Biosorption of nickel by *Bacillus cereus* and *Stenotrophomonas maltophilia* isolated from Bayto River, Zambales

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Heavy metal contamination of water systems is a global environmental concern and biosorption of these heavy metals by using bacteria offers a more potent and cost-effective solution compared to conventional methods. In this study 139 nickel-resistant isolates were obtained from the water samples collected from Bayto River, Santa Cruz, Zambales, Philippines. The metal-resistance profiles of the isolates were determined by using the Kirby-Bauer disc diffusion method. Found to tolerate the highest concentration of nickel tested, i.e., 10,000 ppm, were 16 isolates, and they were subjected to multimetal resistance assays. Out of the 16 most nickel-resistant isolates, 4 were able to tolerate 7,500 parts per million (ppm) copper, and 10,000 ppm chromium and lead. These isolates (S2Q1, S1I2, S3Z1, S2P1) were subjected to biosorption assay. Biosorption of nickel by these isolates was determined by inoculating 16-hour-nutrient broth (NB) cultures to NB supplemented with 1000 ppm nickel. The metal-microbe suspensions were incubated at room temperature in a rotary shaker at 150 rpm for 24 hours. Afterward, the NB from each setup was centrifuged, and the supernatants were analyzed by using atomic absorption spectrophotometry (AAS). The S1I2 exhibited the highest biosorption percentage at 92.27%, followed by S3Z1 (91.67%), S2Q1 (91.36%), and S2P1 (89.78%). The 4 isolates were identified via 16S rRNA sequencing. S1I2 and S2Q1 were identified as *Stenotrophomonas maltophilia*, while S3Z1 and S2P1 were identified as *Bacillus cereus*. These isolates can be utilized in the development of facilities for the treatment of contaminated waters.

**KEYWORDS**

Biosorption, heavy metals, *Stenotrophomonas maltophilia*, *Bacillus cereus*, Bayto River

**INTRODUCTION**

Anthropogenic activities such as chemical industries, agriculture and mining have generated increasing amounts of toxic metals in the environment throughout the 20th century (Francois et al. 2011). The mining industry, however enormous its social and economic benefits, has long-term adverse effects on the environment and public health which cannot be overlooked (Fashola et al. 2016). In Santa Cruz, Zambales, Philippines, the hazards that mining has caused to both the communities and the environment have been extremely evident.

Santa Cruz is a first-class municipality with rich fertile soils and abundant aquatic systems (coastal waters and river channels) suitable for farming (Environmental Justice Atlas 2015). Several rivers in the municipality are established as operational river irrigation systems (RIS) among the 10 National Irrigation Systems in Region III. Bayto River (also known as Cabaluan River) supports the Bayto RIS covering the municipalities of Candelaria and Santa Cruz. The river has a total area of 11,260 hectares supporting farmlands and fishponds in the area (National Irrigation Administration 2015). However, large-scale mining, especially nickel mining, operations which have been running in the municipality since 2006 have led to the degradation of the natural resources (Ayroso 2016).

According to the Environmental Council of British Colombia (2010) as cited by Ochieng et al. (2010), Water has been called “mining’s most common casualty” (Environmental Council of British Colombia 2010, as cited by Ochieng et al. 2010). Water...
pollution problems by mining include acid mine drainage, metal contamination, and increased sediment levels in streams (Coelho et al. 2011). Heavy metal contamination, as a result of human interventions such as mining, has become a serious threat to the safety of the environment, pressurizing soils, atmosphere, water streams, and living systems (Vishan et al. 2016). Unlike many organic pollutants which can be biologically or chemically degraded, heavy metals are not easily removed from the environment; thus they are considered widespread, persistent, inorganic pollutants which pose a worldwide environmental problem (Singh and Lal 2015). Normally, heavy metals with concentrations above acceptable levels have adverse effects on the functional activities of microbial communities in different ecosystems; otherwise, microorganisms exposed to higher concentrations of these toxic metals may evolve tolerance (Issazadeh et al. 2013).

In this pioneering microbiological study of the Bayto River, we conducted the isolation and identification of indigenous bacterial candidates with biosorption potential.

MATERIALS AND METHODS

Collection of water samples
Sterile glass amber bottles were used to collect water samples from Bayto River in Santa Cruz.

Ten water samples (approximately 25 ml each) were collected from each of three sites (0-meter mark, 50-meter mark, 100-meter mark) along the 100-meter length downstream of the Bayto River. Sampling was done once in July 2017. The coordinates of the 0-meter mark, 50-meter mark, and 100-meter mark were 15°43’2”N-119°57’59”E, 15°43’1”N-119°57’59”E, and 15°43’0”N-119°58’0”E, respectively. The ten samples from each site were then pooled into one sample (approximately 250 ml), resulting in three separate samples for the three sites. All the samples were stored in a cooler and were transported to the microbiology laboratory of Cavite State University, Indang, for the isolation of bacteria.

Likewise, a six-liter sample was collected from the same sites (two liters each for the 0-meter, 50-meter, and 100-meter mark pooled into one six-liter water sample). It was then transported to Cavite Water and Wastewater Testing Laboratory of the Department of Science and Technology for determining the presence of and the respective concentrations of metals such as nickel, cadmium, copper, chromium, and lead. Also, the physicochemical properties of the water sample such as pH, total dissolved solids (TDS), conductivity and salinity were determined in situ using a portable water testing equipment.

Isolation of bacteria
Bacteria were isolated from water samples by using the serial dilution technique. Serial dilutions of up to $10^8$ were done with sterile distilled water as the diluents. From each dilution 100 µL was spread plated to nutrient agar (NA) plates with 50 ppm nickel and NA plates without nickel to determine the % population of nickel-resistant bacteria.

The plates were incubated for 24 hours at room temperature until noticeable colonies appeared. The setups were accomplished in duplicates. Colonies varying in morphology (color, shape, and size) on NA plates with nickel were selected for purification. Repetitive plating of the bacterial isolates was done until isolated colonies were purified. Pure cultures were maintained on NA slants and stored at 4°C.

Table 1: Physicochemical properties of Bayto River (July 2017).

<table>
<thead>
<tr>
<th>SAMPLING SITE</th>
<th>SALINITY (mg/l)</th>
<th>CONDUCTIVITY (µS)</th>
<th>TDS (mg/l)</th>
<th>pH</th>
<th>TEMPERATURE (°C)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>104</td>
<td>206</td>
<td>140</td>
<td>6</td>
<td>25</td>
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<tr>
<td>2</td>
<td>103</td>
<td>206</td>
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<td>101</td>
<td>206</td>
<td>136</td>
<td>6</td>
<td>25</td>
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</table>

**VALUE LIMITS FOR CLASS C WATERS***

<table>
<thead>
<tr>
<th>DESIGNATION</th>
<th>DESCRIPTION</th>
<th>Cd (mg/l)</th>
<th>Ni (mg/l)</th>
<th>Cr (mg/l)</th>
<th>Cu (mg/l)</th>
<th>Pb (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>River Water</td>
<td>In glass container; approx. 6L in volume; turbid w/ settled particulates</td>
<td>&lt; 0.002</td>
<td>&lt; 0.05</td>
<td>&lt; 0.010</td>
<td>&lt; 0.02</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

*Value limits according to the WQG (Water Quality Guidelines) established by the Philippines’ Department of Environment and Natural Resources under Administrative Order 2016-08.

TDS = total dissolved solids

Table 2: Concentration of selected heavy metals in Bayto River (July 2017).

<table>
<thead>
<tr>
<th>SAMPLE DESIGNATION</th>
<th>DESCRIPTION</th>
<th>Cd (mg/l)</th>
<th>Ni (mg/l)</th>
<th>Cr (mg/l)</th>
<th>Cu (mg/l)</th>
<th>Pb (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>River Water</td>
<td>In glass container; approx. 6L in volume; turbid w/ settled particulates</td>
<td>0.01</td>
<td>1</td>
<td>0.02</td>
<td>0.04</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Value limits according to the WQG (Water Quality Guidelines) established by the Philippines’ Department of Environment and Natural Resources under Administrative Order 2016-08.

**TDS** = total dissolved solids

**SALINITY** = salinity

**CONDUCTIVITY** = conductivity
Screening for metal resistance among bacterial isolates

The Kirby-Bauer disc diffusion method (Bauer et al. 1996) with minimal modification was employed for screening metal resistance among the bacterial isolates. Twenty-four-hour-old pure bacterial isolates from NA slants were inoculated into 0.85% saline solution, and the turbidity was adjusted against 0.5 MacFarland standard to approximate the bacterial density to be used in the assay. Isolates were each swabbed on Mueller-Hinton agar (MHA) plates. Sterile filter paper discs (Whatman No. 2) were then placed aseptically on each of the swabbed plates at appropriate distances from one another.

Twelve μl of nickel solution from 100 ppm up to 10,000 ppm were each pipetted onto the sterile discs by using a 20 μl micropipettor, while sterile distilled water was used as the control (disc at the center of the plate). The plates were then incubated at room temperature for 24 hours and were observed afterward. The concentrations of the metal solutions used were 100, 200, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 6000, 7500, and 10,000 ppm.

Bacterial isolates which grew in the presence of 1000 ppm nickel were assayed further by using the 1500-5000 ppm range. Those which showed no zones of inhibition in these metal concentrations were again assayed for metal tolerance by using a 6000, 7500, and 10,000 ppm Ni solution. Bacterial isolates which survived the 10,000 ppm Ni solution were assayed for resistance to copper (Cu), chromium (Cr), and lead (Pb) at 1000, 5000, 7500, and 10,000 ppm concentrations. The results from the metal-resistance assays using Cu, Cr, and Pb were then used as the basis for the selection of the most-promising isolates. The experiment was done in duplicate.

Table 3: Percent population of metal-resistant bacteria.
The resulting sequences were phylogenetically analyzed by using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) to compare similarities among known bacterial DNA sequences.

### RESULTS AND DISCUSSION

#### Physicochemical properties and heavy metal contents

Bayto River is classified as class C surface water which may be utilized for fisheries (for the propagation of fish and other aquatic resources), for recreation (boating, fishing, etc.), and/or for agriculture, irrigation, and livestock watering based on Water Quality Guidelines (WQG) established by the Philippines’ Department of Environment and Natural Resources (DENR). According to Administrative Order 2016-08, the temperature range, pH range, and amount of TDS must be 25°C-31°C, 6.0-9.0, and 80 mg/l, respectively. By contrast, the acceptable concentration limits for cadmium (Cd), Ni, Cr, Cu, and Pb are 0.01, 1, 0.02, 0.04, and 0.1 mg/l, respectively (https://www.denr.gov.ph).

The values for the physicochemical properties of the water samples from the Bayto River are summarized in table 1. The temperature and pH of the river water passed the WQG for primary parameters issued by the DENR and summarized in Administrative Order 2016-08 (table 1). No specified permissible values for salinity, conductivity, and TDS were indicated in the administrative order. Furthermore, the results of the water analysis of the pooled sample (table 2) using AAS indicated that the levels of Cd, Ni, Cr, Cu, and Pb present in the Bayto River water sample are likewise still within the acceptable limits for these heavy metals as per Administrative Order 2016-08.

Despite the proximity of Bayto River to the mines in Santa Cruz, Zambales, the quality of the river (at least for the tested parameters) is still appropriate for its intended beneficial use. However, note that water quality of rivers varies with the season (Lawson 2011), and so the results gathered may be applicable only to the present study and may not be used to generalize the conditions of Bayto River. Moreover, there is the possibility of the heavy metals accumulating in the sediments (Parungao et al. 2007).

#### Biosorption experiment

Isolates which showed resistance to multiple metals (Ni, Cu, Cr, Pb) at the highest concentrations were used in the nickel biosorption experiments. Nickel was selected for the biosorption experiments because the mines near the Bayto River mainly extract nickel. Biosorption experiment procedure (Syed and Chinthala 2015) with minimal modification was used. The bacterial isolates were grown in NB for 12 hours in a rotary shaker (150 ppm). The absorbance of each broth culture was then determined by using a spectrophotometer at 600 nm, and the turbidity of each was adjusted to result in similar absorbances among the isolates.

Biosorption of nickel by the most promising isolates was assayed in Erlenmeyer flasks containing 90 ml of NB supplemented with nickel solution to a final concentration of 1000 ppm. To this, the 10 ml turbidity-adjusted 12-hour-old NB cultures were added. The metal-microbe suspension was incubated at room temperature in a rotary shaker (150 rpm) for 24 hours. A control without bacterial cultures was also maintained. The biosorption potential was measured as the amount of metal removed from the medium by determining the residual metal concentration using Atomic Absorption Spectrophotometry (AAS). The atomic absorption spectrophotometry analysis was processed by General Society of Surveillance Philippines.

<table>
<thead>
<tr>
<th>ISOLATE</th>
<th>% QUERY COVER</th>
<th>E VALUE</th>
<th>% IDENTITY</th>
<th>ACCESSION NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenotrophomonas maltophilia (S112)</td>
<td>99%</td>
<td>0</td>
<td>99.52%</td>
<td>MK968634</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia (S2P1)</td>
<td>98%</td>
<td>0</td>
<td>98.58%</td>
<td>MK968810</td>
</tr>
<tr>
<td>Bacillus cereus (S3Z1)</td>
<td>97%</td>
<td>0</td>
<td>98.19%</td>
<td>MK968814</td>
</tr>
<tr>
<td>Bacillus cereus (S2Q1)</td>
<td>95%</td>
<td>0</td>
<td>97.80%</td>
<td>MK968813</td>
</tr>
</tbody>
</table>

% Population of Nickel-resistant Bacteria is given by: (Population of Ni-resistant/Total Population) x 100

### Table 4: BLAST results for the most promising isolates.

<table>
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Figure 4: Resistance to selected heavy metals of the most nickel-tolerant isolates

Figure 5: S2Q1(A), S1I2(B), S3Z1(C) and S2P1(D) showing resistance to copper at concentrations 1000 to 10,000 ppm (from black arrow following a counterclockwise direction, respectively) and chromium at 1000 to 10,000 ppm (from red arrow following a counterclockwise direction, respectively); disc at the center is the control (sterile distilled water).

Figure 6: S2P1(A), S1I2(B), S3Z1(C) and S2Q1(D) showing resistance to lead at concentrations 1000 to 10,000 ppm (from black arrow following a counterclockwise direction, respectively); disc at the center is the control (sterile distilled water).

Nickel resistance among the bacterial isolates

The results from the bacterial isolation revealed a low percentage of Ni-resistant bacteria (relative to the viable count in cfu/ml obtained from the setup without Ni supplementation), with the highest percentage only at 4.86% (table 3). The result can be attributed to the fact that although heavy metals are required by biological systems such as microorganisms, they are required only in trace amounts, and are noxious in excess and with persistent exposure (Ghorbani et al. 2002; Vishan et al. 2016). Heavy metal pollution affects the growth, morphological characteristics, and metabolism of microorganisms through denaturation of proteins, functional disturbance, or destruction of the integrity of the cell membranes (Ghorbani et al. 2002).

Indigenous microorganisms such as bacteria in contaminated environments such as surface waters rapidly adapt and therefore become more tolerant to elevated concentrations of heavy metals compared to those isolated from unpolluted areas (Maitra 2016). These microorganisms have evolved coping mechanisms in order to exist in environments with excessive levels of drugs and heavy metals; hence these can be used as bioindicators for detection of heavy metal contamination in the environment (Chihomvu et al. 2014). The survival of these microorganisms against heavy metals such as nickel relies on their intrinsic structural, biochemical, physiological properties and genetic makeup as well as genetic adaptations (Alboghobeish et al. 2014; Chihomvu et al. 2014).

Nickel is the 24th most abundant element on earth. Thus, it can be found in all environments such as air, soil, sediment, and water (Iyaka 2011). Nickel in low amounts is essential to various biological processes (Alboghobeish et al. 2014) but is toxic.
concentration for a majority of the bacteria (66 out of 139) nickel may be considered as the minimum inhibitory (fig. 1). By inference the specific concentration (6000 ppm) of nickel from 100 ppm up to 10,000 ppm. The number of surviving bacteria gradually decreased as the concentration of nickel increased until a major decline was observed at 6000 ppm nickel (fig. 1). By inference the specific concentration (6000 ppm) of nickel may be considered as the minimum inhibitory concentration for a majority of the bacteria (66 out of 139) studied. Minimum inhibitory concentration is established as the lowest concentration of an antimicrobial or any substance that will inhibit the visible growth of a microorganism after overnight incubation (Andrews 2001). Able to survive 10,000 ppm of nickel were 16 out of the 139 isolates (11.5%) (fig. 1). The top nickel-resistant isolates were S1E2, S2X1, S2W1, S1F2, S1I1, S2P1, S1J1, S2Q1, S2N1, S3Z1, S1S1, S1B1, S1I2, S1O1, S3F1, S1F1. These were the ones subjected to multitemal resistance assays.

Resistance to copper, chromium, and lead of the most nickel-resistant isolates

The isolates which survived the highest concentration of Ni were further tested for their resistance to Cu, Cr, and Pb by utilizing the modified Kirby-Bauer disc diffusion method. In the study 16 isolates were resistant up to both 5000 ppm of Cu and Cr. As the concentrations of the two metals were increased, the number of surviving isolates decreased (fig. 4). No isolate was able to tolerate 10,000 ppm Cu.

By contrast, only 4 isolates were able to tolerate 1000 ppm up to 10,000 ppm of Pb. Since these 4 isolates (S2P1, S1I2, S3Z1, S2Q1) were able to tolerate up to 7500 ppm Cu as well as 10,000 ppm Cr and Pb, they were considered the most promising isolates.

Identities of the four most promising isolates

The NCBI BLAST was used to find regions of similarity between sequences by comparing primary biological sequence information. S1I2’s and S2P1’s top BLAST hit were both Stenotrophomonas maltophilia while S3Z1’s and S2Q1’s sequences showed closest similarity to Bacillus cereus (table 4; figs. 7-10).

B. cereus is a spore-forming, Gram-positive, rod-shaped, motile bacterium with a saprophytic life cycle. The natural environmental reservoir for this species consists of fresh and marine waters, vegetables and fomites, decaying organic matter, and intestinal tract of invertebrates (Paraskevas et al. 2004; Bottone, 2010).

S. maltophilia is a Gram-negative, obligate aerobic, nonsporulating bacillus with cells that are straight or slightly curved. It occurs singly or in pairs and is motile due to several polar flagella. This nonfermentative bacterium is found in a wide variety of environments and geographical regions; it thrives ubiquitously in aquatic sources, including rivers, wells, hypereutrophic lakes, and sewage (Parungao et al. 2007).

Biosorption assay

Biosorption is defined as the removal or binding of substances from an aqueous solution by a biological material. Such substances can be organic and inorganic and are either soluble or insoluble forms (Michalak et al. 2013). In our study, the biosorbents (the solid surface to which the desired substances adhere) used are bacteria.

Before subjecting the most promising isolates to biosorption, the turbidities of the broth cultures were adjusted to have similar absorbance at 600 nm (table 5). The cfu/ml of each isolate was then determined. Subsequent plating and determining the cfu/ml of each isolates were done. This was to ensure that nearly equal concentrations of biosorbents will be used in the assay. Likewise, it was to guarantee accuracy of the biosorption assay results.

The result of the assay (table 6) confirmed the biosorptive capability of the most promising isolates. S1I2, despite having a relatively low absorbance level and subsequent average viable

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Figure 7: Dendrogram showing S12's sequence similarity with related microorganisms.
Figure 8: Dendrogram showing S2P1's sequence similarity with related microorganisms.
count (which can be translated to relatively lower concentration of biomass), still displayed a high biosorption percentage.

Biomass concentration greatly affects biosorption such that higher biosorbent concentration leads to interference between the binding sites thus restricting the access of metal ions to these sites (Abbas et al. 2014).

Several mechanisms have been associated with the biosorptive capacity of bacteria and other biological systems. The mechanism of biosorption is complex, mainly involving ion exchange, chelation, adsorption by physical forces, entrapment in inter- and intrafibrillar capillaries and spaces of the structural polysaccharide network as a result of the concentration gradient and diffusion through cell walls and membranes (Rao and Prabhakar 2011).

Likewise, several factors affect the biosorption capacity of microorganisms and these include temperature, characteristics of biomass, acidity, biomass concentration, initial metal concentration, and metal affinity to the biosorbent (Abbas et al. 2014; Sulaiman 2015; Rao and Prabhakar 2011).

Various studies on resistance and biosorption of heavy metals have produced evidence demonstrating the potential of S. maltophilia as a bioremediating agent (Mukherjee and Roy 2016; Jackson et al. 2012; Pages et al. 2008; Alonso et al. 2000; Parungao et al. 2007). Likewise, the capability of B. cereus in the biosorption of nickel and other heavy metals has also been widely explored (Naskar et al. 2015; Syed and Chintala 2015; Singh et al. 2010; Trihadiningrum et al. 2014).

CONCLUSION

Nickel-resistant Stenotrophomonas maltophilia and Bacillus cereus present in Bayto River can tolerate up to 10,000 ppm Ni, 10,000 ppm Pb, 7500 ppm Cu and 7500 Cr, indicative of their prolonged exposure to these heavy metals. Furthermore, these isolates exhibited superior biosorptive capability that ranged from 89% to 92%. These isolates, upon further studies, have great potential in the bioremediation of heavy metal-contaminated water systems.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors of the study have no conflict of interest of any sort.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

The primary author conceptualized the study and conducted the necessary experiments for its completion, while the second author guided the primary author as to the direction of the study and with the interpretation of the results.

REFERENCES


REFERENCES


Bottonne EJ. Bacillus cereus, a volatile human pathogen. Clinical Microbiology Reviews 2010; 23(2), 382-389.


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Daware V, Wasudev G. Mechanism of arsenic tolerance in Klebsiella pneumonia (HQ857583). International Journal of


