Removal of heavy metals from aqueous solution by biofilm-forming bacteria isolated from mined-out soil in Mogpog, Marinduque, Philippines

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Heavy metal pollution evokes scary health scenarios which should prompt moves for proper treatment to prevent further heavy metal contamination and spread. In this study, heavy metals from synthetic solution were removed through bisorption using biofilm-forming bacteria. Eight out of 90 bacterial isolates obtained from mined-out soil in Mogpog, Marinduque exhibited biofilm formation and tolerance to Cu, Pb, Zn and Cd. Isolates NV1A and NV2A formed the thickest biofilm and isolate NV1A showed the highest degree of tolerance to the four heavy metals. All eight isolates reduced the Cu content with 15-68% efficiency using 100 mg·L-1 Cu in solution at six hours of contact time. The amount of Cu removed by five isolates (NV1A, NV2A, NV11, NV22, and NV112) decreased when 10 mg·L-1 Cu, 0.5 mg·L-1 Cd, 5 mg·L-1 Pb, and 200 mg·L-1 Zn were present in aqueous solution. In contrast, amounts of Cu removed by the isolates NV17 and R11 from a multimetal solution increased compared to that of the Cu only solution while the amount remained the same for isolate NV211. Identification of the isolates by *16s rDNA* sequencing revealed that NV1A, NV22 and NV112 have 98 to 100% sequence similarities with *Rhodococcus equi and R. opacus*; NV2A, 100% sequence similarity with *Pseudomonas aeruginosa*; R11, 99% sequence similarity with *Bacillus megaterium*; NV11, 97% sequence similarity with *Solibacillus silvestris* and *Lysinibacillus sphaericus*; NV211, 97% sequence similarity with *Enterococcus faecium* and NV17, 96% sequence similarity with three species of *Bacillus*. The isolates identified as promising should be studied further for potential application in the removal of heavy metals in actual industrial wastewaters. Any emerging technology from such studies that would prove successful could lead to significantly lesser cost of heavy metal remediation.

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INTRODUCTION

Heavy metal pollution is one of the most pressing environmental concerns associated with industrialization. Heavy metals (HM), such as mercury, lead, cadmium, arsenic, chromium, zinc, copper, and manganese are incorporated into soils and waters through industrial, agricultural and domestic effluents (Jjemba 2004). When concentrations exceed normal levels, they become potentially hazardous. As they are not metabolically-degradable, concentrations increase as they progress through the food chain. Excessive amounts in humans can cause toxicity, cellular function disorders, long-term debilitating disabilities, and eventually death (Naja et al. 2010).

In the Philippines, HM-contaminated areas include abandoned mined-out sites and river systems adjacent to industrial sites

Table 1: Relative growth of biofilm-forming bacteria isolated from soil of an abandoned, mined-out site in Mogpog, Marinduque grown in Trypticase Soy broth supplemented with different concentrations of a mixture of heavy metals incubated under ambient room conditions for 48 hours

Isolates	Concentration of Heavy Metals*					
	C0	C1	C2	C3	C4	C5
NV1A	+	+	+	+	+	+
NV2A	+	+	-	-	-	-
NV11	+	+	+	-	-	-
NV17	+	+	+	-	-	-
NV22	+	+	+	+	-	-
NV112	+	+	+	+	-	-
NV211	+	+	+	+	-	-
R11	+	+	+	+	-	-

***C0**: no heavy metals; **C1**: 170 mg·L^{-1} Pb, 1.6 mg·L^{-1} Cd, 72 mg·L^{-1} Cu and 280 mg·L^{-1} Zn;
C2: C1 x 2; **C3**: C1 x 3; **C4**: C1 x 4; **C5**: C1 x 5; **+** = with growth ; - = without growth

such as an abandoned copper mined-out site in Mogpog Marinduque, reported to be contaminated with high levels of copper (Raymundo 2006; Llamado et al. 2013). The Meycauayan, Bulacan river system, likewise, has been reported as heavily-polluted with HM with the jewelry making , gold smelting, battery recycling, pyrotechnic, and tanneries effluents (Mendoza 2012). In tilapia fishponds, mercury and cadmium were reportedly present in the surface water and that traces of these two HM have been detected in the fish as well. The shells used for feeding prawns and ducks were found to have chromium (pureearth.org/project/marilao-industrial-waste-contamination). These findings evoke scary health scenarios which should prompt moves for proper treatment to prevent further HM contamination and spread.

The removal of heavy metals from industrial wastewater is generally done through physico-chemical methods, consisting of chemical precipitation, chemical oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, ion exchange and membrane technologies (Hussein et al. 2004). However, for solutions containing 1 - 100 mg·L-1 dissolved heavy metal ions, these processes maybe ineffective or expensive (Volesky 2001). Biological methods, such as microbial remediation, may provide cheaper and yet effective alternatives.

Microorganisms have high affinity for metals. The microbial cells' surfaces are negatively- charged due to the presence of various anionic structures and consequently are capable of binding metal cations. This specific microbial property is capitalized in biosorption which is the most prominent emerging alternative technology for removing HM as it allows the accumulation of the pollutants in both living or dead microbial cells via metabolically-independent pathways (Atlas and Philip 2005). The advantages of biosorption over physico-chemical methods include low capital outlay, metal selectivity, high efficiency, minimal chemical and biological sludge, no additional nutrient requirement, regeneration of biosorbent, and the possibility of metal recovery (Volesky 2001). Biosorption, as a means of removing toxic heavy metals, is especially suited as a 'polishing' wastewater treatment step as the quality of the outcome is close to the level of potability. From initial metal concentrations of 1 - 100 mg·L-1 it can be reduced to < 0.01 - 0.1 mg·L-1, especially in packed bed flow-through applications (Naja et al. 2010).

Biofilm formation is one important characteristic if bacteria are to be utilized in heavy metal removal through biosorption. Biofilm's most essential constituent with ion sequestration capability is extracellular polysaccharides or exopolysaccharide, which can exist either as capsule or slime (Gupta and Diwan 2017).

This study was conducted with the aim of isolating bacteria from mined-out soil from Mogpog, Marinduque, screening them for heavy metal tolerance and biofilm formation and using them in removing heavy metals in solution through biosorption.

MATERIALS AND METHODS

Isolation of bacteria from soil samples

Soil samples, as source of bacterial isolates, were collected from an abandoned, copper mined-out site geographically (N13029'54" and S121052'12"E) located in Brgy. Capayang, Mogpog, Marinduque, Philippines. Isolation was done by resuspending 10 grams of composite soil sample in 90 mL sterile phosphate buffered saline. About 100 µL of the soil extracts was spread-inoculated on Tryptic Soy Agar (TSA) plate supplemented with Nystatin at a final concentration of 1,000 units/mL. Inoculated plates were incubated at ambient room condition for 2-3 days or until colony growth was visible. All colonies obtained from the isolation plates were further purified by streaking on TSA plates. In addition, 25 bacterial isolates used in a previous biodiversity study (Llamado et al. 2013) of the same site were included in the subsequent experiment to increase the chances of selecting and identifying biofilm-forming heavy metal-resistant bacteria.

Screening bacterial cultures for biofilm formation

The assay procedure of O'toole et al. (2005) for biofilm formation was followed with some modifications. Briefly, each of the 96 flat-bottomed wells of a microtiter plate was filled with 50 µl fresh TSB and 50 µL of an overnight bacterial culture. After 72 hours of incubation at ambient room conditions, planktonic cells were removed from the wells, followed by thorough washing with sterile distilled water. Biofilm formation was detected by filling the wells with 125 µL of 0.1% aqueous crystal violet. After 10 minutes, the crystal violet solution was removed and the wells were washed with distilled water and air-dried. The presence of blue/violet color in the wells indicated biofilm formation.

The degree of thickness of the biofilm formed was qualitativelyevaluated by comparing it with *Pseudomonas aeruginosa* (BIOTECH 136**8**), a known biofilm former (Kievit 2009; Teitzel and Parsek 2003; O'Tootle and Kolter 1998). The more intense the color is after crystal violet staining, the thicker the biofilm becomes.

Screening bacterial cultures for heavy metal tolerance

The heavy metal tolerance level of the eight selected biofilm-forming isolates was determined using Pb, Cd, Cu and Zn. These four heavy metals were selected based on the results of the previous studies (Llamado 2010; Llamado et al. 2013) which showed that these four heavy metals were present in the soil collected from Mogpog, Marinduque. Five mixtures of the four heavy metals were prepared with each component in increasing concentrations; the first to fifth levels consisted of metal concentrations that were 2-10x the amount of acceptable metal contents of environmental samples, particularly soils (Table 1), based on the Dutch standards (Chen 2000).

About 100 µL of 24-hour culture broth of the bacterial isolates were inoculated into 5 mL TSB supplemented with the aforementioned concentrations of the four heavy metals. All inoculated broths were incubated at ambient room conditions for 48 hours after which growth was observed by qualitatively checking for turbidity.

Removal of copper in single metal aqueous solution by the biofilm-forming isolates

The two bacterial isolates, NV1A and NV2A, which were observed to form the thickest biofilm, were selected for the determination of the contact time needed to remove the highest possible amount of heavy metal in solution through biosorption. They were individually-grown in 450 mL TSB for 72 hours with shaking at ambient room conditions. The cells were harvested by centrifugation at 12,000x g for 15 minutes and were resuspended in 250 mL sterile distilled water thereafter to make the biomass stock. About 5 mL from this biomass stock was centrifuged as above after which the cells were dried at 55 °C for 24 hours to determine their dry weights. The amount of biomass stock needed for the assay was computed based on the resulting dry weight at a final concentration of 2.0 grams biomass per liter of heavy metal solution.

About 50 mL each of Cu solutions of 10 mg·L-1 and 100 mg·L-1 concentrations were dispensed in polyethylene terephthalate bottles and added with pre-determined amount of biomass stock. The cell-aqueous metal mixtures were incubated with shaking under ambient room conditions after which samples were collected after 6, 12, 18 and 24 hours. Following incubation, the samples were centrifuged as above to remove the cells. The resulting supernatant was collected for analysis of copper content through atomic absorption spectroscopy at the Central Analytical Services Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH), UP Los Baños, College, Laguna.

After establishing that the 100 mg·L-1 copper in aqueous solution and 6 hours of contact time were the best conditions for copper removal, the biosorptive capacity of the eight isolates was determined using the same conditions.

Removal of heavy metals in multi-metal aqueous solution by the biofilm forming isolates

Since the potential application of any technology developed from the present study would be for wastewaters the most commonly encountered levels of Cu, Cd, Pb and Zn in industrial settings, obtained from existing literature, were considered in succeeding biosorptive experiments. These levels were as follows: Cu content from 1 to 100 mg·L-1 (Onundi et al. 2010, Lai and Lin 2003; You et al. 2001), Cd content of around 10 mg·L-1 (United States Environmental Protection Agency website), Pb content of 1 to 1.5 mg·L-1 (Onundi et al. 2010, You et al. 2001), and Zn content of around 160 mg·L-1 (Blocher et al. 2003). Water quality guidelines as set by the DENR were also considered. The guidelines for Class C were as follows: Cd level of 0.005 mg·L-1, Cu level of 0.02 mg·L-1, Pb level of 0.05 mg·L-1 , and Zn level of 2 mg·L-1 (Department of Environment and Natural Resources website). Based on the aforementioned values, the mixture of metals for the succeeding biosorptive experiments were determined to be those levels at least 100% increase over the DENR guidelines. A multi-metal aqueous solution containing 10 mg·L-1 Cu, 0.5 mg·L-1 Cd, 5 mg·L-1 Pb, and 200 mg·L-1 Zn was prepared for the biosorption experiment using the eight selected biofilm-forming heavy metal-tolerant isolates. The bacterial biomass stock was prepared as described above. Bacterial biomass was added to 50 mL of multi-metal solution to a final concentration of 2.0 grams per liter and incubated at ambient room conditions with shaking for 6 hours. The supernatant was subsequently analyzed for remaining metal content using atomic absorption spectroscopy at BIOTECH, UP Los Baños, College, Laguna.

Cultural, morphological and physiological characterization of the best isolates

Cultural appearance and morphological characteristics of the isolates were described following standard microbiological methods (Raymundo et al. 2015). Observations under the scanning electron microscope were also made to confirm the attached growth and excretion of polymeric substances by the isolates.

The Biolog Gen III MicroPlate™ ID kit (Biolog California USA) was utilized in determining the physiological abilities of the biofilm-forming isolates following the manufacturer's protocol.

Identification of the isolates through 16S rDNA sequence analysis

The genomic DNA of each biofilm-forming isolate grown in TSB for 24 to 48 hours was extracted using ZR Fungal/Bacterial DNA Kit™ (Zymo Research California USA) according to the manufacturer's protocol. Polymerase Chain Reaction (PCR) procedure was done using primers 11F (5'GTTTGATCMTGGCTCAG 3') and 1492R (5'TACGGCTACCTTGTTACGACTT3') (Green et al. 2004) with 1 µL of undiluted genomic DNA extract as template. The PCR reagent mixture for a 25 µL reaction was based on the work of Llamado et al. (2013). The components were as follows: 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM mixed dNTPs, 0.1 µM of each primer and 0.5 U of *Taq* Polymerase (In vitrogen). The cycle conditions based on the PCR profile employed by Ellis et al. (2003) were utilized.

Agarose gel electrophoresis was employed to detect amplification of the *16S rDNA* of the biofilm-forming isolates. The amplified products were sequenced at the Macrogen Sequencing facility in Korea and sequences were compared with those stored in the Genbank databases of the National Center for Biotechnology Information available on-line using pairwise alignment or BLAST algorithm. To more accurately determine the evolutionary similarity based on 16S rDNA sequence of the biofilm-forming isolates and those in the GenBank databases, a phylogenetic tree was constructed using Clustal X alignment.

The 16S *rRNA* sequence data derived from this study have been deposited to the GenBank database under the following accession numbers:
SUB1311058NV1AKU561906;
SUB1311058NV2AKU561907;
SUB1311058NV11KU561908;
SUB1311058NV17KU561909;
SUB1311058NV22KU561910;
SUB1311058NV112KU561911;
SUB1311058NV211KU561912;
SUB1311058 R11KU561913

Table 2: Amount of copper removed through time by bacterial cells of biofilm-forming isolates from aqueous metal solution incubated with mixing under ambient room conditions

Time (H)	10 mg·L-1 Copper Solution		100 mg·L-1 Copper Solution	
	Final copper concentration (mg·L^{-1})	% Copper removed	Final copper concentration (mg·L^{-1})	% Copper removed
Isolate NV1A				
0	9.84	0	92.81	0
6	4.59	53.4	47.68	48.6
12	5.22	47.0	51.88	44.1
18	5.61	43.0	55.73	40.0
24	5.33	45.9	59.58	35.8
Isolate NV2A				
0	9.84	0	92.81	0
6	4.59	53.4	29.84	67.9
12	3.93	60.1	56.08	39.6
18	4.35	55.8	52.23	43.7
24	3.47	64.7	59.93	35.4

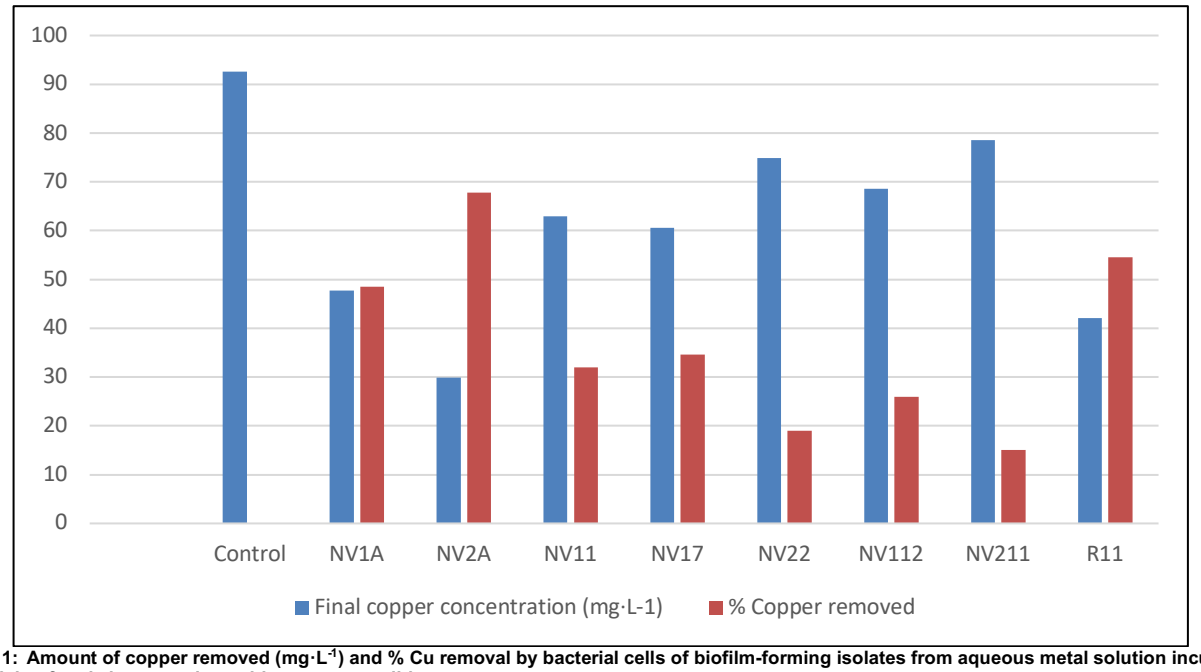

Figure 1: Amount of copper removed (mg·L^{-1}) and % Cu removal by bacterial cells of biofilm-forming isolates from aqueous metal solution incubated with mixing for six hours under ambient room conditions

RESULTS AND DISCUSSION

Biofilm formation and heavy metal tolerance of the isolates

Biofilm formation is a desirable characteristic of bacteria being utilized in heavy metal removal through biosorption. Bacteria, having negatively-charged cell surfaces, have high affinity for positively-charged heavy metals (Atlas and Philip 2005). Of the 90 isolates assayed, eight were found to be biofilm formers. These isolates were designated as NV1A, NV2A, NV11, NV17, NV22, NV112, NV211 and R11. Isolate NV1A formed the thickest biofilm as indicated by deep violet color, the intensity of which was similar to that of the control organism.

All the eight isolates were able to grow in the presence of the four heavy metals tested. Isolate NV1A exhibited the highest tolerance level as it showed growth in TSB supplemented with 8 mg·L-1 Cd, 360 mg·L-1 Cu, 850 mg·L-1 Pb and 1400 mg·L-1 Zn (Table 1). These concentrations are ten times higher than the amount of acceptable metal contents of environmental samples, particularly soils, based on the Dutch standards (Chen 2000). The group of isolates NV22, NV112, NV211 and R11, and the pair of isolates NV11 and NV17, respectively, tolerated levels six times and twice the acceptable levels of heavy metals in the soil. Isolate NV2A grew at the concentration Level I containing 170 mg·L-1 Pb + 1.6 mg·L-1 Cd + 72 mg·L-1 Cu + 280 Zn which is twice the acceptable metal content of environmental samples. All isolates tolerated heavy metal concentrations higher than those of the heavy metals found in the soil collected from Mogpog, Marinduque, which based on soil analysis had 0.68 mg·L-1 Pb, 0.03 mg·L-1 Cd, 70 mg·L-1 Cu and 4.31 mg·L-1 Zn (Llamado et al. 2013).

Isolate NV1A, which formed the thickest biofilm, was the most tolerant to the four heavy metals tested. In the development of biofilm, cells secrete extracellular polymeric substances (EPS) that bind the cells and extracellular materials together, leading to high density of cells that may bring about rapid bioremediation (Sheng et al. 2010; Singh et al. 2006). The EPS also serves as a protective coat of the whole biofilm by binding the heavy metals and retarding their diffusion within the biofilm, providing the cells in a biofilm with a higher degree of adaptation and survival than planktonic microorganisms (Singh et al. 2006).

Teitzel and Parsek (2003) reported that biofilm was more resistant to copper, zinc and lead than either the stationary and logarithmic phases of planktonic cells of *Pseudomonas aeruginosa*. Microscopy further revealed that the exterior of the biofilm was killed after exposure to high concentrations of copper while most of the living cells were found near the substratum. Many indigenous bacteria isolated from heavy metal-contaminated sites possessed tolerance to HM toxicity as reported by Xie et al. (2010). Two bacterial isolates from a long-term copper mine tailing in China were found to tolerate high concentrations of zinc, cadmium, copper, nickel and lead.

To exert a toxic effect, heavy metals must first enter the cell. Since some heavy metals are necessary in small amounts for enzymatic reactions for bacterial growth, uptake mechanisms of cells allow the entrance of metal ions. However, exposure to high amounts of heavy metals results to evolution of different mechanisms of tolerance to the uptake of metal ions. These mechanisms, which maybe plasmid-determined, include efflux of the metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal to a less toxic state (Nies 1999; Tsai 2006). These are utilized for detoxification and removal of heavy metals from contaminated wastewaters (Ahmed et al. 2005).

Removal of copper in single metal aqueous solution by the biofilm forming isolates

Tolerance to high copper concentration may indicate the ability of metal uptake but this does not necessarily mean the capacity to remove the heavy metal from the solution. In order to evaluate the potential of the bacterial isolates as tools for bioremediation, the biosorptive capacity of free cells needs to be determined before further effectiveness studies can be done.

The highest amount of Cu removed by isolate NV1A was 53.4% after 6 hours of contact time between the cells and 10 mg·L-1 Cu in solution. The percent Cu removal decreased with increasing contact time, that is, 47, 43 and 46 % after 12, 18 and 24 hours of contact time, respectively (Table 2). When 100 mg·L-1 of Cu in solution was used, a similar trend was observed; however, the % Cu removal was lower compared to that of the 10 mg·L-1 solution. The percentages of Cu removed in solution were 48, 44, 40, and 35 % after 6, 12, 18 and 24 hours of contact time, respectively (Table 2). The same trend was observed with isolate NV2A. The higher Cu removal at the initial contact time maybe due to the abundance of binding sites on the bacterial cell wall. These binding sites are chemical functional groups that include carboxyl, phosphonate, amine and hydroxyl groups (Doyle et al., 1980; van der Wal et al., 1997). With increasing contact time, the bacterial biomass becomes saturated with heavy metals resulting in fewer remaining binding sites. As the availability of the functional groups diminishes, the biomass would exhibit reduced sorption capacities (Vijayaraghavan and Yun 2008). However, a higher amount of copper was removed by the biomass in 100 mg·L-1 (67.9 %) than in the 10 mg·L-1 (53%) (Table 2). In both cases, the highest amount of copper was removed after 6 hours of contact time. According to Vijayaraghavan and Yun (2008), one of the factors influencing bisorption is the initial concentration of solute.

Previous studies reported that higher initial solute concentration leads to higher uptake of solutes as, at lower initial solute concentrations the ratio of the initial moles of solute to the available surface area is low (Ho and McKay, 1999; Ho and McKay, 2000; Binupriya et al., 2007). In the present study, it is possible that higher Cu concentration and corresponding abundance of binding sites led to percent removal higher than at lower Cu concentration. Following on this assumption, the capability of the eight selected metal-tolerant biofilm-forming isolates for Cu removal was determined using 100 mg·L-1 Cu in solution and 6 hours of contact time. All eight isolates were able to reduce the copper content in the range of 15.10 to 67.80% efficiency (Figure 1). The highest percentage of Cu removal was obtained with isolate NV2A which was consistent with the initial value of 67.9 % (Table 2), an observation that is attributed to NV2A being a thick biofilm-former. Fast sorption of copper by the bacterial isolates, as indicated by the decrease in copper concentration in the solution after 6 hours, was observed.

Leung et al. (2001), in a study of *Pseudomonas pseudoalcaligenes* as biosorbent, reported similar reduction of the amount of copper and lead in the solution. Heavy metal equilibrium concentrations were attained after about 500 min (~8 hours) to 800 min (~13 hours). In the present study that used 6 hour interval to determine the point of biosorption saturation, it was observed that biosorption was generally highest at 6 hours of contact time with no significant increase in the succeeding hours. This may indicate that after 6 hours, saturation of the biomass may have been achieved resulting in metal ions being able to attach to all the binding sites on the cell (Vijayaraghavan and Yun 2008).

Corollary to biosorption are desorption tests to remove and recover the metal ions from the bacterial cell for the possible reuse of the cells in subsequent biosorption. In the study of Wong et al. (2001), wherein desorption was performed, and designed when the biomass had reached equilibrium after 12 hours, it was observed that repeated biosorption and desorption of copper using 0.05 M sulfuric acid resulted in a slight increase of copper uptake of the bacterium, *Micrococcus* due apparently to the increase in surface area of the biomass brought about by the desorption medium creating more binding sites to the metal ions.

Physical and chemical processes may be ineffective in treating aqueous solutions in the presence of metal ions consisting of 1-100 mg per liter (Volesky 2001). The limitation of these processes is the strength of bioremediation since the residual concentrations of contaminants that are above regulatory guidelines can be treated and reduced to acceptable levels. Physical and chemical remediation methods, followed by bioremediation techniques that use microorganisms can be a faster and more cost-effective method of cleaning up heavy metal-contaminated wastewaters (Sharma 2008).

Biofilm-forming bacteria are extensively-used in biosorption studies of different heavy metals. Quintelas et al. (2007) showed that the biofilm of *Bacillus coagulans* was effective in the biosorption of Cr (VI) and removal of the heavy metal from industrial wastewater. *Pseudomonas putida,* in the presence of EPS, was found to bind a large amount of cadmium concentration from solution. Although the amount did not significantly differ from that of Cd removed by EPS-free *P. putida,* the presence of EPS effectively increased the viability of cells in high concentrations of cadmium (Ueshima et al. 2008). In the Philippines, Opulencia et. al (2015) identified a copper-resistant biofilm-forming *Bacillus megaterium. B. megaterium* was immobilized as biofilm on a 30-liter fixed-bed upflow bioreactor using polyvinyl chloride corrugated pipe as carrier and tryptic soy broth combined with alcohol distillery slop (75:25) as growth medium and subsequently used to treat copper-containing wastewater effluent from a semiconductor company. Results showed that it reduced copper by as much as 65.95% making it a potential bacterial remediator in cleaning up copper-contaminated wastewater.

Removal of heavy metals in multi-metal aqueous solution by the biofilm- forming isolates

The amount of copper removed by planktonic cells of isolates NV1A, NV2A, NV22 and NV112 decreased when other heavy

Table 3: Amount of heavy metals removed by bacterial cells of biofilm-forming isolates from aqueous solution of mixed metals incubated with mixing for six hours under ambient room conditions

Isolates	Cu		Pb		Zn		Cd	
	[Final] ($mg \cdot L^{-1}$)	% Removal	[Final] ($mg \cdot L^{-1}$)	% Removal	[Final] ($mg \cdot L^{-1}$)	% Removal	[Final] ($mg \cdot L^{-1}$)	% Removal
Control	10.40	0.00	1.10	0.00	51.18	0.00	0.69	0.00
NV1A	6.93	33.40	<DL	>96.60	5.20	89.90	0.06	90.83
NV2A	8.70	16.40	1.41	0.00	24.55	52.00	0.43	37.89
NV11	5.65	45.70	0.79	28.20	12.33	75.90	0.16	77.32
NV17	2.16	79.20	<DL	>96.60	4.73	90.80	0.15	78.33
NV22	8.86	14.80	1.06	4.00	14.26	72.10	0.31	54.89
NV112	8.37	19.50	1.01	8.00	12.80	75.00	0.26	62.11
NV211	8.70	16.40	0.84	24.10	14.68	71.30	0.32	54.50
R11	4.44	57.40	0.10	90.60	7.90	84.60	0.12	82.72

<DL - less than the detectable level

Table 4: Physiological characteristics of biofilm-forming bacterial isolates from the soil of an abandoned, mined-out site in Mogpog, Marinduque determined using Biolog Gen III MicroPlate™

Test	Isolate							
	NV1A	NV2A	NV11*	NV17*	NV22	NV112	NV211	R11*
Utilization of:								
Sucrose	V	V	-	-	-	-	-	-
Raffinose	V	-	-	-	-	-	-	-
Lactose	V	-	-	-	-	-	-	V
Glucose	V	+	-	V	V	-	-	-
Sorbitol	V	V	-	-	-	-	-	-
Mannitol	V	+	-	-	-	-	-	-
Gelatin	-	V	-	+	-	-	-	+
Alanine	-	+	+	+	-	-	-	V
Arginine	-	+	-	-	-	-	-	+
Inhibition by:								
pH 5	-	V	+	+	V	3	V	-
4% NaCl	-	V	-	-	+	+	+	-
Na lactate	-	-	-	-	-	-	-	-
Vancomycin	-	-	+	+	+	+	+	+
Nalidixic acid	-	-	-	-	-	-	-	-
Lithium chloride	-	+	-	-	-	V	+	-

V= variable *results may be false positive

metals were present in the aqueous solution (Table 3) partly explained by competition of the metal ions in the mixture for binding sites on the cell surface (Claessens and Van Cappellen 2007; Fowle and Fein 1999). In contrast, amounts of copper removed by isolate NV17 and R11 from the multi-metal solution apparently increased more than in the copper only solution. Generally, the amounts of different metals removed by these two isolates in the multi-metal solution were all high. The rates of removal of NV17 were 79.20% for Cu, 96.60% for Pb, 90.80% for Zn and 78.33% for Cd while those of isolate R11 were 57.4% for Cu, 90.60% for Pb, 84.60% for Zn and 82.72% for Cd (Table 3). These results indicate that the bacteria remove heavy metals better when the ions are in mixture. A more lucid explanation of this phenomenon is not possible in this study but the direction taken shows promise in bioremediation of industrial wastewaters contaminated with several heavy metals.

Cultural, morphological and physiological characterization of the selected best isolates

The cultural characteristics of bacterial isolates observed on TSA after 48 hours of incubation under ambient room conditions were as follows: NV1A and NV2A were translucent with the latter showing soluble greenish pigmentation; NV11, opaque white; NV17 and R11, dry and flat while V17 was golden yellow and R11 was cream; NV22, NV112, and NV211, light peace to peach, mucoidal. Mucoid appearance is a specific characteristic of many biofilm-forming microorganisms. Morphologically, isolates NV1A, NV22 and NV112 appeared to be gram-positive cocco-bacilli. Cells of NV211 are cocci in short chain arrangements while isolate NV11 cells are very short rods with both being gram-positive. Isolates NV17 and R11 are both long rods and gram-positive, although the cells of the latter are much larger than those of the former. Cells of NV2A, on the other hand, are gram-negative short rods. Scanning electron photomicrographs after at least three days of undisturbed growth using TSB incubated under ambient room conditions showed that the cells of all isolates were embedded in varying thickness of extracellular substances making them capable of attached growth. These results confirmed observations in the biofilm production assay.

With the use of Biolog Gen III MicroPlate™, isolate NV2A demonstrated versatility in terms of utilizing macromolecules, particularly glucose, mannitol, alanine and arginine (Table 4). Vancomycin effectively inhibited growth of the biofilm-forming isolates except isolates NV1A and NV2A while lithium chloride hindered the growth of isolates NV2A, NV211 and to some extent that of NV112.

16S rDNA sequence-based identification of the selected best isolates

A single band of approximately 1.5 kb was detected after agarose gel electrophoresis of the PCR amplified products using 16s rDNA specific primers. Nucleotide sequencing analyses revealed that isolates NV1A, NV22 and NV112 had 98 to 100% similarity in 16S rDNA sequence with two species of

Table 5: Bacterial species in GenBank databases that have significant alignment using BLAST algorithm with 16S rDNA of the biofilm-forming bacterial isolates from the soil of an abandoned, mined-out site in Mogpog, Marinduque

Isolate	Organism with Sequences of Significant Alignment	Genbank Accession	% Similarity
NV1A	*Rhodococcus equi* 103S	NC_014659.1 FN563149.1	98
	Rhodococcus opacus B4	NC_012522.1 NC_018080.1	98
NV2A	*Pseudomonas aeruginosa*	NC_017549.1 NC_008463.1 CP003149.1 CP002496.1	100
NV11	*Solibacillus silvestris* StLB046	NC_018065.1 AP012157.1	97
	Lysinibacillus sphaericus C3-41	NC_010382.1 CP000817.1	97
NV17	*Bacillus pumilus* SAFR-032	NC_009848.1	96
	Bacillus megaterium DSM 319	NC_014103.1	96
	Bacillus amyloliquefaciens CAU B946	NC_017061.1	96
NV22	*Rhodococcus equi* 103S	NC_014659.1	100
	Rhodococcus opacus B4	NC_012522.1	100
NV112	*Rhodococcus equi* 103S	NC_014659.1	100
	Rhodococcus opacus B4	NC_012522.1	98
NV211	*Enterococcus faecium* DO	NC_017960.1	97
R11	*Bacillus megaterium* WSH-002	NC_017138.1	99

Rhodococcus (Table 5). *Rhodococcus* is an aerobic, non-motile, gram-positive coccus that produces thick capsules and exhibits pleiomorphism, characteristics which are very similar to the cocco-bacilli morphology exhibited by isolates NV1A, NV22 and NV112. In this study, *Rhodococcus* was a moderate biofilm-producer. Compared to that of other well-studied biofilm-formers such as *Pseudomonas*, the build-up of biomass on solid surface was slower which is a drawback for isolates intended for wastewater clean-up. However, *Rhodococcus* has been reported to be resistant to many environmental stresses such as desiccation. (Bell et al. 1998).

Isolate NV2A showed 100% sequence similarity with 16S rDNA of *Pseudomonas aeruginosa.* Many species of *Pseudomonas,* in general, are able to degrade a wide variety of complex compounds that are considered environmental pollutants like aromatic hydrocarbons and synthetic organic substances including pesticides (Madigan et al. 2015). *P. aeruginosa* can produce large amounts of extracellular polymers that contribute to its fast rate of biofilm build-up on many substrates (Yahr and Parsek 2006). This ability has made it useful agent for accumulating metals from wastewaters (Hussein et al. 2004). It can also produce different types of pigments, including soluble forms that diffuse into its growth medium. Production of greenish pigment was observed in isolate NV2A.

Isolate R11 has 99% similarity with *Bacillus megaterium* which was recently identified by Opulencia et al. (2015) as a potential heavy metal bioremediator in the Philippines. The rest of the isolates had relatively lower sequence similarity percentages of 96 to 97%. In particular, the 96% similarity in 16S rDNA sequence between isolate NV17 and several species of *Bacillus* suggests the possibility that NV17 is a completely different and novel species, or an already discovered species. However, its DNA sequence is not in the databases used in the study. Isolates NV17 and R11 were observed to be moderate to fast biofilm-formers. *Bacillus,* being a common soil bacterium able to produce highly resistant endospores, can survive in the soil environment with continual changing conditions temporally and spatially (Slepecky and Hemphill 2006). This is a desirable characteristic as it gives the organism a competitive advantage when introduced into a new environment. *Bacillus* species have,likewise, been reported as tolerant to high concentrations of metals and consequently are being used to remove these cation contaminants in waste waters (Cheung and Gu 2005).

Other gram-positive bacilli that have shown similarity with isolate NV11, a moderate biofilm- former, are *Lysinibacillus* and *Solibacillus*. The former has been reported as one of the genera involved in extracellular biosorption of mercury in water (He et al 2011) and in volatilizing mercury and subsequently removing it in aqueous solution. *Solibacillus*, on the other hand, is an amylolytic bacterium associated with compost and has a

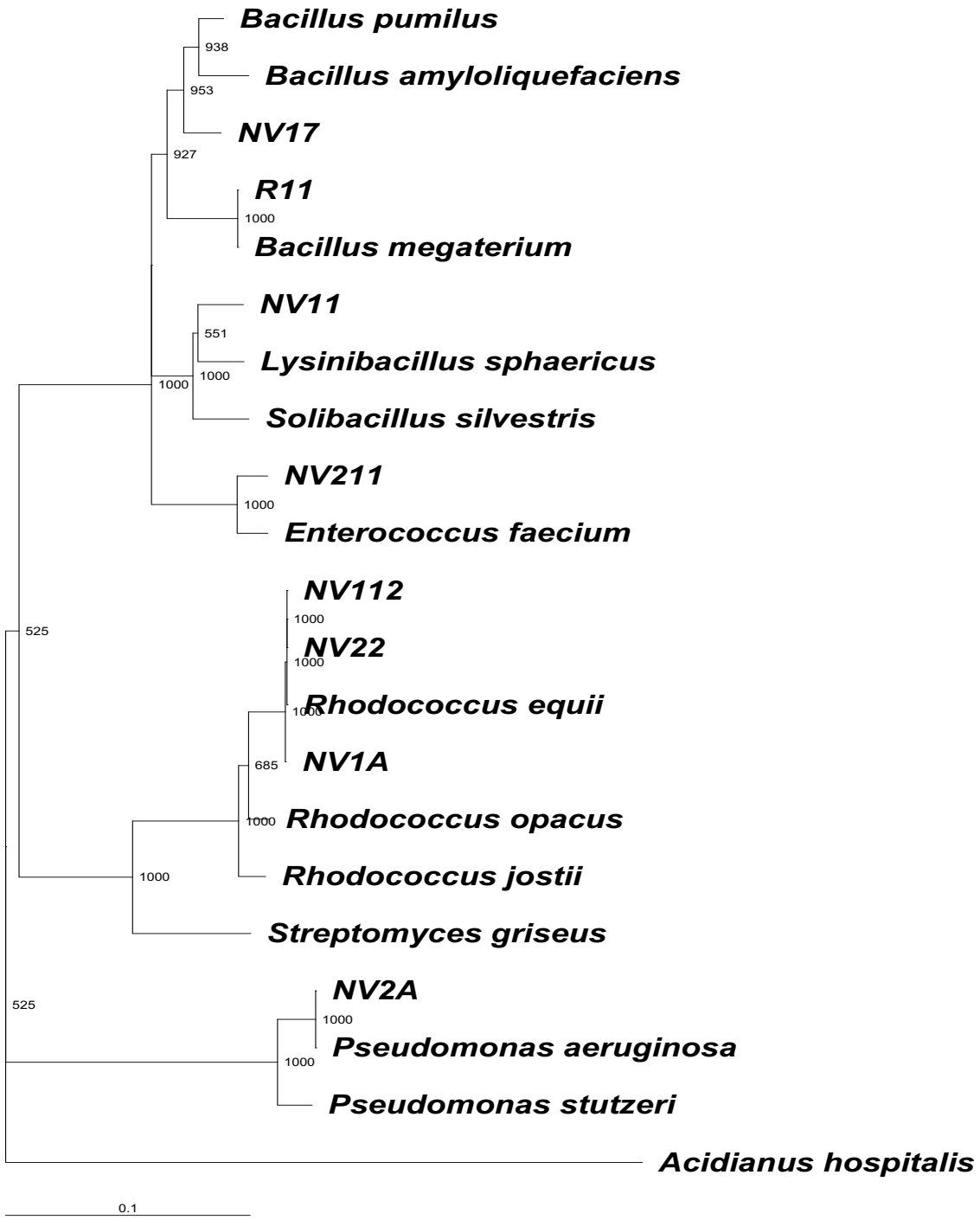

Figure 2: Neighbor-joining tree generated using Clustal X 1.81 alignment program showingthe phylogenetic relationship of 16S rDNA of the biofilm-forming isolates aligned with that of representative members of the domain Bacteria. The archaea Acidianus hospitalis served as the outgroup. Numbers at nodes indicate percent bootstrap values (1000 replicates). Bar indicates 10% estimated sequence divergence

potential as a biocontrol agent against certain plant pathogens (Morohoshi et al. 2012). Isolate NV211, another moderate biofilm-former, has shown 16S rDNA sequence similarity to *Enterococcus faecium* which is a non-motile, non-spore-forming, gram-positive cocci. It is a normal inhabitant of the gastrointestinal tracts of humans and animals and is very rarely associated with disease. Presently, *E. faecium* belongs to the group of normally harmless bacteria that occasionally cause nosocomial infections usually acquired by immuno-compromised patients in a hospital environment (LeBlanc, 2006). Its biofilm-forming ability is relatively weaker thereby requiring more nutrients from the growth medium.

A phylogenetic tree was constructed to establish evolutionary similarity based on 16S rDNA sequences of the biofilm-forming isolates and those in the GenBank databases. Using Clustal X alignment, isolate NV2A was shown to be very similar to *Pseudomonas,* isolates NV1A, NV22 and NV112 were grouped closely with *Rhodococcus*, while isolates NV22 and NV112 had a higher degree of similarity to *R. equi* (Figure 2). Isolate R11 was very similar to *B. megaterium* while isolate NV17 was closely-grouped with several species of *Bacillus*. Geneally, the clusters gave remarkably high percent bootstrap values using 1000 iterations, which confirmed the significant alignments obtained using the BLAST algorithm (Table 5).

Heavy metal tolerant biofilm-forming microorganisms with potential application in cleaning up heavy metal-contaminated wastewater can be isolated from environments contaminated with the same, such as the mined-out soil in Mogpog, Marinduque used in this study. These isolates can further be studied and developed for application in heavy metal removal in actual industrial wastewaters. Any technology that will emerge and prove successful should lead to significantly lesser cost of heavy metal remediation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTION OF INDIVIDUAL AUTHORS

LCVillegas and ALLamado are the projects' principal investigators. CVCatsao was an undergraduate student who performed portions of the project. AKRaymundo was the project's consultant.

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