24 hours and colonies were counted and recorded. The colony-forming unit was computed to measure the number of bacteria that grew per medium. Colonies were randomly selected on 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions. Only colonies differing in morphological characteristics were chosen. These colonies were streaked on TGYA to purify or obtain single type colonies.

Identification of Bacteria

Identification of isolated bacteria was carried out using conventional biochemical tests according to Bergey's Manual of Determinative Bacteriology and Identification Flow Charts from Protocol Online-Bergey's Manual of Determinative Bacteriology (retrieved from http://www.protocol-online.org). Isolates were identified by gram stain reaction, motility test, catalase test, oxidase test, indole and hydrogen sulfide productions, oxygen requirement, citric acid utilization, urea hydrolysis, utilization of sugar, MRVP test, lysine decarboxylase test, and ornithine decarboxylase test.

Metal Tolerance Testing

Metals used in this study include zinc (zinc sulfate, $ZnSO_4$), lead (lead acetate, $Pb(C_2H_3O_2)_2$), copper (copper sulfate, $CuSO_4$), mercury (mercuric chloride, $HgCl_2$), manganese (manganese sulfate, $MnSO_4$), and iron (iron sulfate, $FeSO_4$). The isolates were grown in master plates and replica plated into metal supplemented NA with 100, 150, 200, 250, 400, 800, and 1600 ppm concentrations. The plates were incubated at room temperature for 48 hours. Isolates that grew, regardless of the size of growth, were considered tolerant.

Analysis of Data

The percentage of metal tolerant isolates was calculated by dividing the number of metal tolerant isolates by the total number of isolates and multiplied by 100. The multiple metal tolerance was obtained by analyzing the number and kind of metals in which the isolates were tolerant to and the correlation of the physico-chemical characteristics of the water samples to bacterial count was determined by Pearson Correlation analysis.

Results and Discussion

Physico-chemical Characteristics of the Water Samples

Good quality of water resources depend on a large number of physico-chemical parameters and biological characteristics (Thirupathaiah *et al.*, 2012). Any alteration of these parameters can affect the quality of water as well as the lives of aquatic organisms (Kaur & Verma, 2014).

The physico-chemical characteristics (pH, dissolved oxygen, biological oxygen

Table 1

Physico-Chemical Characteristics of the Water Samples Collected from the Nine rivers of Cavite

RIVER	WATER PARAMETER			
	pH	DO (mg/L)	BOD (mg/L)	TDS (mg/L)
UPLAND				
Ikloy	6.11	8.4	8.5	188.4
Lit-Litan	6.29	10.1	10.3	123.8
Kaong	6.98	2.3	18.77	197.2
Maragondon	6.54	6.28	7.63	213.8
LOWLAND				
Pulunan	4.81	9.1	8.1	212.1
Puting Tubig	7.32	5.36	8.42	208.5
Ylang-Ylang	6.94	8.9	9.23	341.3
Cañas	7.42	10.6	10.38	228.2
Dasmariñas	7.68	7.3	14.31	245.2
Standard*	**6.0-9.0**	**2(minimum)**	**1-15**	**Not to exceed 1000**

**Based on DENR Standard (DAO 2016-08)*

demand, and total dissolved solids) of the nine rivers in this study are shown in Table 1.

Power of hydrogen (pH). The pH values of the nine rivers ranged from 4.81 to 7.68, acidic to slightly alkaline. Almost all the rivers have pH values within the DENR standard except for Pulunan River (4.81). Low pH can be due to the amount of plant growth and organic material within a body of water and the amount of acid precipitation that falls in the watershed. The low pH of Pulunan River can be harmful to the immature fish and insects living there and it can speed up the leaching of heavy metals present in the river (Oram, 2014).

Dissolved oxygen (DO). Dissolved oxygen concentration is a general indicator to assess organic pollution. High concentration of dissolved oxygen is favorable to aquatic organisms while concentrations lower than 2 mg/L in a prolonged period could be detrimental leading to asphyxiation of fishes (Perez *et al.*, 2015). The dissolved oxygen of the nine rivers ranged from 2.3 mg/L to 10.6 mg/L. The DO values of the nine rivers were higher than the DENR standard which is 2 mg/L (minimum) indicating better water quality (Greenpeace, 2007). Therefore, the nine rivers used in this study could still be considered of good quality in terms of DO level.

Biological Oxygen Demand (BOD). The biological oxygen demand of the nine rivers ranged from 7.63 mg/L to 18.77 mg/L. The BOD values obtained from Ikloy, Lit-litan, Maragondon, Pulunan, Puting Tubig, Ylang-Ylang, Cañas, and Dasmariñas Rivers were within the permissible range set by the DENR while Kaong River exceeded the 15 mg/L limit. The high BOD level of the Kaong River (18.77 mg/L) could be due to voluminous amount of organic matter like decaying plants and fallen leaves (Perez *et al.*, 2015). High BOD levels indicate a decline in DO because the available oxygen in water

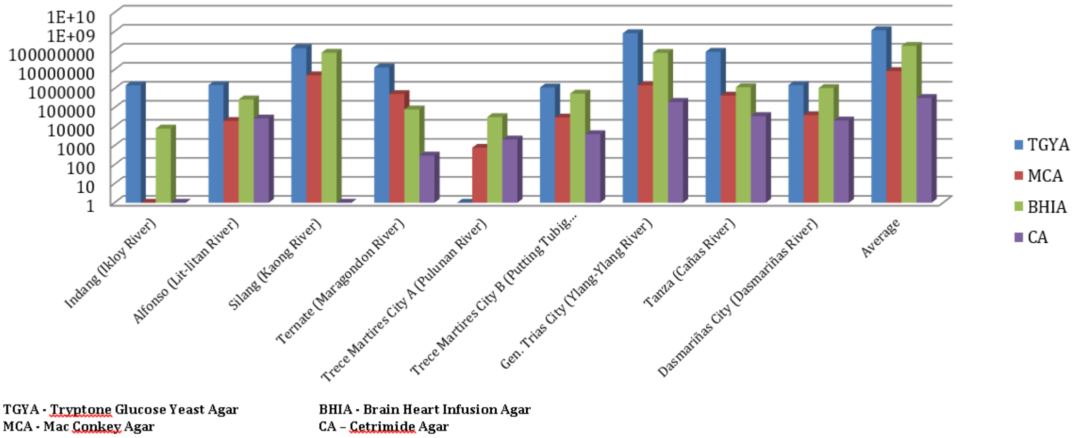

Figure 1. Bacterial count of water samples (cfu/ml) obtained from the nine rivers of Cavite

is being consumed by the bacteria for the decomposition of organic matter leading to the inability of aquatic organisms to survive (Waziri & Ogugbuaja, 2010) and signalling pollution of water (Perez *et al*., 2015).

Total Dissolved Solids. The total dissolved solids of the nine rivers ranged from 123.8 mg/L to 341.3 mg/L. TDS values of the nine rivers did not exceed the 1,000 mg/L limit set by the DENR. Differences in the TDS values may be due to the mixing of pollutants in the river from different anthropogenic activities (Vinod *et al*., 2013). Moreover, according to the group of Vinod (2013), higher TDS level in surface waters increases biological and chemical oxygen demand and eventually depletes the level of dissolved oxygen in water. On the other hand, low TDS level contributes to the palatability of water (Gupta, 2016).

Based on the DENR standard for the five parameters used in this study, Ikloy, Maragondon, Puting Tubig, Cañas, and Dasmariñas Rivers could be considered with good water quality. Lit-litan and Ylang-Ylang Rivers could also be considered of good water quality in terms of pH, BOD, DO, TDS but not in terms of temperature. Pulunan and Kaong Rivers also had good water quality but not in terms of pH and BOD, respectively.

Bacterial Count of the Water Samples

Figure 1 shows the bacterial count of water samples taken from the nine rivers of Cavite. Four media were used in order to isolate different bacteria in the rivers namely: Tryptone Glucose Yeast Extract Agar (TGYA), Mac Conkey Agar (MCA), Brain Heart Infusion Agar (BHIA), and Cetrimide Agar (CA). Since TGYA is a basal or general culture medium used for the cultivation of less fastidious bacteria, it had the highest average count (1.18 x 10^9 cfu/ml) among the four media used in this study. Sample from Gen. Trias City (Ylang-Ylang River) had the highest count in this medium with 8.2 x 10^8 cfu/ml while sample from Trece Martires City A (Pulunan River) had the lowest count (6.1x 10^4 cfu/ml). High bacterial count means high bacterial load in the rivers which can compromise the safety of water for consumption.

BHIA is an enriched medium used for the isolation of fastidious bacteria. In this study, growth in BHIA had an average count of 1.76 x 10^8 cfu/ml. Sample from Silang (Kaong River) had the highest count (7.7 x 10^7 cfu/ml) in this medium. This indicated that majority of the bacteria isolated from this river were facultative anaerobes. Sample from Indang (Ikloy River) had the lowest count of 8.0 x 10^3 cfu/ml.

Table 2

Correlation of Physico-Chemical Characteristics and Bacterial Count of the Water Samples

WATER PARAMETER	MEDIUM					
	TGYA			MCA		
	Pearson-R	P-Value	Remark	Pearson-R	P-Value	Remark
pH	.154	.442	NS	.117	.563	NS
Dissolved Oxygen (DO)	-.751	.000	HS	.171	.393	NS
Biological Oxygen Demand (BOD)	.826	.000	HS	-.126	.530	NS
Total Dissolved Solids (TDS)	-.098	.627	NS	.808	.000	HS

HS – Highly significant ; S – Significant ; NS – Not Significant

TGYA – Tryptone Glucose Yeast Extract Agar ; MCA – Mac Conkey Agar

MCA is being used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae (Aryal, 2015). Presence of Enterobacteriaceae indicates fecal pollution in bodies of water (Figueras & Borrego, 2010). In this medium, an average count of 8.36 x 10^6 cfu/ml was observed. Sample from Silang (Kaong River) had the highest count of 5.0 x 10^6 cfu/ml. This indicated that most of the bacteria isolated from this river were enteric bacteria. Trece Martires City A (Pulunan River) gave the lowest count with only 7.8 x 10^2 cfu/ml and no growth was observed in this medium from sample obtained in Indang (Ikloy River). No bacterial growth in this medium from sample obtained in Ikloy River did not indicate that this river was not contaminated with fecal wastes. The fact that there were bacterial colonies that grew from sample taken in this river in TGYA and BHIA implied that it was polluted with fecal wastes.

CA is used for selective isolation and presumptive identification of *Pseudomonas aeruginosa* (Rijal, 2015). The presence of *Pseudomonas* poses health risk since it causes different kinds of diseases. CA had an average count of 3.23 x 10^5 cfu/ml. Ylang-Ylang River had the highest count with 2.0 x 10^5 cfu/ml. On the other hand, sample from Ternate (Maragondon River) had the lowest count with 3.0 x 10^2 cfu/mL. No growth on this medium was observed from samples obtained in Indang (Ikloy River) and Silang (Kaong River).

Correlation of the Physico-chemical Characteristics and Bacterial Count of the Water Samples

Correlation of the physico-chemical characteristics and bacterial count of the water samples is shown in Table 2. Bacterial count in TGYA had a positive significant correlation with biological oxygen demand (BOD), a negative significant correlation with dissolved oxygen (DO) and no significant correlation with pH, and total dissolved solids (TDS). This significant correlation indicates that an increase in the value of BOD will lead to an increase in the bacterial count and vice versa. On the other hand, an increase in value of DO will result to the decrease of bacterial count and vice versa. BOD is a measure of the oxygen used by microorganisms to decompose organic wastes. If there is a large quantity of organic waste in the water supply, there will also be a lot of bacteria present to decompose this waste. In this case, the demand for oxygen will be high, so the BOD level will be high (retrieved from http://www.polyseed.com/misc/BODforwebsite.pdf).

Bacterial count in MCA had a positive significant correlation with TDS and no significant correlation with pH, dissolved oxygen (DO), and biological oxygen demand (BOD). This implies that as the value of TDS increases, bacterial count also increases and vice versa.

Bacterial Isolates

A total of 190 bacterial isolates which belonged to 16 different genera were isolated from the nine rivers of Cavite, Philippines. These bacterial isolates differ in their morphological characteristics. Seventy-eight isolates were from upland Cavite and 112 from lowland Cavite (Table 3). Twenty-eight (14.74%) isolates were obtained from Ternate (Maragondon River). This was the highest number of bacterial isolates among the nine sampling sites. The least number of isolates was obtained from Indang (Ikloy River) with only 14 (7.37%) isolates. The number of isolates varied because of the differences in the bacterial count per river as shown in Figure 1.

Only colonies differing in morphological characteristics were selected and used for further studies. This variation in the number of isolates implied that bacteria present in the rivers were of different kinds.

Table 3 also shows the different genera of bacteria isolated from the nine rivers of Cavite.

The Gram-positive rod isolates were identified as *Bacillus* and *Corynebacterium* with one remaining as unidentified. Gram-positive coccus isolates were identified as *Micrococcus* and *Staphylococcus*.

Most of the isolates were Gram-negative rods belonging to family Enterobacteriaceae and identified as *Pseudomonas*, *Aeromonas*, *Serratia*, *Klebsiella*, *Enterobacter*, *Yersinia*, *Shigella*, *Proteus*, *Providencia*, *Morganella*, *Citrobacter*, and *E.coli*. Members of family Enterobacteriaceae are common and widespread in the environment. They are used as indicator organisms in evaluating fecal pollution in various bodies of water (Figueras & Borrego, 2010). Thus, the presence of these bacteria particularly *Serratia, Klebsiella, Enterobacter, Yersinia, Shigella, Proteus, Providencia, Morganella, Citrobacter,* and *E.coli* in rivers is a clear indication of fecal contamination.

The microbial composition differs in lowland and upland water samples. The genera *Bacillus, Corynebacterium*, *Pseudomonas*, *Aeromonas*, *Serratia, Enterobacter*, *Yersinia*, *E.coli*, and *Staphylococcus* were present in water samples taken from both upland and lowland Cavite rivers. On the other hand, *Klebsiella*, and *Micrococcuss* were only present in water samples taken from upland rivers while *Shigella*, *Proteus*, *Providencia*, *Morganella*, and *Citrobacter* were only found in samples taken from lowland rivers.

Table 3

Number and genera of bacterial isolates obtained from the nine rivers of Cavite

LOCALITY	NUMBER OF BACTERIAL ISOLATES	PERCENTAGE	GENUS FOUND
UPLAND			
Indang (Ikloy River)	14	7.37	**Gram (+) rod**
Alfonso (Lit-litan River)	18	9.47	*Bacillus*
Silang (Kaong River)	18	9.47	*Corynebacterium*
Ternate (Maragondon River)	28	14.74	Unidentified
			Gram (+) coccus
			Micrococcus

			Staphylococcus
			Gram (-) rod
			Pseudomonas
			Aeromonas
			Serratia
			Klebsiella
			Enterobacter
			Yersinia
			E.coli
LOWLAND			
			Gram (+) rod
Trece Martires City A (Pulunan River)	24	12.63	*Bacillus* *Corynebacterium*
Trece Martires City B (Puting Tubig River)	16	8.42	**Gram (+) coccus** *Staphylococcus*
General Trias City (Ylang-Ylang River)	20	10.53	**Gram (-) rod** *Pseudomonas*
Tanza (Cañas River)	25	13.16	*Aeromonas*
Dasmariñas City (Dasmariñas River)	27	14.21	*Enterobacter* *Yersinia*
			Shigella
			Proteus
			Providencia
			Morganella
			Citrobacter
			E.coli
TOTAL	**190**	**100.00**	

Table 4

Percentage of Bacteria Tolerant to different Concentrations of Heavy Metals

CONCENTRATION (ppm)	METAL/PERCENTAGE OF TOLERANT BACTERIA					
	Lead	Copper	Mercury	Manganese	Iron	Zinc
100	100.00	98.42	86.84	100.00	100.00	99.47
150	100.00	97.37	83.68	100.00	100.00	99.47
200	100.00	94.74	80.53	100.00	100.00	99.47
250	100.00	88.95	79.47	100.00	100.00	98.42
400	98.95	80.00	77.37	100.00	100.00	94.74
800	94.74	40.00	30.00	100.00	100.00	88.42
1600	81.58	0.00	0.00	81.05	76.84	42.11

Total number of bacterial isolates: 190

Heavy Metal Tolerance of Bacterial Isolates

In this study, the bacterial isolates exhibited tolerance to lead, copper, mercury, manganese, iron, and zinc at varying concentrations (Table 4). All the isolates were able to tolerate lead up to 250 ppm and manganese and iron up to 800 ppm. Meanwhile, a decrease in the number of tolerant isolates was observed as the concentration of copper, mercury, and zinc increased. No isolate was also able to tolerate 1600 ppm of copper and mercury. The results indicated that increasing concentrations of metals inhibit bacterial growth. The sustainable growth in heavy metal environment of microorganisms depends on their tolerance to heavy metal. The optimum concentration of heavy metal is a critical factor that influences the survival of microorganisms (Prasanth & Mahesh, 2016).

Among the metals tested, iron and manganese were the most tolerated. All isolates could grow on media containing as high as 800 ppm of these metals, and more than three fourths of the isolates could also grow at 1600 ppm concentration. Iron is a component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions (Todar, 2012). Manganese functions in cofactoring enzymes and providing protection against reactive oxygen species (Zeinert *et al.*, 2018). Lead and zinc were also tolerated followed by copper and mercury. However, at 1600 ppm of copper and mercury, no bacterial growth was observed. Mercury and its compounds bind to the sulfhydryl groups of proteins and enzymes, thus inactivating vital cell functions (Mirzaei *et al.*, 2008). Copper damages the microbial DNA, alters bacterial protein synthesis as well as the membrane integrity (Warnes *et al.*, 2010; Grass *et al.*, 2011; Chaturvedi & Anderson, 2014).

Most industrial discharges contain a huge amount of heavy metals which contaminate nearby bodies of water and continually increase the concentrations of metals. Because of the slowly increasing concentrations of toxic metals, bacteria can develop resistance against it. However, presence of heavy metals in large amount enables the bacteria to develop additional strategies to cope up with a higher concentration stress (Oves & Hussain, 2016).

Table 5 shows the percentage of bacteria per genus tolerant to heavy metals at 800 ppm concentration. Considering the genera with at least ten isolates, *Serratia* was observed to be the most tolerant genus to lead, copper, zinc, manganese, and iron while the least tolerant to mercury. Meanwhile, the genus *Bacillus* was observed to be the least tolerant to almost all the metals used in this study except to mercury. *Serratia* being most tolerant to lead implies that this bacterial genus has plasmid-borne resistant gene or pump effluxing system which involves a protein transporter localized in the cytoplasmic membrane that requires a source of energy, either ATP or proton gradient (Nageswaran *et al.*, 2012). Its being the most tolerant to copper may probably due to evolution of some types of mechanisms to resist copper toxicity. These systems may include compartmentalization and efflux systems. Compartmentalization is done by the proteins found in the periplasm while efflux mechanism is the process whereby copper is removed from the soil through the plasmid-bound genes (Spain *et al.*, 2003). High tolerance to zinc may be attributed to the two efflux mechanisms which transport zinc ions across the cytoplasmic membrane through ATP hydrolysis and one which transports zinc across the cell wall of gram-negative bacteria through proton-gradient (Nies, 1999).

Interactions between microbes and metals have important environmental and health implications. It can be useful as bacteria can be utilized to clean up metal-contaminated sites. On the other hand, it

Table 5

Percentage of bacteria per genus tolerant to heavy metals at 800 ppm concentration

GENUS NAME	TOTAL NO. OF ISOLATES	METAL/PERCENTAGE OF TOLERANT BACTERIA					
		Lead	Copper	Mercury	Manganese	Iron	Zinc
Aeromonas	49	97.96	30.61	32.65	100.00	100.00	89.80
Bacillus	21	76.19	9.52	28.57	42.86	42.86	71.43
Citrobacter	1	100.00	0.00	0.00	100.00	100.00	100.00
Corynebacterium	22	86.36	31.82	22.73	100.00	100.00	81.82
E.coli	7	100.00	85.71	71.43	100.00	100.00	100.00
Enterobacter	5	100.00	60.00	60.00	100.00	100.00	80.00
Klebsiella	1	100.00	0.00	0.00	100.00	100.00	100.00
Micrococcus	3	100.00	0.00	0.00	100.00	100.00	66.67
Morganella	2	100.00	100.00	100.00	100.00	100.00	100.00
Proteus	3	100.00	100.00	66.67	100.00	100.00	100.00
Providencia	1	100.00	100.00	100.00	100.00	100.00	100.00
Pseudomonas	32	96.88	31.25	25.00	100.00	100.00	90.63
Serratia	16	100.00	81.25	12.50	100.00	100.00	100.00
Shigella	2	100.00	0.00	50.00	100.00	100.00	100.00
Staphylococcus	15	100.00	66.67	40.00	100.00	100.00	86.67
Unidentified	1	100.00	0.00	0.00	100.00	100.00	100.00
Yersinia	9	88.89	33.33	0.00	100.00	100.00	88.89

can be disadvantageous as the presence of metal tolerance mechanism may contribute to the increase in antibiotic resistance (Spain, 2003).

The bacterial isolates also exhibited multi-metal tolerance at 800 ppm concentration. More than one-third (34.74% and 38.42%) of the isolates were tolerant to four and five metals, respectively. This indicated that more than half of the isolates had the ability to tolerate either four or five metals. Meanwhile, more than one-eighth (13.68%) of the isolates were able to tolerate the six heavy metals tested.

Heavy metals are similar in their toxic mechanism thus, multiple tolerances are common phenomena among heavy metal tolerant bacteria (Sinha *et al.*, 2013). In addition, different groups of bacteria with a potential to tolerate a variety of toxic heavy metals suggest that resistance to many types of toxicants may be present in the same organism thus have high potential for biotechnology purposes (Keramati, Hoodaji, & Tahmourespour, 2011).

Metal Tolerance Patterns of Bacterial Isolates

The bacterial isolates exhibited four heavy metal tolerance patterns (see Appendix Table 1). Seventy-three out of 190 isolates displayed penta-metal tolerance pattern with three different tolerance combinations. The same result was obtained by Chauhan *et al.* (2015) and Sabry *et al.* (1997) in their studies. Tetra-metal tolerance pattern was exhibited by 66 isolates with four different combinations. Meanwhile, only 26 isolates and 25 isolates displayed hexa-metal tolerance pattern and tri-metal tolerance pattern respectively with two different tolerant ccombinations.

Bacterial resistance to toxic metals reflects the degree of environmental contamination with these substances and may be directly related to exposure of bacteria to them (Suriya, Bharathiraja & Rajasekaran, 2013). In addition, variations in the patterns of metal tolerance among the bacterial isolates may be due to the difference in the concentrations of different heavy metals in the rivers.

Microorganisms possess a variety of mechanisms to deal with high concentrations of heavy metals and often are specific to one or a few metals (Nies, 2003). Therefore, from environmental point of view, bacteria with multiple heavy metal tolerance are of great importance. They have great potential in heavy metal remediation by different processes including metal uptake (Achal *et al.*, 2012).

Conclusions and Recommendations

The study aims to isolate, identify and analyze heterotrophic metal tolerant bacteria in selected rivers of Cavite, Philippines. The results could indicate the rivers' degree of contamination with heavy metals and assess the rivers' present status as well.

Based on the results of this study, the pH of the rivers is within the DENR standard except for Pulunan River. The low pH of Pulunan River can be due to the amount of plant growth and organic material within the river and the amount of acid precipitation that falls in its watershed. DO and TDS of all the nine rivers are within the limit set by the DENR. BOD is also within the DENR standard which is 1-15 mg/L except for Kaong River. An increase in BOD leads to an increase in the bacterial count in TGYA and vice versa while an increase in DO results in the decrease of bacterial count and vice versa. As TDS increases, bacterial count in Mc Conkey Agar also increases and vice versa.

Bacterial population differs from river to river and types of media used. Bacterial isolates present in the rivers belong to the genera *Bacillus, Corynebacterium, Pseudomonas, Aeromonas, Serratia, Klebsiella, Enterobacter, Yersinia, Shigella, Proteus, Providencia, Morganella, Citrobacter, E. coli, Micrococcus,* and *Staphylococcus.* These isolates can tolerate lead, copper, mercury, manganese, iron and zinc with *Serratia* as the most tolerant to heavy metals. These bacteria can tolerate at least three metals. They also exhibit four metal tolerance patterns with penta-metal tolerance as the main pattern. Heavy metals, at high concentrations, are toxic to microorganisms and yet several bacteria are able to tolerate them. This phenomenon is alarming because of its health implications to humans. However, these bacteria may have other uses. These microorganisms could be tapped for their cleaning-up potential. If they could tolerate very high concentrations of metals then, these bacteria could be tested for their ability to accumulate these metals and recover/recycle them for other purposes.

The study does not cover the determination of metal concentration in water samples from the rivers and the sources of metal pollutants. Thus, studies regarding those areas must be conducted. In addition, mechanisms of metal tolerance of the bacterial isolates, confirmation of the identity of the isolates by molecular techniques like 16S RNA sequencing and analysis of genes responsible for metal tolerance can be explored.

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Appendix Table 1

Heavy metal tolerance patterns of bacterial isolates based on 800 ppm metal concentration

TOLERANCE PATTERN	ISOLATE IDENTITY	HEAVY METAL TOLERANCE*							
		NO. OF ISOLATES	%	Copper	Mercury	Zinc	Lead	Manganese	Iron
Hexa – T	*Serratia*	2	1.05	+	+	+	+	+	+
	Providencia	1	0.53	+	+	+	+	+	+
	Staphylococcus	5	2.63	+	+	+	+	+	+
	Corynebacterium	3	1.58	+	+	+	+	+	+
	Aeromonas	4	2.11	+	+	+	+	+	+
	Enterobacter	1	0.53	+	+	+	+	+	+
	Bacillus	1	0.53	+	+	+	+	+	+
	Pseudomonas	1	0.53	+	+	+	+	+	+
	E.coli	4	2.11	+	+	+	+	+	+
	Proteus	2	1.05	+	+	+	+	+	+
	Morganella	2	1.05	+	+	+	+	+	+
	Total	**26**	**13.70**						
Penta – T	*Aeromonas*	10	5.26	+	-	+	+	+	+
		11	5.79	-	+	+	+	+	+
	Pseudomonas	9	4.74	+	-	+	+	+	+
		7	3.68	-	+	+	+	+	+
	Serratia	10	5.26	+	-	+	+	+	+
		1	0.53	-	+	+	+	+	+
	Staphylococcus	4	2.11	+	-	+	+	+	+
		1	0.53	+	+	-	+	+	+
	Corynebacterium	2	1.05	+	-	+	+	+	+

Appendix Table 1 (cont'd)

Heavy metal tolerance patterns of bacterial isolates based on 800 ppm metal concentration

TOLERANCE PATTERN	ISOLATE IDENTITY	HEAVY METAL TOLERANCE*							
		NO. OF ISOLATES	%	Copper	Mercury	Zinc	Lead	Manganese	Iron
		2	1.05	-	+	+	+	+	+
	Bacillus	5	2.63	-	+	+	+	+	+
	Enterobacter	2	1.05	-	+	+	+	+	+
		1	0.53	+	-	+	+	+	+
	E.coli	2	1.05	+	-	+	+	+	+
		1	0.53	-	+	+	+	+	+
	Yersinia	3	1.58	+	-	+	+	+	+
	Shigella	1	0.53	-	+	+	+	+	+
	Proteus	1	0.53	+	-	+	+	+	+
	Total	**73**	**38.43**						
Tetra – T	*Citrobacter*	1	0.53	-	-	+	+	+	+
	Bacillus	5	2.63	-	-	+	+	+	+
		1	0.53	+	-	+	-	+	+
	Corynebacterium	8	4.21	-	-	+	+	+	+
		1	0.53	+	-	-	+	+	+
		1	0.53	+	-	+	-	+	+
	Pseudomonas	11	5.79	-	-	+	+	+	+
	Aeromonas	18	9.47	-	-	+	+	+	+
		1	0.53	-	+	-	+	+	+
		1	0.53	+	-	+	-	+	+
	Serratia	3	1.58	-	-	+	+	+	+

Appendix Table 1 (cont'd)

Heavy metal tolerance patterns of bacterial isolates based on 800 ppm metal concentration

TOLERANCE PATTERN	ISOLATE IDENTITY	HEAVY METAL TOLERANCE*							
		NO. OF ISOLATES	%	Copper	Mercury	Zinc	Lead	Manganese	Iron
	Micrococcus	2	1.05	-	-	+	+	+	+
	Staphylococcus	4	2.11	-	-	+	+	+	+
	Klebsiella	1	0.53	-	-	+	+	+	+
	Enterobacter	1	0.53	+	-	-	+	+	+
	Yersinia	4	2.11	-	-	+	+	+	+
		1	0.53	+	-	+	-	+	+
	Shigella	1	0.53	-	-	+	+	+	+
	Unidentified	1	0.53	-	-	+	+	+	+
	Total	**66**	**34.78**						
Tri – T	*Bacillus*	5	2.63	-	-	-	+	+	+
		4	2.11	-	-	+	-	+	+
	Corynebacterium	3	1.58	-	-	-	+	+	+
		2	1.05	-	-	+	-	+	+
	Micrococcus	1	0.53	-	-	-	+	+	+
	Pseudomonas	3	1.58	-	-	-	+	+	+
		1	0.53	-	-	+	-	+	+
	Staphylococcus	1	0.53	-	-	-	+	+	+
	Aeromonas	4	2.11	-	-	-	+	+	+
	Yersinia	1	0.53	-	-	-	+	+	+
	Total	**25**	**13.18**						

**(+) – Tolerant; (-) – Not-tolerant*