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Biosorption of Copper, Cadmium and Lead by Copper-Resistant Bacteria Isolated from Mogpog River, Marinduque

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Isolation and selection of copper-resistant bacteria were carried out from a collected water sample of Mogpog River. The sample was analyzed for copper (Cu), Cadmium (Cd) and lead (Pb) content for simulation purposes. The selected most copper-resistant bacteria, *Stenotrophomonas maltophilia*, was exposed to 50, 10, and 20 ppm of Cu, Cd, and Pb, respectively, in primary (single metal) and ternary (mixed metals) solutions. Bacterial cells were separated from solutions by centrifugation and the supernatants were analyzed for remaining metals in solution using Atomic Absorption Spectrophotometry. Biosorption profile was determined to be Cu>Pb>Cd and Pb>Cu>Cd in primary and ternary solutions, respectively. Biosorption of the three heavy metals was higher in primary solutions than in ternary solutions. Approximately 22 % of Cu, 24% of Cd, and 42.75% of Pb were removed from primary solutions; 16% of Cu, 8% of Cd, and 35% of Pb, were removed from ternary solutions by *S. maltophilia*.

Key Words: *Stenotrophomonas maltophilia*, biosorbents, efflux, heavy metals

INTRODUCTION

Many mechanisms of resistance are known in bacteria for maintaining intracellular homeostasis of metal ions (Silver 1996; Nies 1999). Two of which are the metabolically-independent binding of these ions unto the cell surface (Binkley & Simpson 2003) and metabolic production of binding proteins (Samuelson et al. 2000). The cell walls of gram-positive bacteria naturally carry a negative charge because of their phosphate groups and teichoic acids that bind and regulate the movement of cations across the membrane. Also, the outer membrane of gram-negative bacteria, which consists of lipopolysaccharides, lipoproteins, and phospholipids carries a strong negative charge (Tortora et al. 2005). Because the cell surface of bacteria carries a net negative charge due to the presence of carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups (Tortora et al. 2005), it can adsorb appreciable quantities of positively charged cationic metals (e.g. Cd) (Scott & Palmer 1990). Furthermore, heavy metal resistance through production of binding proteins enhances the suitability of bacteria for biosorption of heavy metals dissolved in solution (Higham 1984).

In this study, we evaluated the ability of the copper-resistant bacteria *Stenotrophomonas maltophilia* isolated from copper-contaminated Mogpog River in Marinduque, Philippines (Oxfam Australia 2004), to biosorb Cu, Cd, and Pb from synthetic primary and ternary heavy metal solutions.

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MATERIALS AND METHODS

Water sampling

At least 10 water samples from randomly selected sites along a 100-meter length upstream the Mangamnan Bridge in Mogpog River, Marinduque, Philippines were collected in sterile sample bottles. The samples were collected from 3 locations at each site—near each bank and from the center of the river. The collected water from each location was pooled into a single sample bottle. Proper coding and identification of the sampling bottles were done. All the bottles used at each site were rinsed thrice with the river water prior to collection of the sample. The samples were stored in an ice cooler and transported chilled.

Screening for the most copper-resistant bacteria

Water samples from Mogpog River, Marinduque were mixed with phosphate buffer solution (PBS, pH: 7) in a 1:9 volume ratio to make a 100 mL inoculum source. Approximately 3mL inoculum (10 % rate v/v) were pipetted into 6 flasks containing 27 mL of nutrient broth supplemented with copper nitrate [$Cu(NO_3)_2$] of concentrations 0, 15, 30, 55, 80, and 140 ppm, respectively. The cultures were incubated for 1 week in an orbital shaker under ambient temperature conditions. After 1 week of incubation, the cultures were drop-plated onto corresponding copper-supplemented nutrient agar plates. Drop-plating was done by carrying out serial dilutions of up to 10-6 on nutrient broth cultures using 0.85% saline solution as diluent. Miles and Misra method of drop-plating was used (Miles & Misra 1938). Experiments were done in triplicates.

Bacterial plates were incubated for 24 to 72 h at ambient temperature until observable colonies appeared. The plates were examined and surveyed for distinguishable colonies. The bacterial colonies that survived in the plate with the highest copper concentration (most resistant) were isolated, purified, and preserved using glycerol and subsequent transfer techniques.

For multiple bacterial species isolated from the highest concentration (i.e. 140 ppm) setup, each species in-plate were purified further, separately incubated in flasks, and plated in media supplemented with higher copper concentrations (i.e. 275, 300, 330, 360, 390 ppm) until 3 bacterial species differentiated based on colony growth morphology in NA plates were determined to be the most copper-resistant.

The 3 most copper-resistant species were submitted to Microbiology and Infectious Disease Center (MIDC) in Muntinlupa City, Philippines for confirmation of pathogenicity and for pre-identification purposes.

Characterization of isolates

The most copper-resistant bacterial isolates were submitted to the Philippine National Collection of Microorganisms (PNCM) and Electron Microscopy Service Laboratory (EMSL) of the National Institute of Molecular Biology and Biotechnology (BIOTECH) in University of the Philippines Los Baños, Laguna, Philippines for characterization and identification up to the species level. Transmission electron microscope (Hitatchi H-300, 80 kV) was used for image observation at 15,000× – 20,000× magnification.

River sample analysis of Cu, Cd, Pb

The concentrations of copper, cadmium, and lead in the stored river sample from which the copper-resistant bacteria were isolated were measured using an atomic absorption spectrophotometer (AAS, Shimadzu Model AA-6300). Official AAS analysis methods of AOAC International were used in measuring the heavy metal concentrations (AOAC 1995). The samples were acidified to pH 2 with nitric acid [HNO_3] after isolation of copper-resistant bacteria for preservation and storage.

Biosorption experiments

Biosorption of copper, cadmium, and lead were evaluated by allowing a known bacterial biomass to interact with metal ions in solution.

Bacterial pre-cultures were prepared by inoculating a loopful of bacterial growth into 50mL Tryptic Soy Broth (TSB) (Merck) and were incubated for 24 h. Approximately 3 mL of pre-culture was inoculated into 10 flasks containing 222 mL Tryptic Soy Broth (TSB) to make a total of 2.25 L working culture.

Bacterial cells were harvested from the working culture after 24 h by centrifugation (EC B-22M Programmable Centrifuge) at 10,000 rpm delivering a centrifugal force of 7,826 × g for 10 min at 4° C.

Wet biomass fractions were accurately weighed on an analytical balance (Sartorius Model CP224S). After weighing, the biomass fractions were oven-dried at 100°C for 24 h. The final weights after drying were determined and used to calculate its dry matter content.

Roughly 0.5-gram weights of wet biomass were added into 3 flasks containing 25 mL of approximately 50 ppm, 10 ppm and 20 ppm of Cu, Cd, and Pb in primary solutions, respectively. Primary solutions contain only a single dissolved metal. The same weight of wet biomass was added to a fourth flask containing 25 mL of a ternary solution containing all three dissolved metals. The concentrations of each metal present in the solution,

Cu, Cd and Pb, were approximately 50 ppm, 10 ppm, and 20 ppm, respectively. The heavy metal concentrations of the solutions were based on the Mogpog River analysis results for simulation purposes. Nitrate salts of the metals [$Cu(NO_3)_2$, $Cd(NO_3)_2$, and $Pb(NO_3)_2$] were dissolved in distilled water to prepare the primary and ternary solutions.

The solutions were adjusted to pH 5 using 0.1 M sodium hydroxide [NaOH]. The bacterial suspensions were shaken for 2 h using an orbital shaker and were incubated under ambient temperature. Centrifugation was done after the defined contact time to separate the bacterial cells. The supernatants were analyzed using an atomic absorption spectrophotometer for remaining heavy metals in solution.

Percentage metal removal or biosorption and specific metal uptake (Q) were calculated (Volesky & May-Phillips 1995) as:

1. Percentage metal removal or biosorption:
   % Biosorption = $[(Ci - Cf) \div Ci] \times 100$
2. Specific metal uptake or metal biosorbed by the tested biomass (mg metal/g dry biomass weight):
   $Q = [V \times (Ci - Cf)] \div [1000 \times M]$

Where:

Q = specific metal uptake (mg metal/g biosorbent)

V = volume of metal solution (mL)

Ci = initial concentration of metal in solution (mg/L)

Cf = final concentration of metal in solution (mg/L)

M = mass of biosorbent (g)

Results were expressed as mean of experiments done in 6 replicates.

Statistical Analysis

Data gathered were subjected to one-way analysis of variance (ANOVA) at 5% level of significance. Mean values were then compared using Tukey's Test. Statistical Package for the Social Sciences (SPSS) for Windows (Standard Version Release 10.0.1 Copyright © SPSS Inc., 1989-1999) software was used for statistical analysis.

RESULTS

Screening for the most copper-resistant bacteria

A total of 5 bacterial isolates differentiated based on colony growth morphology were isolated from nutrient agar plates supplemented with 140 ppm Cu during the initial screening for the most copper-resistant bacteria. Further screening eliminated isolate #1, which failed to grow in Nutrient media of 275 ppm Cu concentration. After another round of screening procedures, isolate #2, which failed to grow in NA plates of 390 ppm concentration, was eliminated (Table 1).

TABLE 1. Bacterial growth in copper-supplemented nutrient media

Copper concentration (ppm)	Presence / absence of bacterial growth[a]				
	1	2	3	4	5
0	+	+	+	+	+
15	+	+	+	+	+
30	+	+	+	+	+
55	+	+	+	+	+
80	+	+	+	+	+
140	+	+	+	+	+
275	-	+	+	+	+
300	-	+	+	+	+
330	-	+	+	+	+
360	-	+	+	+	+
390	-	-	+	+	+
410	-	-	-	-	-
550	-	-	-	-	-

[a] (+) represents presence of bacterial growth
(-) represents absence of bacterial growth

Characterization of isolates

Pre-identification results from Microbiology and Infectious Disease Center (MIDC) revealed that isolates #3 and #5 were of the same species. Isolates #4 and #5 were determined to be of the same species and were identified as Stenotrophomonas maltophilia (Good Identification, 99.5% using API 20NE Identification System) by the Philippine National Collection of Microorganisms

(PNCM), BIOTECH.

S. maltophilia was observed to be rod-shaped, occurring singly or in pairs with 1 to several polar flagella under a transmission electron microscope. Diameter was approximated to be 0.5 to 1 μm and length, 1 to 2 μm (Figures 1 and 2).

River sample analysis of Cu, Cd, and Pb

High levels of copper, cadmium, and lead were measured in the water sample from which the copper-resistant bacteria were isolated. This was performed to simulate Mogpog River water conditions in terms of copper, cadmium, and lead content. The concentration of each heavy metal was approximately 50 ppm, 10 ppm, and 20 ppm for Cu, Cd, and Pb, respectively.

Biosorption experiments

S. maltophilia was able to remove 22.43% (0.57 mg/g) of Cu, 23.51% (0.12mg/g) of Cd, and 42.75% (0.41 mg/g) of Pb in primary solutions and 15.67% (0.39 mg/g) of Cu, 7.73% (0.4 mg/g) of Cd, and 35.19% (0.38 mg/g) of Pb in ternary solutions (Figures 3 and 4).

Comparison of biosorption data of the 3 heavy metals, both in primary and ternary solutions, showed significant differences. In primary solutions, biosorption profile was determined to be Cu > Pb > Cd. In ternary solutions, removal of Cu was almost equal to that of Pb, while cadmium removal remained least. Moreover, biosorption of all heavy metals in primary solutions were all higher than biosorption in ternary solutions. Specific heavy metal uptake data showed that the presence of other metals in

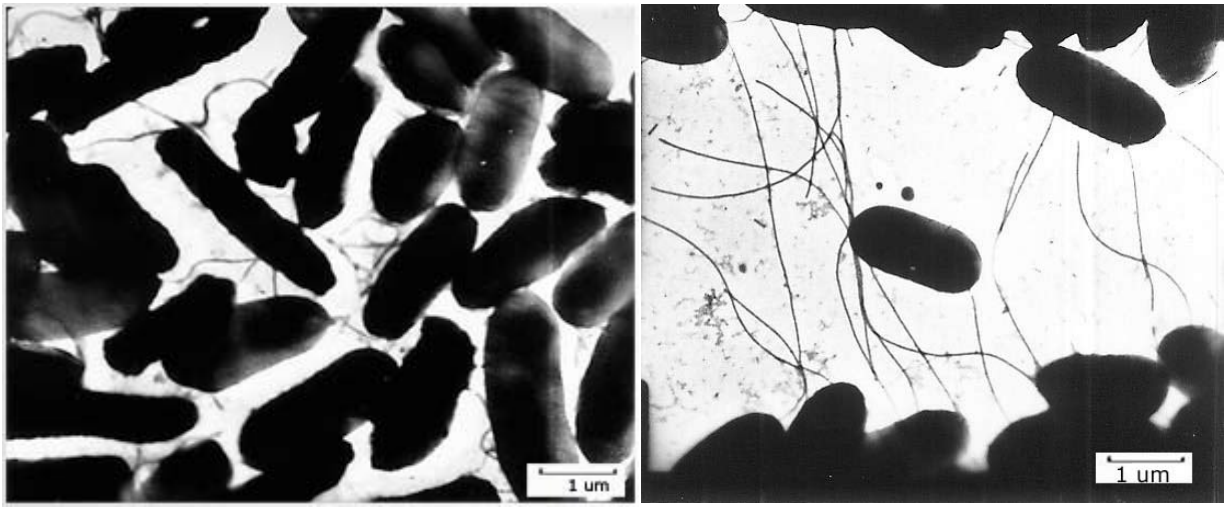

Figure1. TEM (Transmission Electron Microscope) Micrographs of *Stenotrophomonas maltophilia* (15,000X magnification)

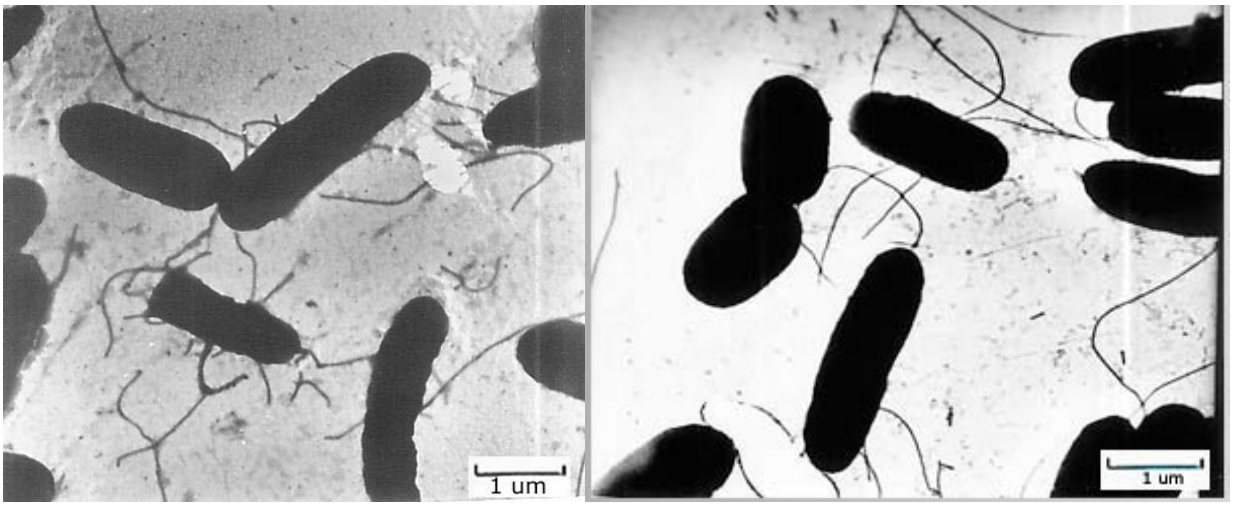

Figure 2. TEM (Transmission Electron Microscope) Micrographs of *Stenotrophomonas maltophilia* (20,000X magnification)

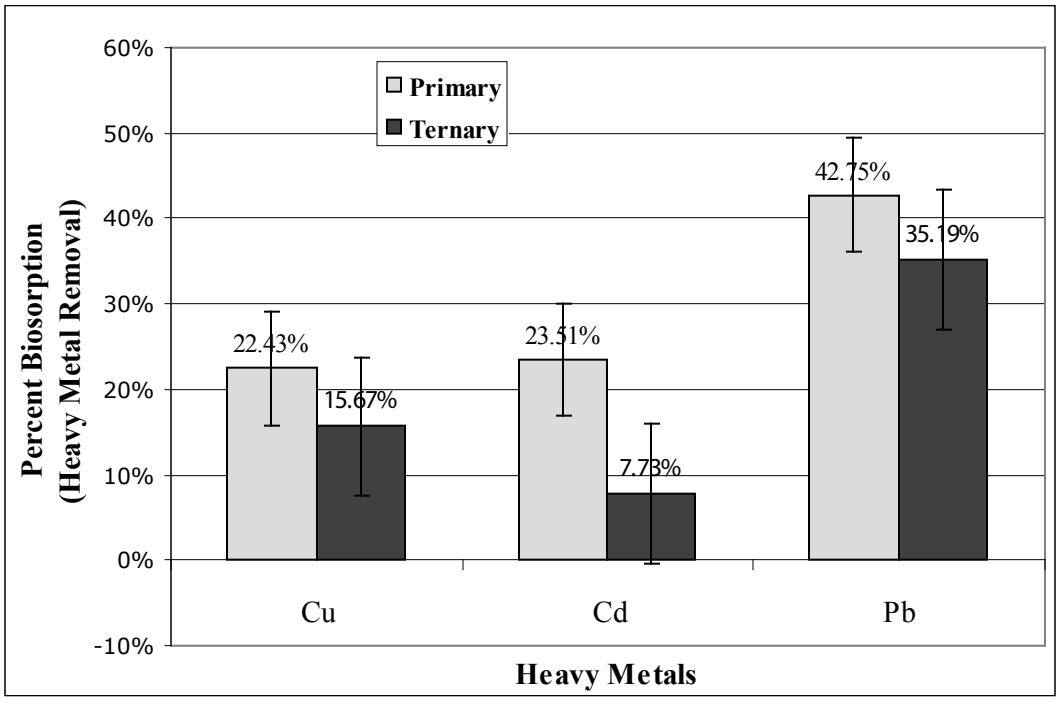

Figure 3. Comparison of Mean Percent Biosorption (% metal removal) of *Stenotrophomonas maltophilia* in Primary and Ternary Solutions (*Bars represent standard error)

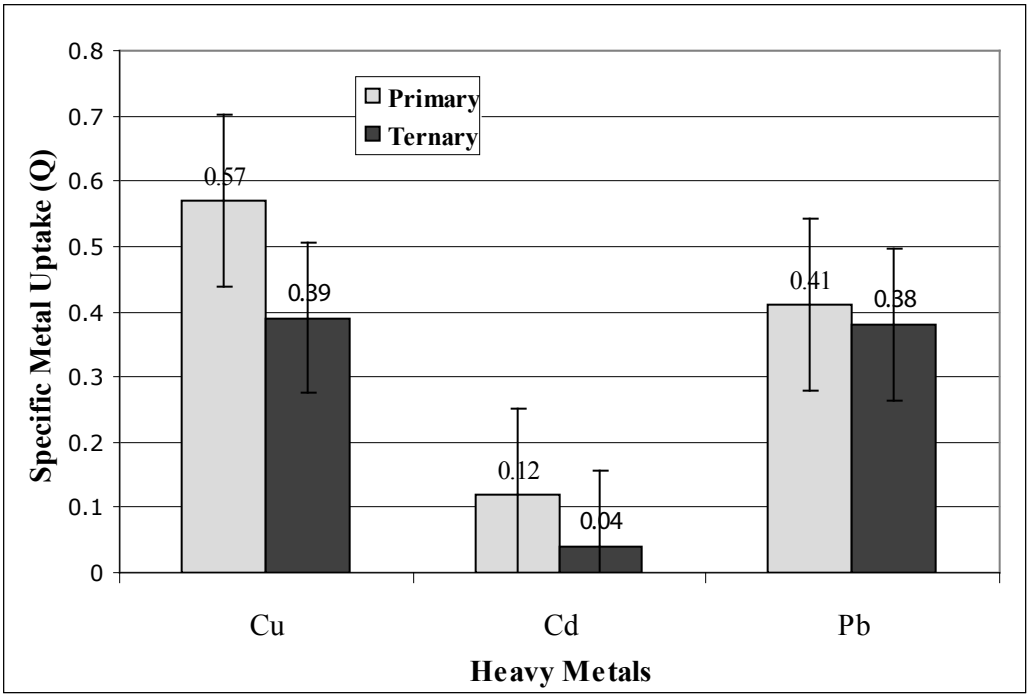

FIGURE 4. Comparison of Mean Specific Metal Uptake, Q (in mg/g) of *Stenotrophomonas maltophilia* in Primary and Ternary Solutions (*Bars represent standard error)

ternary solutions significantly reduced removal of Cu and Cd, whereas Pb removal was relatively less affected (Figures 3 and 4).

DISCUSSION

Screening for the most copper-resistant bacteria

The bacterial isolates are able to survive high copper concentrations in both liquid and solid media due to resistance mechanisms that they passively and actively display in response to the presence of heavy metals in their environment (Bae et al. 2003). The number of surviving bacterial species in the nutrient media decreases as the concentration of copper increase. This happens as the relatively less resistant bacteria are screened-out in the presence of high copper concentration.

The most Cu-resistant bacteria were selected due to previous knowledge that higher resistance infers a better capacity to retard metal diffusion to the inside of the cell by biosorbing heavy metals unto the cell surface, or compartmentalizing the heavy metals inside in case penetration happens (Nies 1999; Ahalya et al. 2003).

Characterization of isolates

S. maltophila or *maltophilia* is a gram-negative obligate aerobe, non-sporulating bacilli with cells that are straight or slightly curved. They occur singly or in pairs and are motile due to several polar flagella. This non-fermentive bacterium is found in a wide variety of environments and geographical regions, and thrives ubiquitously in aquatic sources including rivers, wells, hypereutrophic lakes, and sewage. It was designated initially *Pseudomonas maltophilia* and later moved to genus *Xanthomonas*. Continuing dissatisfaction with the classification of this organism finally gave rise to the proposal in 1993 to create the new genus Stenotrophonas with *S. maltophilia* as the sole member (Denton & Kerr 1998).

River sample analysis of copper, cadmium and lead

According to the Department of Environment and Natural Resources (DENR - Philippines), Mogpog River is classified as class C surface water with beneficial uses for fish propagation, recreation (boating, etc.), and industrial manufacturing processes after treatment. DENR Administrative Order No. 34 (Water Quality) states that the maximum limits for the concentrations of dissolved Cu, Cd, and Pb in class C waters are 0.05 ppm, 0.01 ppm, and 0.05 ppm, respectively (Oxfam Australia 2004). However, the results of this study show that the levels of Cu, Cd, and Pb all exceed the limits the water quality criteria class C for these heavy metals.

According to Oxfam Australia (2004), none of the communities living near the Mogpog River use it as a regular source of drinking water. Locals may come into contact with the river however, from bathing, crossing the stream, and other activities. Indirect contact may also result if fish or other animals are caught for food and through irrigation of crops and consumption by domestic animals.

Exposure to copper has been linked to gastrointestinal, hepatic, and renal effects. Cadmium is known to cause damage primarily to the liver and kidneys while lead, a multi-targeted toxicant, affects the gastrointestinal tract, hematopoietic system, cardiovascular system, central and peripheral nervous systems, kidneys, immune system, and reproductive system (Plumlee et al. 2000).

It is important to note, however, that the measured levels of Cu, Cd, and Pb may only be applicable to the present study because the level of pollutants is likely to vary at any location over time due to varying rates of outflow from the dam, the volume and rate river flows, and other factors like amount of rainfall prior to collection and waste dumping by nearby residents (Oxfam Australia, 2004). It is also probable that significant levels of metals have accumulated in the sediments given that discharge from the dam has been occurring for some years (Oxfam Australia, 2004 and Plumlee et al. 2000).

Biosorption Experiments

In this study, two sets of biosorption data were gathered: percent biosorption (or percent metal removal) and specific metal uptake (Q). Specific metal uptake describes how much heavy metal was removed from solution without reference to how much was present initially in solution. Percent biosorption, however, describes how much heavy metal was removed from solution with reference to how much was present initially. Calculation of Q in mg/g (metal/biomass) unit was based on the study of Al-Garni (2005). Q data were also expressed in μmol/g (metal/biomass).

Since the initial concentrations of the heavy metals in simulated river solutions were not equal, it is easier to understand the biosorption profiles using Q data than percentage removal. With Q data, we can directly compare how much heavy metal was biosorbed by the bacteria from solution irrespective of whether the metal was present in greater or lesser concentration initially.

Percent biosorption data for *Stenotrophomonas maltophilia* showed that Pb removal was highest both in primary and ternary solutions. This was followed by Cu and then Cd (Figure 3). However, Q data showed a

change in trend wherein Cu removal in primary solutions was highest, followed by Pb, and then Cd. It is important to note that in ternary solutions, although Cu uptake was the highest, Pb uptake was almost at the same level. Cadmium remained least removed from ternary solutions (Figure 4).

In the present study, biosorption was found to be higher when the metals are present individually in primary solutions than when present collectively in ternary solutions (Figures 3, 4 and 5). This is because in ternary solutions, more metal ions are in competition against one another for metal-binding sites in the bacteria (Chang and Chen 1998). In the study of Göksungur et al. (2005), competitive biosorption experiments were performed with Cd(2+) and Pb(2+) together with Cu(2+) and they reported that competitive biosorption capacities of the yeast biomass they used for all metal ions were lower than in non-competitive conditions.

In primary solutions, the Q data biosorption profile was Cu > Pb > Cd (Figure 4). This is due to the fact that the initial concentrations of the heavy metals in solutions were Cu > Pb > Cd when compared with each other. Relatively more Cu ions were readily available to occupy the binding sites in the bacteria. On the contrary, Cd, present in lowest concentration initially had the least chance of being removed from solution.

Comparison of the Q data of the 3 metals in ternary solutions (simulated river) showed that despite the lower concentration of Pb compared to Cu, Pb was unexpectedly biosorbed to an almost similar degree as Cu by both isolates (Figure 4). When we take into account the discrepancies of the heavy metals' initial concentrations, we can modify the biosorption profile from Cu > Pb > Cd to Pb > Cu > Cd because Pb was selected over Cu by the biomass' binding sites. Explanations for this phenomenon, that are based either on the properties of the metals or the bacteria, have been laid down by many studies (Chang & Chen 1998; Chang & Huang 1998; Chang & Chen 1999; Puranik & Pakniker 1999; Lu et al 2006; and Lu et al. 2007).

Such biosoprtion profile in our study, Pb > Cu > Cd, has also been reported by Puranik and Pakniker (1999) in a Citrobacter species, Chang and Chen (1998), Chang and Chen (1999) in *Pseudomonas putida*, Chang and Huang (1998) in *Pseudomonas aeruginosa* and Lu et al. (2006) in an indigenous isolate *Enterobacter* sp. Except for the last mentioned, the studies have demostrated that Pb ions strongly inhibited the sorption of Cu and Cd due to ion exchange effect. Lead, being a larger and heavier ion of atomic radius 180 pm and 207.2 g/mol atomic mass, is likely to replace the smaller and lighter Cu and Cd ions of 135 pm and 155 pm atomic radius, respectively and 63.5 g/mol and 112.4 g/mol atomic mass, respectively

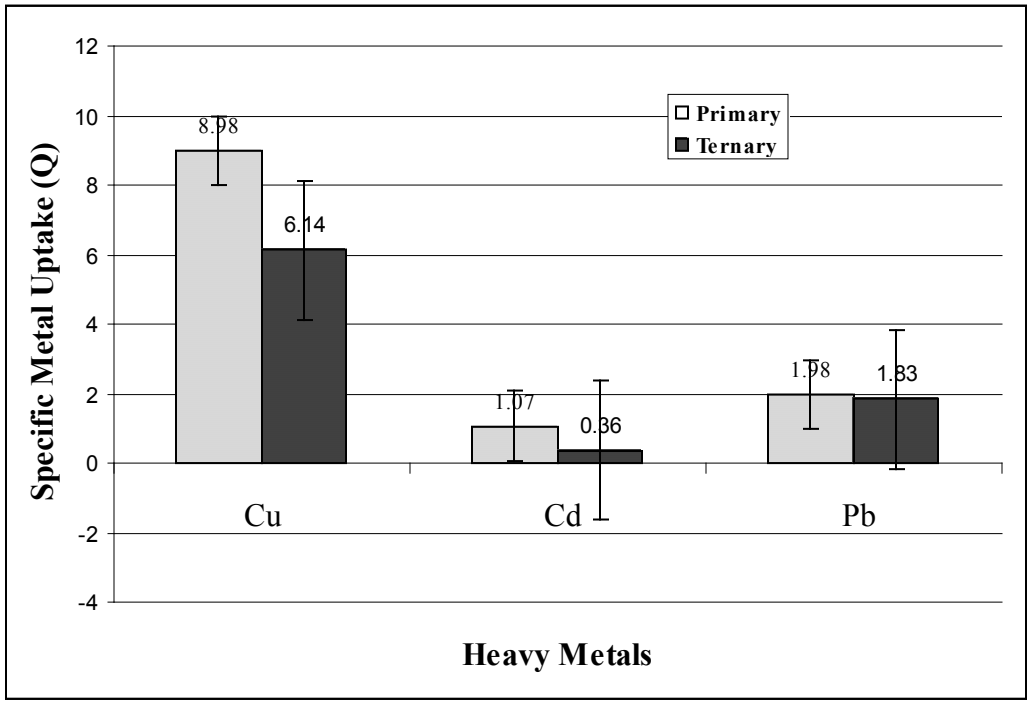

Figure 5. Comparison of Mean Specific Metal Uptake, Q (in µmol/g) of *Stenotrophomonas maltophilia* in Primary and Ternary Solutions (*Bars represent standard error)

(Chang & Huang 1998; and Han et al. 2006). *Enterobacter* sp. was suggested by Lu et al. (2006) to have a different mechanism of lead biosorption. They initially attributed the selectivity to a possible intracellular accumulation during the uptake of Pb. Further exploration by Lu et al. (2007) using a novel experimental design revealed that presence of other metals resulted in a competitive effect. They showed that the influence of the other 2 metals in ternary metal biosorption system can be easily determined by comparing the stray distance from the single metal biosorption.

A similar trend may also be observed in Figure 5. Although biosorption of Pb showed a marked difference compared to Figure 4, the trend is retained because the concentrations of metals do not change despite conversion of units into μmol. This only shows that Pb ions are bigger than Cu and Cd ions that support the explanation as to why Pb selectively replaces the other two ions in binding sites during equilibrium in solution.

In a study of Wong et al. (1993), the presence of Pb(II) greatly reduced the uptake of Cu(II) by fivefold in *P. putida*. Pb and Cu have been shown by Chang and Chen (1998) to replace pre-adsorbed Cd ions from *P. aeruginosa*, but their competition against each other for binding sites was comparative, contrasting the study of Wong et al. in 1993.

Other studies that involved Cu, Cd, and Pb in mixture solutions also reported similar biosorption profiles. Ilhan et al. (2004), Akar and Tunali (2006), Han et al. (2006) and Uslu and Tanyol (2006) reported the biosorption profiles results of their studies to be Pb>Cu for *Staphylococcus saprophyticus*, *Aspergillus flavus*, *Saccharomyces cerevisiae* and *Pseudomonas putida*, respectively. Odokuma and Abah (2003) Tüzün et al.(2005) and Yu et al.(2007) reported Pb>Cd for *Bacillus* sp., *Staphylococcus* sp. and *Pseudomona*s sp. *Chlamydomonas reinhardtii* and cysteine-modified *Saccharomyces cerevisiae*, respectively. Moreover, Chong and Volesky (1995) reported a Cu>Cd profile for *Ascophyllum nodosum*. All of these observations are consistent with our present study.

This biosorption profile, however, is not applicable to all organisms and conditions. Contrasting our study, Da Costa and Duta in 2001 reported a reverse profile in four Bacillus species: Cd > Cu > Pb. Also in contrast are other researchers' biosorption profile report, which was Pb > Cd > Cu, wherein Cd was biosorbed to a greater extent than Cu (Chang et al. 1997; Saeed et al. 2005 and Kim et al. 2007). This can be explained by differences in selectivity of biosorbents for metals in aqueous solutions, which has been more extensively studied by Premuzic et al. in 1991. They studied systematically the comparative capacity for selective uptake of metals by different species of microorganisms under identical experimental conditions. Results of their study showed that in addition to metal selectivity, there was also a species-dependent differentiation in the uptake capacity. Chemical and structural characteristics of cell membranes and cell wall constituents vary with species and thus also influence the behavior of metallic species in the environment (Premuzic et al. 1991).

In the study of Kim et al. (2007) transmission electron microscopy (TEM) and energy dispersive x-ray spectroscopy (EDS) of CPB4 (*Bacillus* spp.) revealed that the CPB4 cell has a variable pattern of biosorption with different heavy metals. In Pb-uptake and Cd-uptake, the electron dense granules were mainly found both on the cell wall and cell membrane. However, in Cu-adsorbed cells, most of the electron-dense granules were found on the outside but located near the cells.

Using atomic force microscopy (AFM) and Fourier transform infrared (FT-IR) spectroscopy, a study conducted by Pan et al. (2006) explained the potential mechanisms involved in the biosorption of metal ions. Moreover, AFM imaging and FT-IR spectra showed a major morphological change occurred after Pb^{2+} biosorption and indicated the binding characteristics of the lead ions involved the carboxyl, hydroxyl, and amino groups in the biomass.

Numerous studies have reported the sorption of Cd from aqueous solutions by microorganisms (Macasckie & Dean 1984; Scott & Palmer 1990; Samuelson et al. 2000; Pardo et al. 2003 & Al-Garni 2005). But in this study, results showed that Cd was not accumulated effectively by *Stenotrophomonas maltophilia* (Figure 4) indicating that resistance to this metal was not through a similar biosorption mechanism that was used for Cu and Pb but, through efflux systems. This may be explained by a cluster of genes involved in heavy metal resistance that has been characterized from the gram-negative bacterium *S. maltophila* by Alonso et al. in 2000. These genes included a cadmium efflux determinant (cad A), together with the gene cad C owing for its transcriptional regulator (Alonso et al. 2000). This is aside from the fact that in this study, Cd was present in lowest concentration in ternary simulated solutions.

It is well recognized that microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms (Malekzadeh et al. 1996). Mogpog River analysis of Cu, Cd, and Pb revealed that levels of these metals are at high levels at the river during the time of sample collection. Since the bacteria used in this study were isolated from the same

heavy-metal contaminated waters, it was assumed that exposure of the isolate to similar levels of heavy metals in simulated solutions did not kill or inactivate the cells to an extent where metabolic activities were halted. Hence, classical adsorption isotherms (e.g. Freundlich and Langmuir models) were not used in this study; owing to the probability that uptake was not restricted to surface adsorption phenomena. Metabolically-dependent mechanisms of heavy metal resistance may have contributed to the uptake (Da Costa & Duta 2001).

In this study, live cells were used because, although metabolic resistance mechanisms are more complex, they may also offer more potential in biosorbing heavy metals since absorption may add to the total biosorption capacity (Mullen et al. 1989). This may result to a higher yield in biosorption than adsorption unto cell surface alone. In the study of Chang et al. in 1997, their results showed that resting live cells were able to uptake higher amounts of Pb than inactivated dead cells of *Pseudomonas aeruginosa*.

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