Biosorption of copper (II) by live biomasses of two indigenous bacteria isolated from copper-contaminated water

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The study investigated the potential of intrinsic bioremediation of copper using indigenous resistant bacterial flora. Screening and isolation of bacteria were carried out from copper-contaminated water samples of Bol River Reservoir, Marinduque, Philippines. Bacterial colonies grew on amended nutrient agar plates containing 50, 100, and 200 ppm copper. Four copper-resistant bacteria were isolated and purified. The biomasses of the least and most resistant isolates, namely *Staphylococcus sciuri* and *Bacillus fastidiosus*, were used as adsorbents for the removal of copper from aqueous solutions. Removal efficiencies depended on pH and biomass dosage, leveled off with time, and decreased with initial copper concentration. The biosorption of copper on both biomasses correlated well with the Langmuir isotherm *(r^2 = 0.95 and 0.99)*. In keeping with its greater resistance, the Langmuir model revealed that *B. fastidiosus* has approximately 27% more adsorption sites on its surface than *S. sciuri*. However, *B. fastidiosus* has weaker affinity for copper than *S. sciuri*. The maximum adsorption capacities obtained for *B. fastidiosus* and *S. sciuri* compare well with those of other microbes reported in the literature. The Scatchard plots indicated that both bacterial surfaces contain multiple binding sites and that the adsorption of copper exhibits negative cooperative behavior. Also, the biomasses removed copper from actual wastewater with up to 90% efficiency, with negative deviations from expected removal efficiencies attributable to the presence of iron and zinc. Unfortunately, the actual use of *S. sciuri* in bioremediation is precluded by its pathogenicity. The same, however, is not true with *B. fastidiosus.* Thus, *B. fastidiosus* can potentially be used as an efficient and non-pathologic bioremediating agent for copper.

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INTRODUCTION

Bioremediation is a field of environmental microbiology that is geared towards degrading toxic organic pollutants and removing heavy metals through the use of microbial systems. Usually, bioremediation is used in treating organic contaminants (Kao and Prosser 1999). Recently, however, progress has been made in the successful utilization of microbes in the removal of heavy metals. This technique has proved efficient in the removal and recovery of heavy metals such as arsenic (Rodríguez et al. 2013), copper (Ghosh and Saha 2013, Parungao et al. 2007), chromium (Anjana et al. 2007), cadmium (Meitei and Prasad 2013), and lead (Abdel-Aty et al. 2013). Some procedures on bioremediation employ indigenous microorganisms (Anjana et al. 2007, Llamado et al. 2013) while others rely on the use of foreign bacterial, fungal, or algal strains (Al-Homaidan et al.

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2014). Currently, studies are exploring the possibility of using genetically engineered microbes for bioremediation (Di Caprio et al. 2014). Intrinsic bioremediation involves the utilization of indigenous plants (Zhang et al. 2014) and microorganisms (Colaka et al. 2011, Clausen 2000) in the removal and degradation of pollutants (van Bemmel 2010). This promising method can be used in the sequestration of highly toxic metals without the threat of possible ecological imbalance since the remediating organism is native to the environment to be treated.

Biosorption is defined as the adsorption of ions, compounds, or pollutants on biological materials. Biosorption, which, in simple terms is physical adsorption on biomass surfaces (Chong and Volesky 1995), has to be differentiated from bioaccumulation, which refers to the uptake of metals and other compounds by microbial cells through processes closely associated with microbial metabolism (Garnham et al. 1992). Adsorption of metals on biomasses results from the ionic and covalent interactions between metal ions and hydroxyl, amino, carbonyl, carboxyl, phosphoryl, and other functional groups present on microbial cell walls (Abdel-Aty et al. 2013, Beveridge 1989, Da Costa 1999). Although virtually all biological materials have significant adsorptive ability, the concept and application of biosorption have largely involved the use of microbial systems. Thus, biomasses from bacteria (Barbosa et al. 2014), algae (Chen et al. 2008, De Guzman and Cao 2010), and fungi (Chhikara and Dhankhar 2008) have been used in the efficient removal of heavy metals from contaminated sources and artificially prepared wastewater.

The Marcopper Mining incident of 1996 has caused severe environmental damages to Marinduque, Philippines. Marcopper plugged concrete into the Tapian pit and converted it into a disposal pond for mining waste after they have exhausted its ore reserve. Seepage was observed until the plug ultimately fractured and caused the discharge of some 1.6 million cubic meters of mine tailings into the Makulapnit-Boac River system. The onrush of tailings inundated low-lying areas, destroyed crop fields, and clogged irrigation channels. The discharge caused experts to declare the Boac River dead (Bennagen 1998). Consequently, the local population, who mainly derive their income from farming and fishing, were severely affected. Pollution of areas close to the mining sites persisted in the years after the disaster. The Bol River Reservoir which receives surface and possibly ground waters from the Tapian pit and other waste dumps shows a green tint, suggesting elevated levels of copper (Plumlee et al. 2000). This work was conducted for the purpose of isolating copper-resistant bacteria from Bol River Reservoir, Marinduque and evaluating their copper-removal efficiencies for potential intrinsic bioremediation.

MATERIALS AND METHODS

Screening and Isolation of Copper-Resistant Bacteria

Water samples from Bol River Reservoir, Marinduque were mixed with phosphate buffered saline (PBS, pH:7) in a 1:9 volume ratio to make 100 mL inoculum. Three mL inoculum was pipeted into each of six flasks containing 27 mL of nutrient broths amended with copper sulfate ($CuSO_4{\cdot}6H_2O$) of Cu^{2+} concentrations 0, 50, 100, 200, 300, and 500 ppm. The cultures were incubated for one week in an orbital shaker at 28 ^{O}C. After one week of incubation, the cultures were drop-plated onto corresponding copper-ammended 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. Bacterial plates were then incubated for one to three days at ambient temperature until observable colonies appeared. After incubation was complete, the plates were examined and surveyed for the number of colony-forming units (CFUs). Distinguishable bacterial colonies were isolated, purified, and labeled accordingly.

Identification of Copper-Resistant Bacteria

The bacterial isolates were identified by the Philippine National Collection of Microorganisms (PNCM) of the National Institute of Molecular Biology and Biotechnology (BIOTECH) at the University of the Philippines Los Banos. It entailed the analysis of cell morphology; biochemical tests including oxygen requirement test, indole production test, methyl red test, Voges Proskauer test, citrate utilization and hydrogen sulfide production test, motility test, gelatin liquefaction test, nitrate reduction test, oxidase and catalase production test, starch utilization test, lysine decarboxylase and arginine dihydrolase test, casein hydrolysis test, tyrosine utilization and lipid utilization test, acid from glucose test, gas from glucose test, sucrose, galactose, lactose, xylose, arabinose, maltose, raffinose tests, and growth in 5%, 10%, 30%, and 40% NaCl tests, as well as endospore staining.

Preparation of Biomass

Bacterial pre-culture was prepared by inoculating 50 mL nutrient broth. Three mL pre-culture was pipeted into each of ten Sakaguchi flasks containing 297 mL nutrient broth to make a total of 3 L main culture. Biomasses were harvested by pooling and collecting the pellets after centrifuging the main culture at 10,000 rpm for 10 min. Wet biomass fractions of weights ranging from 0.1 to 0.5 g were accurately weighed on an analytical balance. After weighing, the biomass fractions were oven-dried at 100 ^{0}C for 1 day. The final weights after drying were used to calculate the dry matter content.

Batch Sorption Experiments

Optimization of pH

Biomasses of 0.5 g weight were separately added into five flasks containing 25 mL of 10 ppm Cu^{2+} solutions of pH 2, 3, 4, 5, and 6. The bacterial suspensions were shaken for 2 hrs at 28 ^{O}C and 150 rpm on an orbital shaker. Centrifugation was carried out after the defined contact-time and the supernatants analyzed for copper using Atomic Absorption Spectroscopy (AAS).

Optimization of Contact-Time

Biomasses of 0.5 g weight were separately added into four flasks containing 25 mL of 10 ppm Cu^{2+} solutions adjusted to optimum pH. The bacterial suspensions were then shaken for 0.5, 1, 2, and 4 hrs at 28 ^{O}C and 150 rpm on an orbital shaker. Centrifugation was carried out after the defined contact-times and the supernatants analyzed for copper.

Optimization of Biomass Dosage

Biomasses of 0.05, 0.10, 0.25, and 0.50 g weight were added into four flasks containing 25 mL of 10 ppm Cu^{2+} solutions adjusted to optimum pH. The bacterial suspensions were then shaken at 28 ^{O}C and 150 rpm on an orbital shaker. Centrifugation was carried out after the optimum contact-time and the supernatants analyzed for copper.

Effect of Initial Copper Concentration

Biomasses of optimum weight were separately added into four flasks containing 25 mL of 10, 20, 40, and 100 ppm Cu^{2+} solutions adjusted to optimum pH. The bacterial suspensions were then shaken at 28 ^{O}C and 150 rpm on an orbital shaker. Centrifugation was carried out after the optimum contact-time and the supernatants analyzed for copper.

Evaluation of Adsorption Parameters

The resulting C_{eq}, equilibrium copper concentration, and Q, adsorption capacity by the biomasses were used in constructing the Freundlich and Langmuir adsorption isotherms and the Scatchard plot. The thermodynamic parameters from those isotherms were evaluated and correlated with observed bacterial copper resistance.

Application to Actual Wastewater

Biomasses of optimum weight were added into two flasks containing 25 mL of Bol and Tapian wastewater, respectively. The bacterial suspensions were then shaken at 28 ^{O}C and 150 rpm on an orbital shaker. Centrifugation was carried out after the optimum contact-time and the supernatants analyzed for copper.

RESULTS AND DISCUSSION

Isolation of Copper-Resistant Bacteria

Bacterial growth was only observed on nutrient agar (NA) plates containing 200 ppm copper or less. Copper-resistant bacteria were isolated from 50, 100, and 200 ppm amended plates. Pure bacterial cultures grew in the presence of 50 and 200 ppm Cu, while a mixed culture of two bacteria was observed on 100 ppm Cu NA. Photographs of the bacterial cultures on amended NA plates are shown in Figure 1. Expectedly, as can be seen in Figure 2, the population of bacteria in the amended nutrient broths decreased with the concentration of copper. This occurred as none or relatively less resistant bacteria were screened out in the presence of increasingly high copper concentrations.

Identification of Copper-Resistant Bacteria

The results of the tests conducted by the Philippine National Collection of Microorganisms on the identification of the least (isolate 4) and most (isolate 1) copper-resistant isolates, the bacteria that grew on 50 ppm Cu and 200 ppm Cu, respectively, are detailed in Tables 1 to 4. Isolate 1 is a spore-forming, rod-shaped, gram-positive, motile, facultative anaerobe that has been identified as *Bacillus fastidiosus*, while isolate 4 is a nonspore-forming, spherical, gram-positive, immotile, facultative anaerobe that has been identified as *Staphylococcus sciuri* (Philippine National Collection of Microorganisms 2004).

B. fastidiosus, having an average length of 2.59 µm, is larger than *S. sciuri*, which has an average diameter of 0.70 µm (Figure 3). The S. sciuri electron micrograph, however, does not show the clustered morphology of this bacterium.

Only these two isolates were used for biosorption of copper.

Batch Sorption Experiments

Effect of pH

Figure 4 shows the increase in Cu removal with an increase in pH for both biomasses. This can be attributed to the increase in the number of available adsorption sites. Increasing the pH causes the deprotonation of phosphate, carboxyl, hydroxyl, and other potential lone-pair donors (Tipping 2002), thereby making them available for interaction with copper ions. Furthermore, hydronium ions are reported to compete with cations for the adsorption sites on the surface of the biomass; therefore, the 100-fold decrease in concentration of this ion from pH 2 to 4 may account for the increased removal of copper (Gadd 1988). As can be seen in the figure, the plots for the removal of copper against pH by the biomasses are generally similar. In addition, removal of copper by both bacteria was optimum at pH 4. This similarity in pH profiles can be attributed to the existence of identical functional groups on the two biomass surfaces as the two bacteria were both gram-positive. Adsorption experiments at pH greater than six were not carried out because copper starts to form insoluble species at these pH values (Goksungur et al. 2003).

Effect of Contact-Time

Adsorption of heavy metals on biomasses is relatively fast (Puranik and Paknikar 1997) and involves three steps: bulk transport, film transport, and actual binding (Weber 1985). Bulk transport of metal ions in the aqueous phase is usually fast because of continuous mixing (Gadd 1988). Film transport involves the diffusion of ions across a hypothetical boundary. Lastly, the actual binding of cations on adsorption sites is a rapid, reversible reaction (Tsezos et al. 1988). According to Figure 5, adsorption of copper by *B. fastidiosus* and *S. sciuri* approximately reached equilibrium after 2 hrs and 30 minutes of continuous shaking, respectively. Actually, it is possible that adsorption of copper by *S. sciuri* was already complete even before the 30-minute-contact-time has elapsed since the surveyed data points do not show the time behavior of copper adsorption at contact-times shorter than 30 min. However, the optimum contact-time chosen for *B. fastidiosus* and *S. sciuri* were 4 and 2 hrs respectively, which were well beyond the time needed to achieve sorption equilibrium in order to provide allowances that would take into account the accompanying difficulty in repeatedly simulating experimental conditions involving live microbial species (Jianlong et al. 2001).

Effect of Biomass Dosage

As can be seen in Figure 6, the efficiency of copper removal from aqueous solutions increased as the amount of biomass increased for both *B. fastidiosus* and *S. sciuri*. Since adsorption is a physico-chemical equilibrium process, its

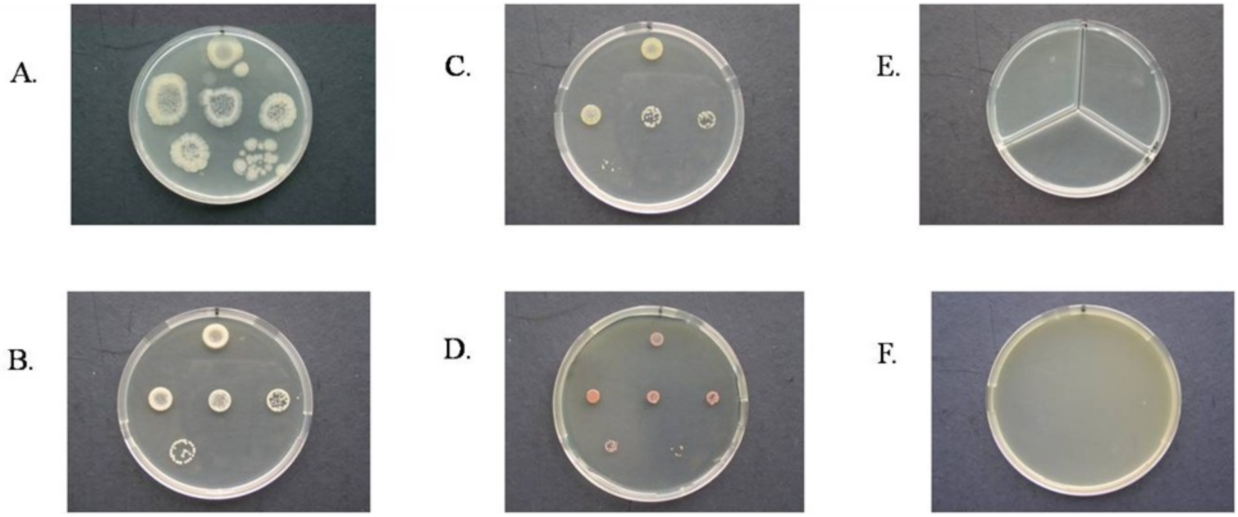

Figure 1. Bacterial cultures on nutrient agar plates containing A) 0 ppm Cu^{2+}, B) 50 ppm Cu^{2+}, C) 100 ppm Cu^{2+}, D) 200 ppm Cu^{2+}, E) 300 ppm Cu^{2+}, F) 500 ppm Cu^{2+}

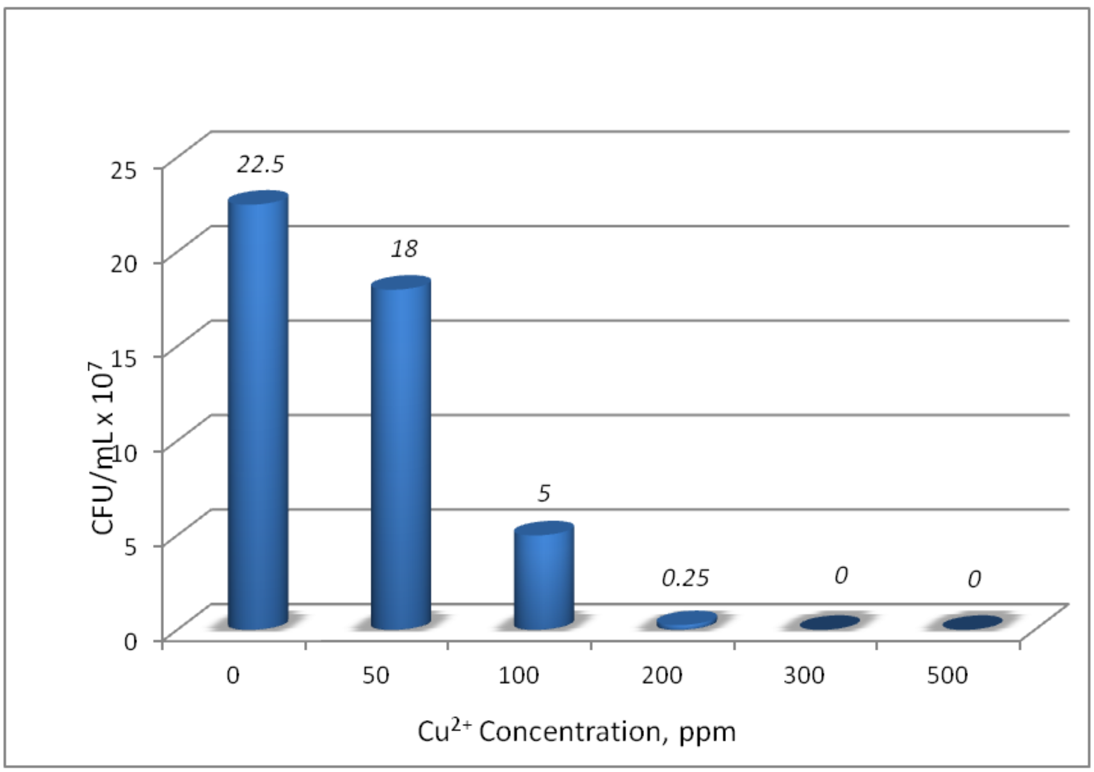

Figure 2. Effect of Cu^{2+} concentration on bacterial population

Table 1. Morphological characteristics of the bacterial isolates

Isolate No.	Colony Morphology	Cell Morphology
1	Pink, shiny, pinpoint, flat to slightly raised colonies with entire margin	Gram positive, short rods, arranged in singles
4	Off-white to cream, translucent, circular, convex colonies with entire margin	Gram positive, coccoid, arranged in singles and clusters

(Philippine National Collection of Microorganisms 2004)

Table 2. Biochemical characteristics of the bacterial isolates (+, Positive; -, Negative)

Test Conducted	Isolate 1	Isolate 4
Oxygen requirement	Facultative anaerobe	Facultative anaerobe
Indole production	-	-
Methyl Red test	-	-
Voges Proskauer test	-	-
Citrate utilization	-	-
H_2S production	-	-
Motility test	+	-
Gelatin liquefaction	-	Weak +
Nitrate reduction	-	+
Oxidase production	+	+
Catalase production	+	+
Starch utilization	-	-
Lysine decarboxylase test	-	-
Arginine dihydrolase test	-	-
Casein hydrolysis	-	Weak +
Tyrosine utilization	-	Not Determined
Lipid utilization	-	-
Acid from glucose	+	+
Gas from glucose	-	-
Sucrose	-acid; -gas	-acid; -gas
Galactose	-acid; -gas	+acid; -gas
Lactose	-acid; -gas	-acid; -gas
Xylose	-acid; -gas	+acid; -gas
Arabinose	-acid; -gas	+acid; -gas
Maltose	-acid; -gas	-acid; -gas
Raffinose	Not Determined	-
Growth in 5% NaCl	+	+
Growth in 10% NaCl	+	+
Growth in 30% NaCl	+	-
Growth in 40% NaCl	+	-

(Philippine National Collection of Microorganisms 2004)

Table 3. Endospore staining results on the bacterial isolates

Isolate No.	Endospore
1	Sub-terminal to terminal oval spores that distend the cell
4	None

(Philippine National Collection of Microorganisms 2004)

Table 4. Identities of the selected copper-resistant bacteria

Isolate No.	Identity
1	*Bacillus fastidiosus*
4	*Staphylococcus sciuri*

(Philippine National Collection of Microorganisms 2004)

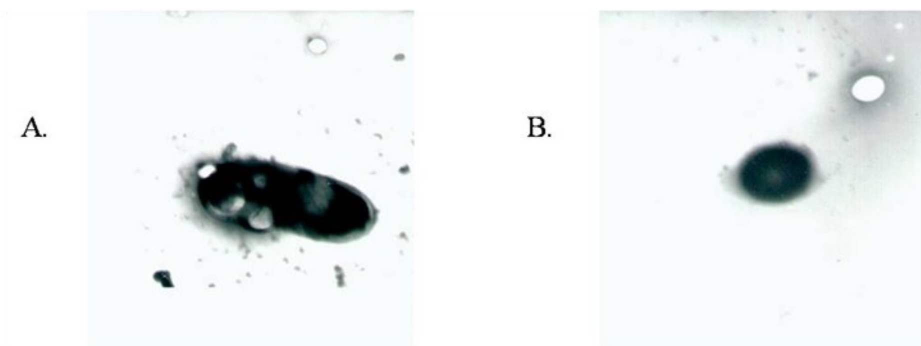

Figure 3. Transmission Electron Micrographs of A) *B. fastidiosus* and B) *S. sciuri*

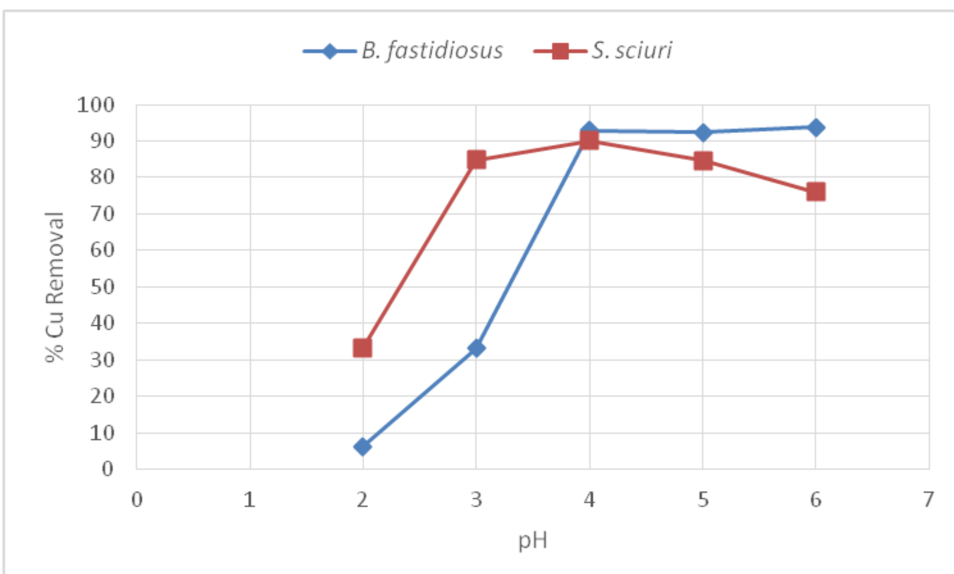

Figure 4. Effect of pH on copper removal by *B. fastidiosus* and *S. sciuri* (C_i = 10 ppm, Biomass dosage = 0.50 g, Contact-time = 2 hrs)

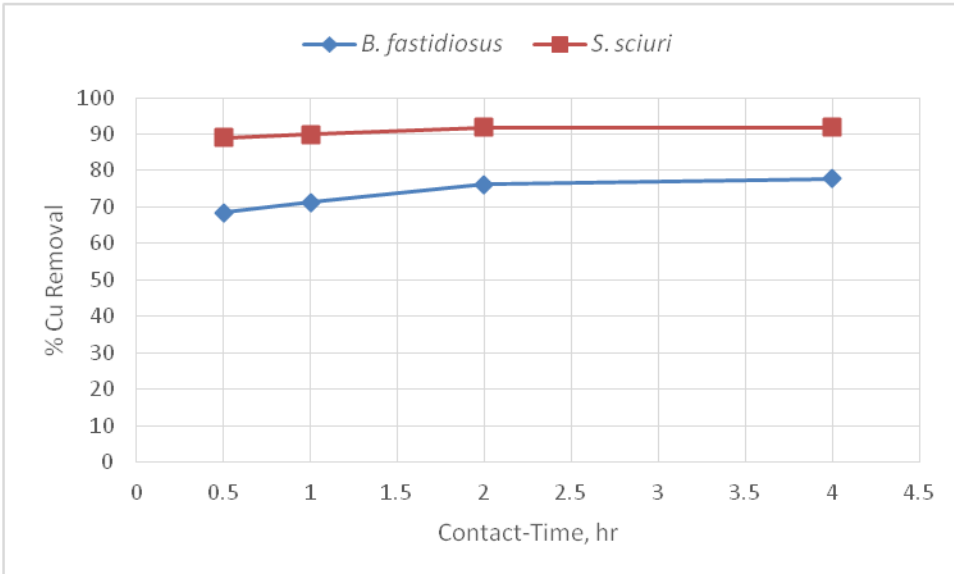

Figure 5. Effect of contact-time on copper removal by *B. fastidiosus* and *S. sciuri* (C_i = 10 ppm, pH = 4, Biomass dosage = 0.50 g)

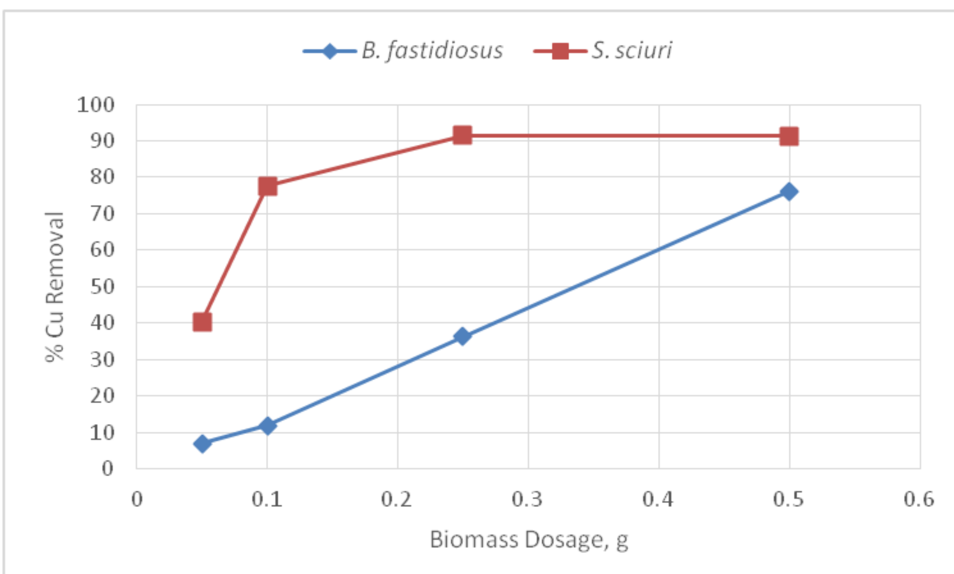

Figure 6. Effect of biomass dosage on copper removal by *B. fastidiosus* and *S. sciuri* (C_i = 10 ppm; pH = 4; Contact-time = 2 hrs *(S. sciuri),* 4 hrs (*B. fastidiosus))*

behavior is subject to the constraints of the Le Chatelier's Principle (Tsezos et al. 1988). If we let **B** be the adsorption site and **B(Cu^{2+})** be the adsorption site-copper complex, the interaction between the adsorbate and the adsorbing species can be written in the form

$$\mathbf{B} + \mathbf{Cu^{2+}} \leftrightarrow \mathbf{B(Cu^{2+})} \quad \mathbf{(1)}$$

The concomitant increase in the number of binding sites brought about by the increase in biomass dosage pushed the equilibrium to the right, which in turn, decreased the amount of copper remaining in the solution; hence, an increase in copper removal was observed. Interestingly, however, as can be seen in the figure, copper removal by *S. sciuri* leveled off starting at a biomass dosage of 0.25 g while that of *B. fastidiosus* was still on the rise at this same dosage. This difference can be explained by the difference in the morphologies of the bacteria. *B. fastidiosus* cells are arranged in singles while *S. sciuri* cells are arranged in clusters. Due to its relatively large size, bacterial clusters of *S. sciuri* can aggregate easily. The accompanying decrease in the effective surface area as a result of aggregation caused a decrease in the number of exposed binding sites. Thus, two opposing factors - the increase in concentration of the biomass and the aggregation of bacterial cells - are responsible for the leveling off observed with *S. sciuri*. On the other hand, *B. fastidiosus* cells, being arranged in singles, did not aggregate to a significant extent at the dosages used. Hence, the same pattern of behavior was not observed with this bacterial biomass. The optimum biomass dosage chosen for *B. fastidiosus* was 0.50 g, as it was the amount of biomass that brought about maximum copper recovery. On the other hand 0.25 g was chosen for *S. sciuri* because no significant increase in copper removal accompanied further increase in biomass above this dosage.

Effect of Initial Concentration

It is evident from Figure 7 that the percentage removal of copper from aqueous solution decreased with initial copper concentration. This was a result of the biomasses becoming increasingly saturated with copper, which led to fewer copper ions being further removed from the solution (Bai and Abraham 2001).

The optimum adsorption properties of *B. fastidiosus* and *S. sciuri* are given in Table 5. The table shows that the adsorption of copper by both bacteria was optimum at pH 4. In addition, copper removal was fast and economical, requiring only 30 minutes to 4 hrs to effect efficient removal. Lastly, relatively minute quantities of bacterial biomasses, 0.25 and 0.50 g, were required to remove copper from 25-mL solutions with 80 to 90 % efficiency.

Adsorption Isotherms and Scatchard Plots

Adsorption isotherms describe the equilibrium between an adsorbate and an adsorbent. Adsorption isotherms can be generated by letting solutions of adsorbate of differing concentrations reach equilibrium with known quantities of adsorbent. The resulting equilibrium concentrations of adsorbate in solution, C_{eq}, are plotted against the concentration of adsorbate in the adsorbent phase, Q. The quantity Q is defined as the ratio of the amount of adsorbate in milligrams to the amount of adsorbent in grams. The measurements are performed at a constant temperature, hence the term isotherms. There are several equilibrium equations that can be used to describe experimental adsorption data (Pagnanelli et al. 2002). Two of such equations are the Freundlich and Langmuir equations.

The Freundlich model is an empirical adsorption model (Freundlich 1906) that describes the heterogeneity of adsorption sites. The Freundlich isotherm is generated by plotting the natural logarithm of the adsorption capacity of the biomass, Q, against the natural logarithm of the equilibrium concentration of the adsorbate in the solution, C_{eq}, according to equation **2**.

$$\ln Q = \ln K + (1/n) \ln C_{eq} \qquad \mathbf{(2)}$$

According to this model, K is a measure of the adsorption ability of the adsorbent, with a high value of K indicative of high adsorption ability and vice versa. The model also suggests that n values greater than one indicate repulsion between adsorbate particles while n values less than one imply attraction (McCabe et al. 1993, Hayward and Trapnell 1964).

The Langmuir isotherm assumes that adsorption only forms a single layer of adsorbed molecules on a uniform surface and that the adsorption enthalpy is identical for each individual binding event (Langmuir 1918). It also assumes that the molecules are immobilized on the adsorbent, unable to move from one part of the surface to another (Alberty 1987). The Langmuir isotherm is generated by plotting the equilibrium concentration of the adsorbate in the solution, C_{eq}, against the ratio of the same quantity to the adsorption capacity, C_{eq}/Q.

$$C_{eq}/Q = 1/bQ_{max} + C_{eq}/Q_{max} \qquad \mathbf{(3)}$$

The Langmuir constant b is the ratio of the adsorption rate constant to the desorption rate constant and, therefore, can be considered a measure of the affinity of the adsorbent for the adsorbate. In addition, the maximum adsorption capacity, Q_{max}, measures the maximum amount of adsorbate molecules that forms a single layer on the adsorbent surface (Atkins and De Paula 2010). This quantity is characteristic of an adsorbent and is a common parameter used for evaluating the adsorption potential of a biomass.

Figures 8 and 9 show that the adsorption of copper on *B. fastidiosus* is better described by the Langmuir equation than by the Freundlich equation, while the adsorption of copper on *S. sciuri* can be equally described by either the Freundlich or the Langmuir isotherm. Adherence to the Langmuir equation implies that copper ions form a single shell of adsorbed particles on the biomass surfaces. It also suggests that the surfaces do not permit the lateral movement of copper ions, that is, the adsorbed copper ions are localized at the binding sites.

A comparison of the ability of *B. fastidiosus* and *S. sciuri* to adsorb copper ions based on the Freundlich model is shown in

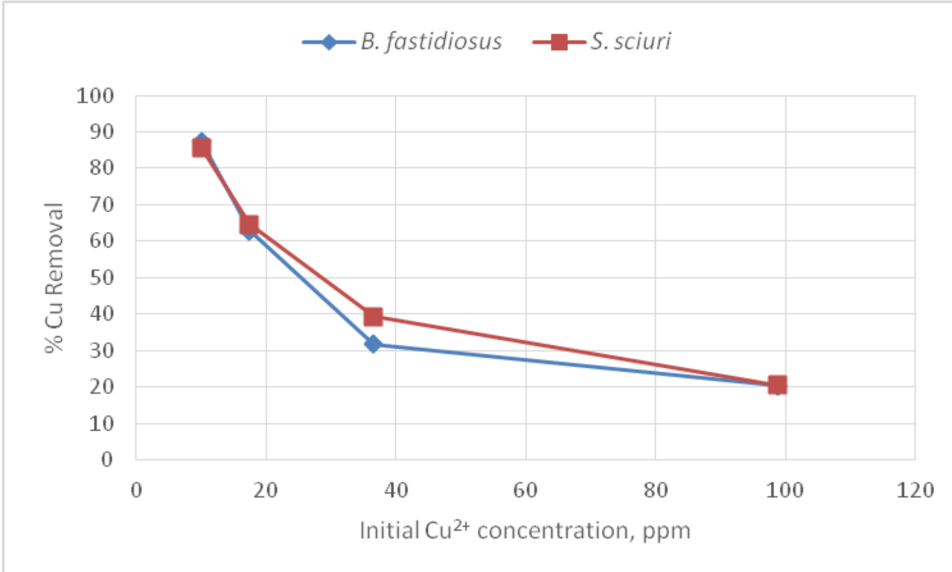

Figure 7. Effect of initial copper concentration on copper removal by *B. fastidiosus* and *S. sciuri* (pH = 4; Contact-time = 2 hrs (*S. sciuri),* 4 hrs (*B. fastidiosus)*; Biomass dosage = 0.25 g (*S. sciuri*), 0.50 g (*B. fastidiosus*))

Table 5. Copper adsorption properties of *B. fastidiosus* and *S. sciuri*

Adsorption Property	Copper-Resistant Bacteria	
	Bacillus fastidiosus	*Staphylococcus sciuri*
Optimum pH	*4*	*4*
t_{eq}	*2 hrs*	*30 mins*
Optimum Contact-Time	*4 hrs*	*2 hrs*
Optimum Biomass Dosage (Wet)	*0.50 g*	*0.25 g*

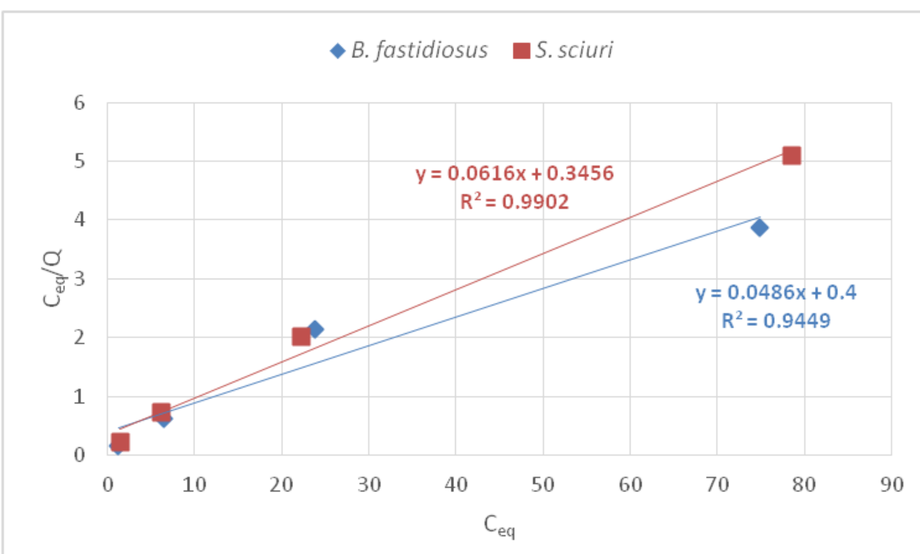

Figure 8. Freundlich isotherm for the adsorption of copper on *B. fastidiosus* and *S. sciuri (*pH = 4; Contact-time = 2 hrs (*S. sciuri),* 4 hrs (*B. fastidiosus)*; Biomass dosage = 0.25 g (*S. sciuri*), 0.50 g (*B. fastidiosus*))

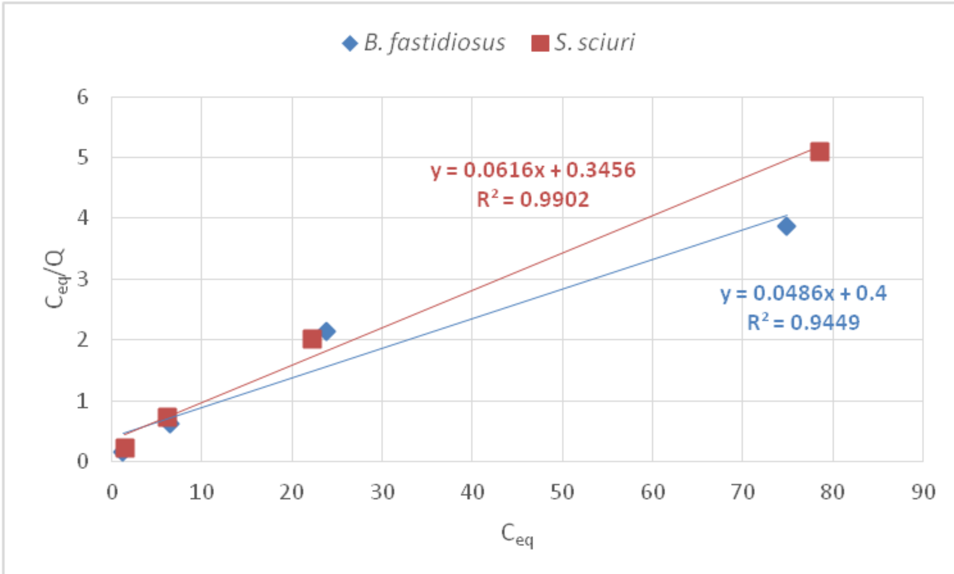

Figure 9 Langmuir isotherm for the adsorption of copper on *B. fastidiosus* and *S. sciuri* (pH = 4; Contact-time = 2 hrs (*S. sciuri),* 4 hrs (*B. fastidiosus)*; Biomass dosage = 0.25 g (*S. sciuri*), 0.50 g (*B. fastidiosus*))

Table 6. Freundlich constants for adsorption of copper on *B. fastidiosus* and *S. sciuri*

Bacteria	*Freundlich Constants*	
	K	*n*
Bacillus fastidiosus	*7.6*	*5.5*
Staphylococcus sciuri	*6.0*	*4.8*

Table 7. Langmuir constants for adsorption of copper on *B. fastidiosus* and *S. sciuri*

Bacteria	*Langmuir Constants*	
	b, ppm	*Q_{max}, mg/g dry biomass*
Bacillus fastidiosus	*0.12*	*20.8*
Staphylococcus sciuri	*0.18*	*16.4*

Table 6. The Freundlich isotherm suggests that *B. fastidiosus* was a more efficient adsorbent than *S. sciuri*, having a greater $\boldsymbol{K}$ value. The $\boldsymbol{n}$ values for *B. fastidiosus* and *S. sciuri*, which were 5.5 and 4.8, respectively, suggest repulsion between adsorbed copper ions in keeping with their positive charges.

Shown in Table 7 are the Langmuir constants for the adsorption of copper on the two biomasses. The smaller value of the Langmuir constant $\boldsymbol{b}$ for *B. fastidiosus* suggests that it binds copper less readily than *S. sciuri*. On the other hand, the $\boldsymbol{Q_{max}}$ of *B. fastidiosus* was greater than that for *S. sciuri* indicating that it can accommodate more copper on its surface.

The greater resistance of *B. fastidiosus* toward copper than *S. sciuri* can be explained by the higher $\boldsymbol{Q_{max}}$ of the former than the latter. It can be inferred from the $\boldsymbol{Q_{max}}$ values that the total number of available binding sites on *B. fastidiosus* was approximately 27% greater than that on *S. sciuri*. As mentioned previously, however, the $\boldsymbol{b}$ values for the two biomasses do not correlate correspondingly with bacterial resistance toward copper. These contradicting results suggest that the greater number of binding sites on *B. fastidiosus* more than compensate for its weaker affinity toward copper. Alternatively, the results may also imply that *B. fastidiosus*, aside from adsorbing copper on its surface, is actively employing bioaccumulation mechanisms for sequestering copper (Chen et al. 2005, De Guzman and Cao 2010).

Table 8 compares the values of $\boldsymbol{Q_{max}}$ for the adsorption of Cu^{2+} on different microbial biomasses. It is apparent that the values of $\boldsymbol{Q_{max}}$ obtained for *B. fastidiosus* and *S. sciuri* are comparable to, if not even better than, those of other bacteria (Lo et al. 2003, Chen et al. 2005), fungi (Romera et al. 2008), green alga (Romera et al. 2008, Sheng et al. 2004), red alga (Romera et al. 2008, Prasher et al. 2004), and lichen (Kılıç et al. 2014). However, it has to be noted that the $\boldsymbol{Q_{max}}$ values for *Pseudomonas sp.* and *Micrococcus sp.* are more than twice those for the bacterial isolates used in this study (Lo et al. 2003). Interestingly, the $\boldsymbol{Q_{max}}$ values of the brown alga are greater than any of the bacterial biomasses shown in the list (Ahmady-Asbchin and Mohammadi 2011, Ahmady-Asbchin et al. 2009, Davis et al. 2003, Sheng et al. 2004, Chen et al. 2008).

Scatchard plots reveal the number of types of binding sites present on an adsorbent as well as cooperative or noncooperative binding behavior (Bordbar et al. 1996). It is constructed by plotting $\boldsymbol{Q}$ against $\boldsymbol{Q/C_{eq}}$. Adsorbents that contain a single type of binding sites and on which adsorption of molecules occurs as independent binding events show linear Scatchard plots, while adsorbents with multiple types of binding sites show curved plots. The Scatchard plots of *B. fastidiosus* and *S. sciuri* shown in Figure 10 are upward curved, indicating the presence of multiple types of binding site on their surfaces. These binding sites include carboxyl and phosphoryl groups present in the complex peptidoglycan layers and teichoic acid polymers that are present in gram-positive bacterial cell walls (Beveridge 1989, Da Costa 1999). Additionally, the curvature of the plots also suggest negative cooperative binding (Bordbar et al. 1996), that is, further adsorption of copper ions on the surface is suppressed by the presence of already bound copper.

Application to Actual Wastewater

The pH of and the concentrations of heavy metals in Bol River Reservoir and Tapian pit are shown in Table 9. It is evident that the water samples from these sources were acidic and were contaminated with elevated levels of copper. In addition, the samples also contained iron and zinc in significant concentrations.

As can be seen in Figures 11 and 12, the use of *B. fastidiosus* and *S. sciuri* biomasses for removing copper from Bol river and Tapian pit wastewater yielded substantial copper removal. However, the experimental copper removal efficiencies were lower than the values expected from Figure 7. These negative deviations from expected efficiencies can be attributed to the presence of competing ions, Fe^{3+} and Zn^{2+}. Fe^{3+} and Zn^{2+}, being trivalent and divalent, respectively, act as potent competitors of Cu^{2+} for the adsorption sites on the biomass surface. This was particularly true for Fe^{3+} as it was removed from the solutions with relatively high efficiency. However, Zn^{2+} was removed to a lesser extent due to its relatively diminutive concentration compared to the other two ions. Lastly, though the natural organic matter content and the amounts of other organic and inorganic substances present in the wastewater were not analyzed in this study, their presence may also account for the observed less than optimal Cu removal efficiencies.

Though the ability of *B. fastidiosus* to assimilate and degrade small organic molecules like uric acid and allantoin (Bongaerts and Vogels 1976) and glycerol (van der Drift et al. 1986) has already been reported in the literature, there is currently no report on its utilization in the degradation of organic pollutants. Also, there is presently no report on its application in the removal of heavy metals. To the best of our knowledge, then, this article is the first study on the potential of *B. fastidiosus* for bioremediation. On the other hand, there are already a handful of studies on the use of *S. sciuri* in bioremediation. For instance, a strain of *S. sciuri* isolated from crude oil contaminated soil has very recently been utilized in the degradation of 1,3,5-trimethylbenzene (1,3,5-TMB), a very toxic, carcinogenic, and mutagenic organic pollutant (Lv and Yu 2014). In addition, phenol- and catechol-degrading strains of *S. sciuri* have also been isolated and identified from a phenol-acclimated activated sludge (Mrozik and Labuzek 2002). Furthermore, resident cadmium-resistant *S. sciuri* strains have been isolated from the gray treefrog, *Hyla chrysoscelis*, in a study on the use of amphibians as bioindicators of heavy metal contamination (AbuBkar and Crupper 2010).

B. fastidiosus and *S. sciuri* are bacteria that appear to be common microbial flora in environmental reservoirs such as soil and water (Kaltwasser 1971, Couto et al. 2000). Whereas *B. fastidiosus* has not yet been implicated in any disease, *S. sciuri*,has been associated with a number of serious infections in humans (Hedin and Widerstrom 1998, Stepanovic et al. 2002, Stepanovic et al. 2003). A more recent article, for instance, cited the prevalence of *S. sciuri* in a bloodstream infection in patients in a hospital in Benin, a country in West Africa (Ahoyo et al. 2013). The

Table 8. Comparison of various literature studies on copper removal by microbial species

	Species	Q_{max}, mg/g dry biomass	pH	Reference
Bacteria	*Bacillus fastidiosus*	20.8	4	This work
	Staphylococcus sciuri	16.4	4	This work
	Bacillus sp.	21	5	Lo et al. 2003
	Pseudomonas sp.	47	5	Lo et al. 2003
	Micrococcus sp.	43	5	Lo et al. 2003
	Pseudomonas putida CZ1	29.9	5	Chen et al. 2005
Fungi	*Aspergillus armata*	21.0	6	Romera et al. 2008
Lichen	*Lobaria pulmonaria*	12.1	5	Kilic et al. 2014
Red Alga	*Chondrus crispus*	40.7	6	Romera et al. 2008
	Palmaria palmate	6.4	6.5-7	Prasher et al. 2004
Green Alga	*Codium vermilara*	17.2	6	Romera et al. 2008
	Spirogyra insignis	19.1	6	Romera et al. 2008
	Ulva sp.	47.7	5	Sheng et al. 2004
Brown Alga	*Fucus vesiculosus*	117.6	5.5	Ahmady-Asbchin and Mohammadi 2011
	Fucus serratus	109.9	5.5	Ahmady-Asbchin et al. 2009
	Sargassum vulgare	59.1	4.5	Davis et al. 2003
	Sargassum fluitans	50.8	4.5	Davis et al. 2003
	Sargassum sp.	62.9	5	Sheng et al. 2004
	Undaria pinnatifida	78.9	4	Chen et al. 2008

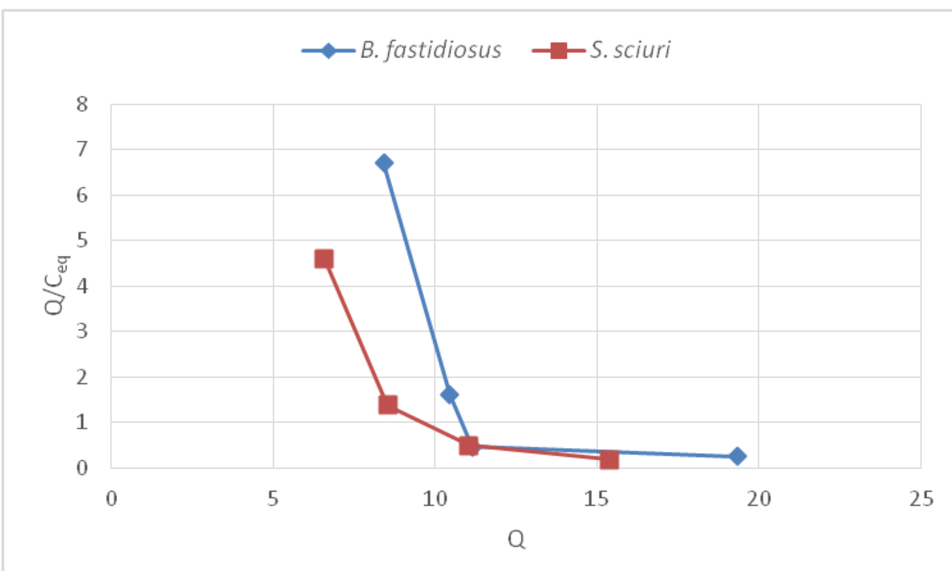

Figure 10. Scatchard plot for the binding of copper on *B. fastidiosus* and *S. sciuri* (pH = 4; Contact-time = 2 hrs (*S. sciuri*), 4 hrs (*B. fastidiosus*); Biomass dosage = 0.25 g (*S. sciuri*), 0.50 g (*B. fastidiosus*))

Table 9. pH of and heavy metal ion concentrations in Bol and Tapian wastewater

	Bol	Tapian
pH	*3.6*	*3.6*
[Cu^{2+}], ppm	*9.1*	*23.6*
[Fe^{3+}], ppm	*1.9*	*12.5*
[Zn^{2+}], ppm	*1.0*	*2.2*

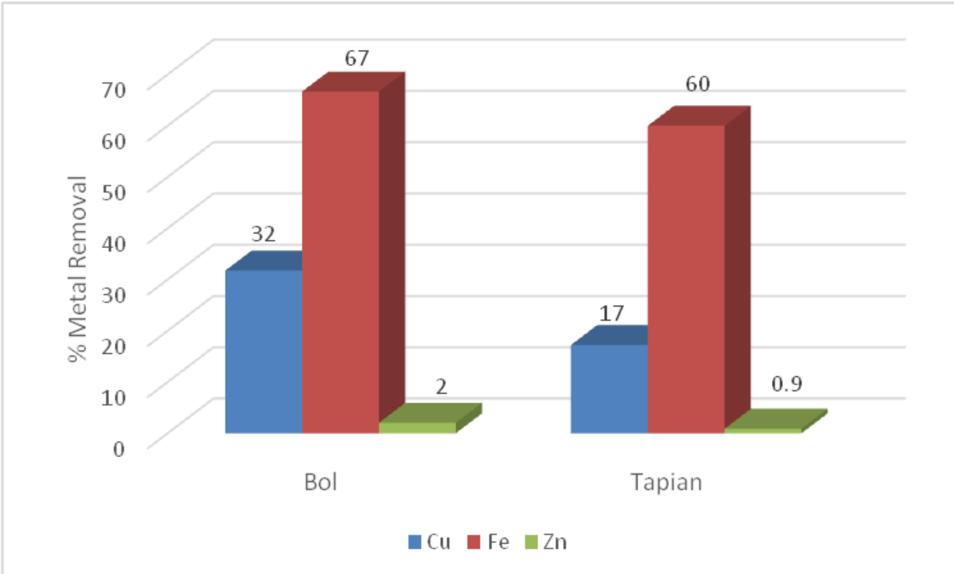

Figure 11. Percent metal removal from Bol and Tapian wastewater by *B. fastidiosus (*Contact-time = 4 hrs, Biomass dosage = 0.50 g)

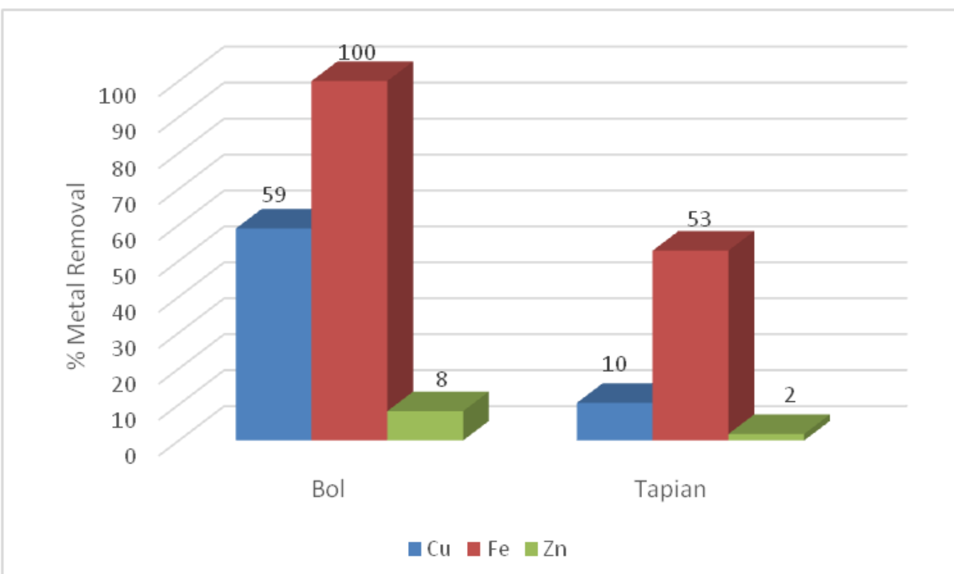

Figure 12. Percent metal removal from Bol and Tapian wastewater by *S. sciuri (*Contact-time = 2 hrs, Biomass dosage = 0.25 g)

pathogenic nature of *S. sciuri*, therefore, precludes its further use in bioremediation.

In summary, four bacterial isolates exhibited resistance to copper. The least and most resistant bacteria, namely *Staphylococcus sciuri* and *Bacillus fastidiosus* grew in the presence of 50 and 200 ppm copper, respectively. No bacterial growth was observed at copper concentrations higher than 200 ppm. The efficiency of copper removal by both bacteria was found to be dependent on pH and biomass dosage, to level off with time, and to decrease with initial copper concentration. The optimum pH, contact-time, and biomass dosage for *B. fastidiosus* were 4, 4 hrs, and 0.50 g, respectively. The same optimum properties for *S. sciuri* were 4, 2 hrs, and 0.25 g. The biosorption of copper on both biomasses correlated well with the Langmuir model. This model suggested that, though the more copper-resistant isolate, *B. fastidiosus*, has more available sites for adsorption of copper on its surface, its affinity for copper is weaker than that of *S. sciuri*. These results imply that either the greater number of binding sites on *B. fastidiosus* more than compensate for its weaker affinity toward copper or that *B. fastidiosus* is actively employing bioaccumulation mechanisms for sequestering copper. The Q_{max} obtained for *B. fastidiosus* and *S. sciuri* compare well with those of other bacteria, fungi, alga, and lichen reported in the literature. The Scatchard plots indicated that both bacterial surfaces consisted of more than a single type of binding site and that the adsorption of copper exhibits negative cooperative behavior. Also, the biomasses efficiently removed copper from actual wastewater, with negative deviations from expected removal efficiencies attributable to the presence of iron and zinc.

This study demonstrated that the two isolated indigenous copper-resistant bacteria, *B. fastidiosus* and *S. sciuri*, were effective in removing copper. Unfortunately, the pathogenic nature of *S. sciuri* complicates its actual and large scale application *in situ*. The same issue, however, is not encountered with *B. fastidiosus*. Thus, *B. fastidiosus* can be used as an efficient and non-pathologic bioremediating agent for copper.

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CONFLICTS OF INTEREST

There is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Christopher Jay T. Robidillo was the student who performed the experiments. Dr. Nelson R. Villarante and Dr. Lorele C. Trinidad were his thesis advisers.

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