Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Copper removal using a heavy-metal resistant microbial consortium in a fixed-bed reactor

Isis E. Mejias Carpio [a], Glaucia Machado-Santelli [b], Solange Kazumi Sakata [c], Sidney Seckler Ferreira Filho [d], Debora Frigi Rodrigues [a,*]

[a] Department of Civil and Environmental Engineering, University of Houston, Houston, TX 77004, USA
[b] Instituto de Ciências Biomédicas, Universidade de São Paulo, CEP 05508-000 São Paulo, SP, Brazil
[c] Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN-SP), CEP 05508-000 São Paulo, SP, Brazil
[d] Departamento de Engenharia Hidráulica e Ambiental, Escola Politécnica, Universidade de São Paulo, CEP 05508-000 São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form
22 May 2014
Accepted 25 May 2014
Available online 4 June 2014

Keywords:
Biosorption
Copper
Metal
Biofilm
Bacterial
Microbial consortia

ABSTRACT

A heavy-metal resistant bacterial consortium was obtained from a contaminated river in São Paulo, Brazil and utilized for the design of a fixed-bed column for the removal of copper. Prior to the design of the fixed-bed bioreactor, the copper removal capacity by the live consortium and the effects of copper in the consortium biofilm formation were investigated. The Langmuir model indicated that the sorption capacity of the consortium for copper was 450.0 mg/g dry cells. The biosorption of copper into the microbial biomass was attributed to carboxyl and hydroxyl groups present in the microbial biomass. The effect of copper in planktonic cells to form biofilm under copper rich conditions was investigated with confocal microscopy. The results revealed that biofilm formed after 72 h exposure to copper presented a reduced thickness by 57% when compared to the control; however 84% of the total cells were still alive. The fixed-bed bioreactor was set up by growing the consortium biofilm on granular activated carbon (GAC) and analyzed for copper removal. The biofilm-GAC (BGAC) column retained 45% of the copper mass present in the influent, as opposed to 17% in the control column that contained GAC only. These findings suggest that native microbial communities in sites contaminated with heavy metals can be immobilized in fixed-bed bioreactors and used to treat metal contaminated water.

1. Introduction

Heavy metal contaminated water is a growing concern because heavy metals accumulate in biological systems and can lead to severe poisoning (Kunhikrishnan et al., 2012). Several industries, such as mining, electroplating, aerospace, energy and fuel production, and catalysis produce effluents containing various heavy metals. Most of the traditional techniques used for removal of heavy metals from aqueous

* *Corresponding author*. Tel.: +1 713 743 1495; fax: +1 713 743 4260.
E-mail address: dfrigirodrigues@uh.edu (D.F. Rodrigues).
http://dx.doi.org/10.1016/j.watres.2014.05.043
0043-1354/

systems require expensive equipment, costly reagents and energy, and can generate large amounts of sludge that require disposal (Chen et al., 2009; Baig et al., 2009; Bessbousse et al., 2008). Some of these technologies also need a long contact time and are more efficient when the metals are present in concentrations higher than 100 ppm (Kratochvil and Volesky, 1998).

Biosorbents, such as yeasts, algae, and bacteria are potential alternatives for the removal of heavy metals in aqueous systems (Monteiro et al., 2011; Romera et al., 2008,Lezcano et al., 2010; Moon and Peacock, 2011). Also, biosorbents are at least one order of magnitude (1/10) cheaper than traditional technologies, and minimize the volume of chemical and/or biological sludge to be disposed (Davis et al., 2003; Volesky, 2007). These properties make biosorbents a very promising solution to treat metal contaminated water.

Bacteria can be economic and eco-friendly biosorbents for water and wastewater treatment processes due to their small size and short generation time, which facilitate rapid adaptation to a wide range of environmental conditions (Wang and Chen, 2009). Such properties are important for engineered systems, where biosorption could be implemented in conjunction with coagulation, clarification, and disinfection to improve the overall efficiency of the treatment process (Rodrigues, 2011). In biological wastewater treatment, microbial consortia are commonly employed, since pure cultures are difficult to maintain in large-scale units and because microbial communities can easily adapt to environmental changes.

In this study, we investigate the use of a metal-resistant microbial consortium immobilized in a fixed-bed column. Prior to the immobilization of the consortium in a fixed-bed column, we analyzed the survival and the mechanisms of copper removal by this microbial community in batch cultures. We also investigate whether the planktonic microbial community can form biofilm and persist in the presence of a metal-rich environment. Then, we investigated the consortium biofilm immobilization onto granular activated carbon (GAC) and its performance for copper removal in a fixed-bed column for future application in water treatment units.

2. Experimental methods

2.1. Sample collection and isolation of the 'Pirapora' microbial consortium

The consortium used in the present study was collected from sediment of a dam located in the Metropolitan Region of São Paulo, Brazil at 23°23′31.81″S and 46°59′47.67″W. The copper concentration in the dam sediment was determined to be 90.8 ppm by using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES Optima 7000DV, Perkin Elmer) after acid digestion as described in the USEPA Method 3050B (USEPA, 1996). The pH of the dam sediment and water were 6.8 and 7.2, respectively. The water total phosphorous, inorganic phosphorous, total kjeldahl nitrogen (TKN), and total organic carbon (TOC) were determined as described elsewhere (Bowman and Delfino, 1982) (Table S1).

The consortium was obtained from the dam sediment by incubating 1 g of the sediment at 30 °C under constant stirring (125 rpm) for 24 h with 40 mL of a growth medium prepared with the river water and the sediment extract ('P medium'). After pre-incubating the inoculum, an aliquot of the solution was transferred to fresh P medium and incubated again at 30 °C under constant agitation (125 rpm) for 48 h. Four transfers to fresh media were performed during subsequent days. Microbial consortium stocks with 25% sterile glycerol were stored at −80 °C.

2.2. Growth conditions and medium preparation for enrichment of the 'Pirapora' microbial community: "P medium"

The growth medium (P medium) was prepared using the sediment and the water collected from the dam. First, 100 g of sediment were mixed with 1.4 L of the dam water, autoclaved for 15 min at 121 °C and 15 psi, and centrifuged at 4000 rpm for 15 min. A volume of 500 mL of the supernatant was mixed with 500 mL of deionized water, 1 g of glucose, 0.5 g of yeast extract and 0.5 g of K_2HPO_4 (Rodrigues et al., 2012). The solution was autoclaved for 15 min at 121 °C and 15 psi.

The metal concentration in the final P medium was analyzed with ICP-OES after digestion using the USEPA Method 3010A (USEPA, 1992). No Cu^{2+} ions were detected in the final P medium, however, the final medium presented 4.6 ppm of Pb^{2+} and 2.8 ppm of Zn^{2+} and other minor metal ions (Table S1). The final pH of the medium was adjusted to 5 to prevent metal precipitation. The total phosphorous, TKN, and TOC concentrations of the medium were 6.31 ± 0.06 mg/L, 1.91 ± 0.80 mg/L, and 428.2 ± 40.3 mg/L, respectively.

For all experiments, 5 mL of cells grown in the broth P medium were grown overnight at 30 °C from a frozen stock at −80 °C. The grown bacterial culture was centrifuged at 4000 rpm for 30 min, and the pellet was washed three times with phosphate buffered saline solution (PBS) and resuspended in P medium to an optical density (OD) of 0.5 at 600 nm, which corresponds to a concentration of 10^8 colony forming units per milliliters (CFU/mL) (Mejias Carpio et al., 2012). The concentration was determined based on plate counts using P medium agar, which consisted of 1 L of P medium with 15 g of agar at pH value of 5.

2.3. Determination of Minimum Inhibitory Concentration (MIC) for the Pirapora consortium

The MIC of copper for the consortium was determined by exposing the consortium to different copper concentrations. A 1000 ppm solution of Cu^{2+} was prepared by dissolving the hydrolyzed salt $CuSO_4 \cdot 5H_2O$ in sterile de-ionized water at pH = 5, filtered with 0.2 μm syringe filter, and mixed with sterile P medium at pH = 5. Further dilution in the P medium was done to obtain metal-rich media with different copper concentrations (0 ppm, 6 ppm, 17 ppm, 23 ppm, 45 ppm, 68 ppm, and 90 ppm).

After growing overnight the consortium in P medium without metals, as described in the Section 2.2, aliquots of 500 μL of this pre-inoculum at $OD_{600nm} = 0.5$ were transferred under aseptic conditions into tubes containing 4.5 ml of P medium with different metal concentrations. The MIC was determined through absorbance readings at OD_{600nm} taken at

different time periods (0 h, 7 h, 24 h, 48 h, 72 h, and 6 d). The lag phase duration of the consortium exposed to each copper concentration was determined as the initial phase prior to the log phase (Table S2). Each lag phase duration was analyzed with a post hoc pairwise LSD test using STATISTICA 8.0.

The MIC, the bacteriostatic, and bactericidal effect of copper were determined after the consortium was grown for 45 h in different concentrations of copper (23 ppm, 45 ppm, 68 ppm, and 90 ppm) (Fig. S1). Plate counts of the cultures exposed to copper were determined on metal-free P medium agar at 30 °C. A control consortium grown in liquid P medium without metals was also plated. The CFU/mL of each condition was determined in triplicates. Averages and standard deviations were calculated from the triplicates.

2.4. Mechanisms of copper removal by the 'Pirapora' microbial consortium

The Fourier transform infrared spectroscopy (FT-IR) was used to determine the heavy metal binding sites in the biomass. Aliquots of 10 mL of the consortium in P medium at $OD_{600nm} = 0.5$ were transferred into flasks containing 90 mL of P medium at pH = 5 with a concentration of 23 ppm of copper. The solutions were incubated at 30 °C under constant agitation (125 rpm) for 72 h. The samples were then centrifuged at 4000 rpm for 30 min and the pellets were collected. The pellets were freeze-dried with a lyophilizer Labconco (Triad, Catalog No. 7400040 Missouri) overnight. The ATR-FTIR spectra were collected on a Bomem MB100 equipped with a DTGS detector from 6500 cm^{-1} to 350 cm^{-1} wavenumbers. All spectra were taken with a nominal spectral resolution of 4 cm^{-1} in absorbance mode. The measurements of the lyophilized cells were obtained under standard temperature and pressure (STP) and dry conditions.

2.5. Effects of exposure time, pH, and copper concentration on copper removal by the consortium

The effects of exposure time, pH, and copper concentration on the ability of the consortium to remove copper was determined in batch cultures. Briefly, after inoculating the consortium in P medium as described in the Section 2.2, the harvested cells were rinsed 3 times with 0.1 M NaCl solutions and suspended in 0.1 M NaCl solutions with 15 ppm copper at $OD_{600nm} = 0.5$ and pH = 4 (Takenaka et al., 2007). To determine the consortium's copper sorption equilibrium, the copper concentration in the supernatant was measured every 15 min for 120 min by collecting the supernatant through centrifugation at 4000 rpm for 30 min. The influence of pH on the removal of Cu^{2+} by the consortium was examined by varying the pH from 2 to 7 of a 15 ppm copper solution with 0.1 M HCl or 0.1 M NaOH. The pH of the aqueous solutions was measured using a pH meter (Orion 720A, Hexis). ANOVA statistical analysis with STATISTICA 8.0 was done with the removal percentages at different pH values. The effect of initial Cu^{2+} concentrations was investigated by varying the amount of metal in the solution (0, 5, 15, 30, 50, 75, 100 ppm) under the optimum conditions (pH = 4 and 100 min sorption equilibrium). All samples were centrifuged at 4000 rpm for 30 min, and the supernatant was digested to mineralize any organic constituents according to the USEPA method 3010A prior to ICP-OES analysis (USEPA, 1992).

2.6. Effects of copper on the consortium biofilm formation

Confocal microscopy was used to assess biofilm formation, thickness and mortality in the presence of copper. Two assays were performed, one assay was performed with biofilm grown with 20 ppm of copper and the other consisted in growing the biofilm in metal-free medium and exposing afterwards to copper for different time periods to determine biofilm detachment. To investigate biofilm formation in the presence of copper, the planktonic cells grown in P medium were washed once with PBS, and resuspended at $OD_{600nm} = 0.5$ in fresh P medium supplemented with 20 ppm Cu^{2+}. The biofilm was allowed to grow for 72 h at 30 °C in the presence of metals.

The effect of copper exposure on the detachment of fully mature biofilm grown in P medium without metals was also determined for different exposure times. Briefly, in this assay 10 mL of PBS-washed planktonic cells, grown overnight in P medium without copper, were transferred to petri dishes with metal-free media containing coverslips as a biofilm substrate. The consortium was then incubated under static condition for 72 h at 30 °C to allow the biofilm to grow. After incubation, the supernatant was removed and the biofilm was washed three times with PBS. Following the washes, the biofilms were then exposed to 0.1 M NaCl solution with 30 ppm of copper for 2 h, 3 h, and 4 h to observe microbial detachment in the presence of copper. The laser scanning confocal microscope used for these analyses was LSM 510 (Zeiss; Jena, Germany) with argon (458 nm, 488 nm, and 514 nm) and helium-neon (543 nm and 633 nm) lasers. The confocal microscopy analysis and image acquisition of the biofilms were carried out using the Zeiss LSM 5 Image software. This procedure, along with the procedure for Live/Dead staining, is described in the supporting information.

2.7. Preparation of fixed bed reactor with the consortium biofilm on granular activated carbon (BGAC)

The biofilm was grown on GAC as a support medium to obtain a biofilm-covered GAC (BGAC) fixed bed reactor. The GAC, purchased from Calgon Carbon Co. (Pittsburgh, PA, characterization found in SI), was sieved using 0.6 mm (ASTM 10) and 2 mm-diameter (ASTM 30) sieves (Bertel Industria Metalurgica Ltda. São Paulo). The carbon was washed with deionized water, sterilized in an oven at 120 °C for 24 h, and placed in a 150 mL Erlenmeyer flask connected to a vacuum pump containing sterile water to release the air inside the pores. Two glass columns (21 cm in height and internal diameter of 2.5 cm) were filled with 51 g of GAC, to obtain an empty bed hydraulic residence time (HRT) of 20 min. One column was set up as control (GAC only) and the other was used for biofilm growth (BGAC). The BGAC column had 9 L of P medium with the consortia at an $OD_{600nm} = 0.5$ pumped through the column at a flow rate of 5 mL/min for 4 d to allow the biofilm growth. The control had deionized water pumped through the column at the same flow rate for 4 d. Finally, $CuSO_4 \cdot 5H_2O$ with a

concentration of 15 ppm Cu^{2+} (C_0) was prepared with deionized water.

2.8. Breakthrough of BGAC fixed bed bioreactor

The metal solution of 15 ppm Cu^{2+} (C_0) in deionized water was passed through the BGAC column at a flow rate of 5 mL/min for 24 d. Effluent concentrations of Cu^{2+} ions (C) from the column were analyzed every day using ICP-OES. The experimental ratio of the effluent copper concentration to the influent copper concentration (C/C_0) was plotted as a function of the number of bed volumes eluted. The number of bed volumes eluted is defined as:

$$\text{No. of bed volumes eluted} = \frac{\text{Volume of solution treated}}{\text{Volume of carbon bed}} \quad (1)$$

The total mass of Cu^{2+} ions passed through the column is given by:

$$m_{in} = \frac{QC_0t_{total}}{1000} \quad (2)$$

The mass of Cu^{2+} ions sorbed to the column, m_{sorbed} (mg), for a fixed initial copper concentration and flow rate, is obtained by integrating the area above the plot (Fig. 7) as a function of time. Therefore, the mass balance is given by:

$$m_{sorbed} = m_{in} - \frac{Q}{1000}\int_{t=0}^{t=t_{total}} Cdt \quad (3)$$

where Q, C_0, C, and t_{total} are the volumetric flow rate (mL/min), the initial copper concentration (ppm), the copper effluent concentration measured (ppm), and the total flow time (min), respectively. So, the removal percent of copper is the ratio of the mass sorbed (m_{sorbed}) to the mass input into the column, and is given by:

$$\text{Removal\%} = \frac{m_{sorbed}}{m_{total}} \quad (4)$$

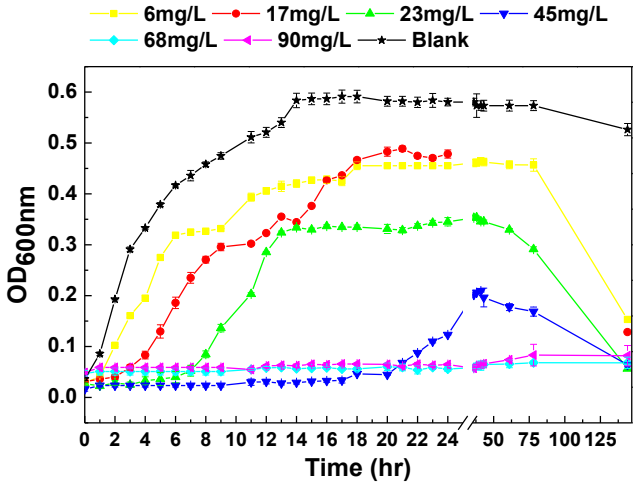

Fig. 1 – 'Pirapora' microbial community growth curves after exposure to $CuSO_4 \cdot 5H_2O$ at different concentrations: 6 ppm, 17 ppm, 23 ppm, 45 ppm, 68 ppm, 90 ppm in P medium.

3. Results and discussion

3.1. Environmental consortium resilience in copper-rich conditions

The use of microorganisms to treat heavy metal-contaminated water requires the assessment of the effect of different metal concentrations on cell growth. The ability of the '*Pirapora*' microbial community to grow and survive in increasing concentrations of copper was investigated in planktonic cells. The results show that the '*Pirapora*' microbial community was able to grow in copper concentrations up to 68 ppm, which was determined to be the MIC for this consortium (Fig. 1). Previous studies have found copper MIC values of 125 ppm (Ozdemir et al., 2003), 190 ppm (Monchy et al., 2007), and 127 ppm (Teitzel and Parsek, 2003) for pure cultures of *Ochrobactrum anthropi*, *Cupriavidus metallidurans*, and *Pseudomonas aeruginosa*, respectively. Although these copper MIC values exceeded the MIC of the consortium used in this work, these pure cultures were grown in rich media containing salts and other components that are known to complex with metal cations, which would result in higher MICs (Teitzel and Parsek, 2003). Conversely, the '*Pirapora*' consortium was grown in a minimum medium prepared with water and sediment from the contaminated dam to simulate removal of heavy metals under similar water chemistries to the contaminated site.

The ability of the consortium to grow after exposure to 68 ppm copper or higher concentrations was investigated by plating on metal-free agar medium. The results show that cells exposed to 68 ppm presented about one log growth removal compared to the control (Fig. S1). On the other hand, no growth on metal-free agar was observed after exposure to 90 ppm copper in "P" broth. This lack of growth in broth containing heavy metal, but high number of live cells after removing the heavy metal and plating in metal-free agar media could be attributed to a bacteriostatic or inactivation effect of copper to some more sensitive species in the consortium (Teitzel and Parsek, 2003).

The concentration of metal also impacted the consortium's lag phase. Microbial growth undergoes several phases: lag, exponential, stationary, and death phase (Fig. S2). The lag phase involves slow growth and a period of acclimation in which the bacteria is adjusting to a new condition in order to successfully grow (Rolfe et al., 2012). The duration of the lag phase of the '*Pirapora*' community increased with higher metal concentrations (Pearson's correlation $r = 0.97$; $p < 0.001$), with an increase in its lag phase for copper concentrations equal to or above 6 ppm (post hoc pairwise LSD tests, $p < 0.001$ in all cases, Table S2). This result suggests that the community can grow with heavy metal; however, it may require a longer adaptation period as the metal concentration increases.

After investigating the resistance of the planktonic microbial community to different concentrations of copper, we investigated the ability of these planktonic cells to form biofilm under copper-rich conditions (biofilm attachment) and also the resilience of fully mature biofilms grown without heavy metal followed by exposure to copper-rich solutions (biofilm detachment), as shown in Table 1. Both of these

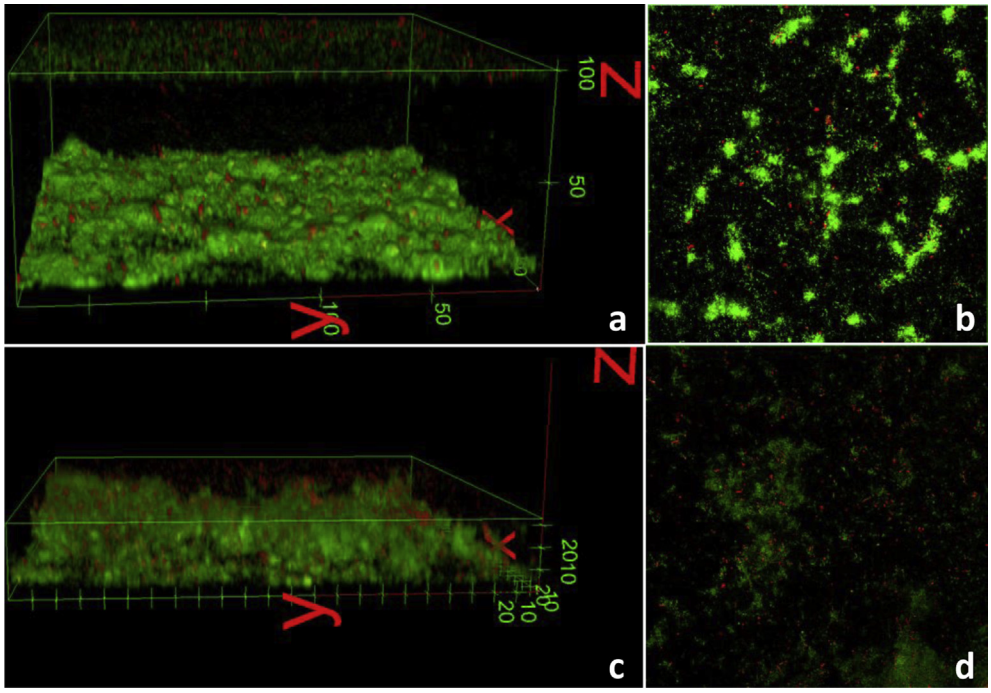

Fig. 2 – Biofilm Detachment Analysis by Confocal Microscopy. a) 3D composition of biofilm grown for 72 h and b) Top layer of biofilm grown for 72 h c) 3D composition of biofilm grown for 72 h after exposure to 30 ppm Cu^{2+} ions for 4 h and d) Top layer of biofilm grown for 72 h after exposure to 30 ppm Cu^{2+} ions for 4 h.

investigations were done by quantifying the biofilm viability and thickness.

The results of the biofilm detachment assays are shown in Fig. 2a (control) and Fig. 2c (biofilm grown in metal-free solution and then exposed to 30 ppm Cu^{2+} ions for 4 h). These results show an increased percentage of dead cells (stained red) on the surface (t-test, $p = 0.001$) (Fig. 2b and d), and in the inner layers (t-test, $p = 0.003$) when compared to the control biofilms. The biofilm inner and outer layers had a 10.1% and 2.6% increase in the percentage of dead cells in the metal-rich environment, respectively (Table 1). However, the thickness of the biofilms exposed to copper was only significantly affected after 4 h of metal exposure. Our results indicate that the metal content in the medium induced up to 16.1 ± 4.4% and 16.2 ± 1.1% death of inner and outer cell layers, respectively.

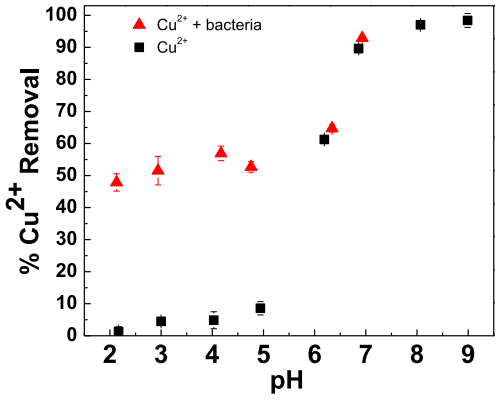

Fig. 3 – pH dependency of a) Cu^{2+} sorption by 'Pirapora' microbial consortium in 0.1 M NaCl solution at different pH values (2–7). Error bars represent standard deviation values. Since copper precipitates as $Cu(OH)_2(s)$ at or above pH = 6, a pH = 4 was chosen for further sorption studies.

In the case of the biofilm formed in the presence of copper, we observed that the thickness of the biofilms (attachment assay) was not statistically different from the control (t-test, $p = 0.42$), but had an increased percent of cell mortality in the inner layers (77.7 ± 15.6%), whereas the fully formed biofilms in heavy metal-free medium followed by a 4 h exposure to metal (detachment assay) were significantly thinner than the control ($p = 0.025$), but the percent mortality of the inner layers was only 16.1 ± 4.4%. These results suggest that biofilms formed without the presence of heavy metals are more robust and resistant to copper, i.e. less cells are inactivated, than biofilms formed in the presence of metals. The resistance of the fully mature biofilm exposed to the heavy metal, compared to the biofilm being formed in the presence of heavy metal, may potentially be attributed to the EPS already formed in the fully mature biofilm that works as a diffusion barrier that slows down the infiltration of metal ions (Stewart and Costerton, 2001; Hentzer et al., 2003).

The EPS contains diverse high molecular weight organic macromolecules, e.g. polysaccharides, proteins, nucleic acids, phospholipids, as well as other low molecular weight non-polymeric components. These molecules can be located at the surface (insoluble EPS) of the microbial cell or secreted to the environment (soluble EPS). The insoluble EPS usually forms a gelatinous matrix that functions to support aggregation between cells, initial attachment of cells to surface, protection from desiccation, and resistance to toxic exogenous

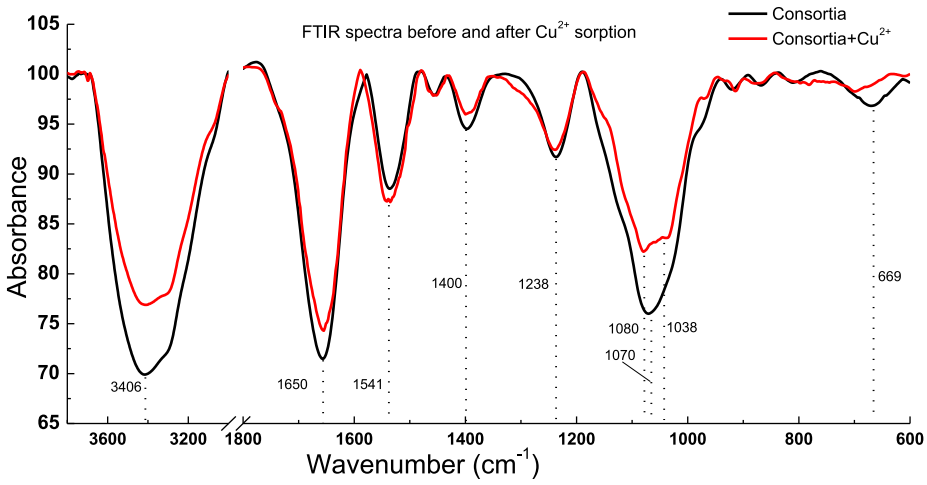

Fig. 4 – FT-IR spectra of 'Pirapora' bacterial community exposed or not to 23 ppm of copper. Black line corresponds to the control (no heavy metal). The red line corresponds to the community exposed to 23 ppm Cu^{2+} for 4 d. Bands in the range of 1070 cm^{-1} are attributed to the C–OH of the carboxyl stretching vibration. The consortium with adsorbed copper ions presented the same vibration with two peaks (1038 and 1080 cm^{-1}), which suggests that only a portion of the carboxyl groups have bonded to the copper ions. The peaks at 4306 and 669 cm^{-1} correspond to OH vibrations, and were also responsible for the copper binding.

constituents, such as metals (Pal and Paul, 2008; Laspidou and Rittmann, 2002). Previous research has shown that high content (up to 38%) of uronic acids in the EPS are responsible for metal binding due to the presence of carbonyl, carboxylic acid, and hydroxyl functional groups (Salehizadeh and Shojaosadati, 2003; Morillo Pérez et al., 2008). Other studies also found that proteins, with negatively charged amino acids such as aspartic and glutamic acids, also constitute a significant portion of the EPS and can effectively bind to positively charged metal ions (Laspidou and Rittmann, 2002).

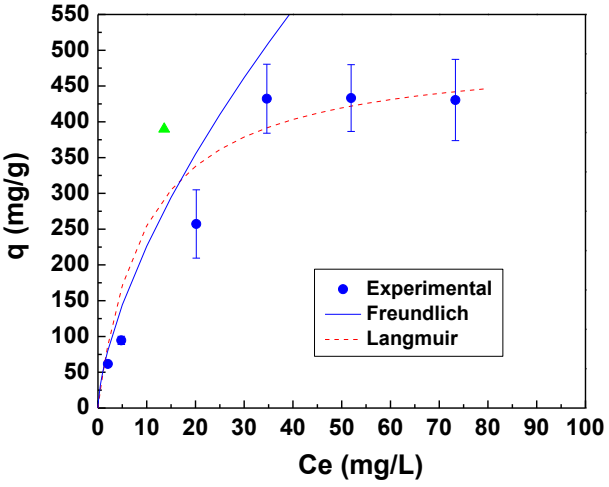

Fig. 5 – Sorption isotherm of Cu^{2+} removal at pH = 4 by 'Pirapora' microbial community grown in P medium. The 'Pirapora' microbial community has a sorption capacity for Cu^{2+} ions estimated at 450.0 mg/g. The ▲ symbol represents the fixed-bed column equilibrium point.

These findings and the better understanding of the behavior and sensitivity of the Pirapora microbial consortium in the presence of copper are essential to determine the optimum conditions needed to form a robust biofilm on the GAC surface for a better set up of the fixed-bed bioreactors.

3.2. *Ability of the Pirapora microbial consortium to remove copper under different pH values*

In the previous section, we showed that the Pirapora microbial community is very resistant to heavy metals and can successfully form biofilms that are resistant to high concentrations of copper. The second step was to determine whether this community is able to remove copper from water, so that it can be used in fixed-bed bioreactors for copper water treatment. Therefore, we used batch dynamic experiments for the present investigation. Batch experiments are critical for biosorption investigation because they provide insights into the mechanisms of sorption reactions and the reaction pathways.

The availability of metals, as well as the adsorption capacity of an adsorbent, is directly dependent on the pH of the solution. The pH plays an essential role in the sorption of metal ions since it affects the sorbent surface charge, the degree of protonation of the surface functional groups, and the precipitation of metal ions in the solution (Peters, 1999; Blais et al., 2008). In microbial biomasses, metal sorption depends on the extent of protonation of the carboxyl groups in the cell walls, given that an increase in pH reduces the competition between H^+ and metal ions (Baig et al., 2009) (Fig. 3). It is critical to highlight that 15 ppm copper starts to precipitate at pH values above 6; hence sorption at lower pH values indicate microbial sorption effects (Lewis, 2010). The ANOVA showed that overall the removal percentages are independent of pH from pH = 1 to pH = 5 ($F(3,7) = 2.9654$, $p = 0.107$). In the present study, the pH = 4 had an average

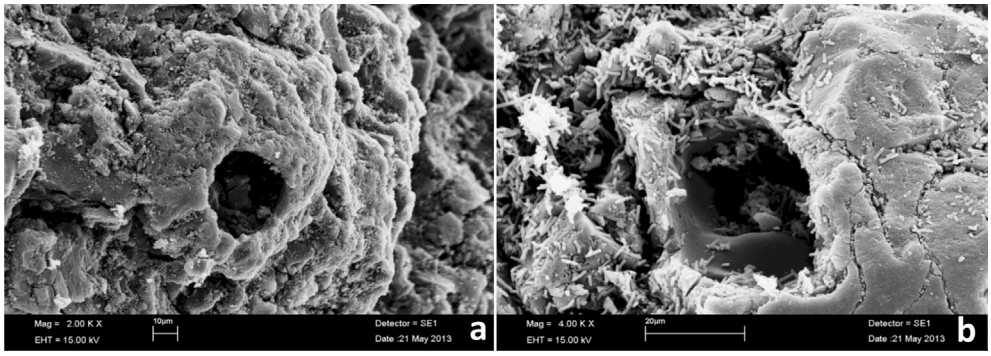

Fig. 6 – SEM imaging of a) BGAC before biofilm growth at 2 K magnification, b) BGAC after biofilm growth at 4 K magnification.

percent removal of 57% (Fig. 3). This pH was chosen for subsequent studies of copper removal, even though the percent removal at this pH was not statistically different than those observed at other pH values lower than 5.

3.3. *Mechanisms of copper removal by the Pirapora consortium*

Microbial sorbents first interact with ions dissolved in aqueous phase through surface complexation (Warren and Haack, 2001). Functional groups present on bacterial cell surfaces can become negatively charged to buffer the surrounding environment by donating protons (Warren and Haack, 2001). For instance, teichoic acids give the negative charge to the Gram-positive bacteria; while in the Gram-negative the lipopolysaccharide and phospholipids provide the primary sites for metal interaction (Wang and Chen, 2009). Thus, deprotonated functional groups on bacterial surfaces can effectively bind metals.

Through the FT-IR spectrum of the '*Pirapora*' community grown in metal-free media (control) and in media with 23 ppm of copper (red), we identified the functional groups

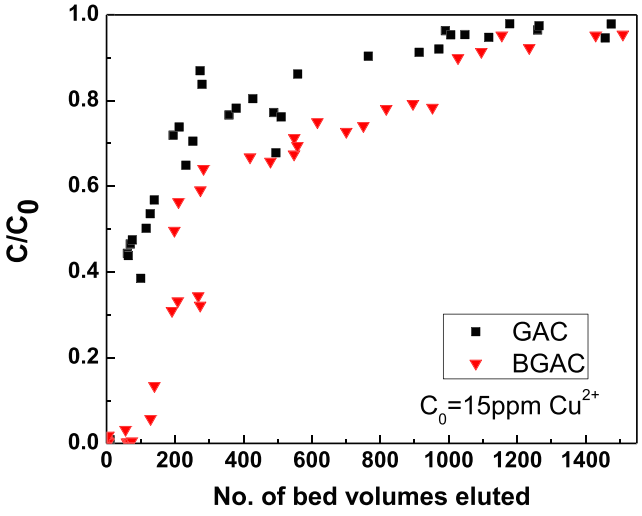

Fig. 7 – Breakthrough curves for copper on a 2.5 cm × 22 cm GAC (black) and BGAC (red) column.

responsible for the metal sorption. In Fig. 4, the control showed a peak at around 1070 cm^{-1} which corresponds to the C–OH stretching vibration of carboxyl groups (Smith et al., 2014). These functional groups are commonly found in different biomacromolecules in bacteria, such as peptidoglycan, teichoic acids, proteins, and lipoproteins (Wang and Chen, 2009). When the community was exposed to Cu^{2+} ions, another peak at 1038 cm^{-1}, which was also related to the C–OH of carboxyl groups appeared, suggesting that the counter ions associated with the carboxyl group had changed due to Cu^{2+} ions uptake (Cayllahua and Torem, 2010). These two peaks indicate that only a portion of the C–OH of the carboxyl groups are binding to the Cu^{2+} ions, since only one peak at 1038 cm^{-1} would be shown if all C–OH were bound to Cu^{2+}. Similar stretching vibrations were found in previous biomass surface characterization studies along with amine, hydroxyl, and phosphate groups which are also responsible for copper sorption into *Pseudomonas stutzeri* biomass (Pistorius et al., 2009; Oh et al., 2009).

The peak at 3406 cm^{-1} in the control represents the normal polymeric OH stretch, whereas the peak at 1400 cm^{-1} denotes a possible tertiary alcohol (Coates, 2000; Schmitt and Flemming, 1998). The peak at 669 cm^{-1} may also been attributed to an OH vibration (Coates, 2000). These three peaks were slightly shifted after the metal sorption to the biomass, indicating complexation of Cu^{2+} to OH groups. Besides the biomass, carboxyl, and hydroxyl groups are also found in uronic acids present in the EPS, which can complex with metal ions (Salehizadeh and Shojaosadati, 2003,Morillo Pérez et al., 2008).

Additionally, secondary and tertiary amine groups are indicated at 1650 cm^{-1} and 1238 cm^{-1} in the control, respectively, and were also changed after the metal sorption. The minor change in such peaks corresponds to the binding of the N–H and C–N groups with copper. Finally, the peak at 1541 cm^{-1} may relate to aliphatic or aromatic nitro compounds (Coates, 2000). The EPS, which contains negatively charged amino acids such as aspartic and glutamic acids, holds amino and hydroxyl groups that can bind metals (Laspidou and Rittmann, 2002).

Our results suggest that the carboxyl, hydroxyl, and amine groups of peptide chains provided a negative charge to the bacterial cell wall. However, among all the functional groups

Table 1 – Confocal Microscopy Data of Biofilm Grown for 72 h in P medium with and without Copper. The attachment analyses represent the biofilm grown with copper for 72 h, while the detachment assay represents fully mature biofilm grown without copper with subsequent exposure to copper for 4 h. Statistically significant differences with respect to the control of $p < 0.05$ and $p < 0.005$ is represented by* and by, respectively.**

Analysis	Parameter	Control (Avg ± Std Dev.)	Exposed to Cu^{2+} (Avg ± Std Dev.)	$[Cu^{2+}]$ (ppm)	Exposure time
Attachment	Biofilm thickness (μm)	109.7 ± 27.3	94.4 ± 11.2	20	72 h
Attachment	Percent mortality (%)-inner layers	40.4 ± 7.5	77.7 ± 15.6**	20	72 h
Attachment	Percent mortality (%)-outer layers	42.1 ± 5.8	82.2 ± 13.1**	20	72 h
Attachment	Cell density (cells/μm^2)	26.3 ± 3.1	56.1±*2.6**	20	72 h
Detachment	Biofilm thickness (μm)	89.4 ± 8.5	58.3 ± 7.4*	30	2 h
Detachment	Biofilm thickness (μm)	76.1 ± 26.7	32.5 ± 3.3	30	3 h
Detachment	Biofilm thickness (μm)	120.1 ± 28.2	40.3 ± 7.42*	30	4 h
Detachment	Percent mortality (%)-inner layers	6.0 ± 3.2	16.1 ± 4.4**	30	4 h
Detachment	Percent mortality (%)-outer layers	13.6 ± 3.3	16.2 ± 1.1**	30	4 h
Detachment	Cell density (cells/μm^2)	0.14 ± 0.01	0.13 ± 0.02	30	4 h

identified in the consortium, carboxyl and hydroxyl groups were the main functional groups responsible for the metal sorption process in the Pirapora microbial consortium.

Further evidence of metal sorption by the Pirapora microbial community was revealed through SEM imaging. Fig. S4 presents a cell from the consortium before (a) and after exposure to 25 ppm copper (b). Fig. S4b shows spots deposited on the cell wall surface and increased cell size, when compared to the control (Fig. S4a). Earlier studies presented similar results for *Mesorhizobium amorphae* CCNWGS0123 (Mohamad et al., 2012), suggesting that copper uptake was due to both cell-surface binding and intra-cellular accumulation of Cu^{2+}. Chen et al. (2000) confirmed the presence of copper on cell mass and changes in cell morphology after copper biosorption by *Desulfovibrio desulfuricans* (Chen et al., 2000), attributing the changes to secretion of EPS during the sorption process. Additional indication of copper sorption to microbial cells is presented by the EDS analysis (Table 2, Fig. S5a and b), which presents direct detection of metals on the biomass. The results showed 32.43 wt% Cu^{2+} on the biomass that was exposed to copper as opposed to the control that presented only 2.07 wt% Cu^{2+}. Therefore the Pirapora microbial community is able to bind heavy metal through adsorption.

3.4. *Pirapora consortium copper sorption kinetics and isotherm*

The design of a large-scale sorption treatment unit requires prior knowledge on the metal removal kinetics and sorption capacity of the adsorbent to achieve maximum metal removal. In the present study, we used the pseudo-second order model to describe the kinetics of copper removal by the '*Pirapora*' microbial community (Ho and McKay, 1999). This model is often used to describe the kinetics of biosorption (Lu et al., 2006, Mudhoo et al., 2012). Correlation parameters were derived from the linear model in Eq. (S1) (Kumar and Sivanesan, 2006) by plotting t/q_t vs t (Fig. S7), and are shown in Table 3. The model (Fig. S8, Table 3) depicted an R^2 of 0.91, which indicates that the model explains most variance. Copper equilibrium uptake rate, q_e, was achieved after 100 min at a pH = 4, which is also consistent with previous bacterial sorption studies. Lu et al., for example, modeled the sorption of copper by live cells of *Enterobacter* sp J1, attaining an equilibrium metal uptake rate after 100 min at a pH = 5 (Lu et al., 2006). Although similar equilibrium times were attained by *Enterobacter* sp. J1 and the Pirapora community, the metal uptake rate obtained during 100 min was higher for the Pirapora community, 303.03 mg/g, than for *Enterobacter* sp J1, 12.3 mg/g. The results suggest that the consortium used in this study may have a higher metal affinity than pure microbial cultures. This key finding implies that the use of bacterial communities isolated from environmental sites might be more efficient to remove heavy metals from water, than the use of pure microbial cultures.

Equilibrium isotherm models describe the solid–liquid equilibrium between the metal concentration bound on the bacteria and the metal concentration in the liquid phase. The equilibrium sorption behavior is important because the quality of any sorbent is determined by how much metal (mg) can be retained per gram of sorbent, or the metal uptake (mg metal/g dry biomass) (Volesky, 2007). We fitted the experimental data of the biosorption of copper by the log-phase '*Pirapora*' consortium into the most common models that describe single metal systems, the Langmuir and the Freundlich models (Wang and Chen, 2009) (Fig. 5). The correlation parameters of these two models are listed in Table 4.

The results show that the Langmuir model is able to explain most of the variance ($R^2 = 0.9$) and presented a higher explanatory power than the Freundlich model ($R^2 = 0.7$) based on the R^2 value. The Langmuir model indicates that the sorption capacity for copper was 450.0 mg/g. Previous studies have shown sorption capacities for Cu^{2+} ions by live, *Thiobacillus thiooxidans*, *Mesorhizobium amorphae* CCNWGS0123, *P. aeruginosa* PU21, and *P. stutzeri* of 39.8 mg/g, 169.7 mg/g, 23.1 mg/g, and 22.9 mg/g, respectively (Mohamad et al., 2012;

Table 2 – Energy Dispersive Spectroscopy (EDS) analysis of consortia exposed to copper.

Element	Control: bacteria		Bacteria +25 ppm Cu^{2+}	
	Mass%	Atom%	Mass%	Atom%
C*	97.13	99.45	67.57	91.68
Cu*	2.07	0.4	32.43	8.32
Zn*	0.8	0.15	–	–
Total	100	100	100	100

Table 3 – Pseudo-Second Order Kinetics of Cu^{2+} uptake by Pirapora microbial community.

	Intercept	Slope	$v_o = kq_e^2$	q_e	k	R^2
			mg g^{-1} min^{-1}	mg g^{-1}	g mg^{-1} min^{-1}	
Cu	0.0442	0.0033	22.62	303.03	0.000246	0.91

Table 4 – Parameters of Langmuir and Freundlich equilibrium models for the sorption of Cu^{2+} onto Pirapora community.

Metal	Langmuir			Freundlich		
	q_{max} (mg/g dry cells)	K_L	R^2	K_F	n	R^2
Cu^{2+}	450.0	0.07	0.93	41.2	1.53	0.70

Liu et al., 2004; Chang et al., 1997; Nakajima and Ueda, 2008). Although dead or inactivated pure cultures of bacteria typically have higher metal sorption capacities than live cells (Wang and Chen, 2009), our study indicates that the Pirapora microbial community has higher copper sorption capacities than dead cells of pure cultures, as depicted in Table S5.

3.5. *Copper removal by the Pirapora consortium biofilm in fixed-bed GAC*

Biosorption of heavy metals by biofilms can be optimized using a support medium for the microorganisms, which can serve as a process unit packing material with the right size, mechanical strength, rigidity, and porosity necessary for use in practical processes (Wang and Chen, 2009). Immobilization of microorganisms on fixed beds is a common procedure in some treatment systems, e.g. nitrifications systems using ammonia-oxidizing bacteria or anaerobic wastewater treatment processes (Haseborg et al., 2010; Cresson et al., 2008). We used a GAC bed as support matrix for the immobilization of microorganisms, and analyzed the GAC surface before and after biofilm formation to determine the amount of time needed for the biofilm to form on this surface (Fig. 6). This image is an example of a series of images taken from various locations of the GAC particle to confirm the presence of biofilm.

The GAC, with the immobilized consortium biofilm, was used as a packing material for a fixed bed column to treat heavy metal contaminated water. The Fig. 7 shows the breakthrough curves for copper sorption onto GAC and BGAC beds. The BGAC curve exhibits a broader leading edge than the GAC curve, which appears to have a greater sharpness, particularly during the initial number of bed volumes eluted.

Table 5 – Column Performance based on Cu^{2+} removal.

Column	Copper removal%	Initial Cu^{2+} concentration (ppm)	Flow rate (mL min^{-1})
GAC	17	15	5.3
BGAC	45	15	5.2

The BGAC curve also shows a broader trailing edge than the GAC curve. Potentially, the BGAC column presents slower intra-particle diffusion within the surface pores before reaching the entire biomass attached to the surface, as previously suggested (Chu, 2004).

The GAC bed exhibited complete breakthrough after 990 bed volumes. The BGAC approximate breakthrough occurred after 1429 bed volumes, but its C/C_0 never reached a value of 1 during sample collection. This longer saturation time exhibited by the BGAC can be attributed to the biofilm immobilized on the carbon's surface. The confirmation of the presence of the biofilm on the GAC after exposure to the metal solution can be found in the supporting information (Fig. S12).

For an initial copper concentration of 15 ppm and a flow rate of 5 mL/min, the BGAC column retained 45% of the copper mass added at $t = 0$ h to the column, whereas the GAC column only removed 17% (Table 5). The BGAC was also modeled using the batch Langmuir uptake rate at 15 ppm. The model showed a sorption capacity of 256.3 mg/g, which corresponds to 28% of the copper mass at $t = 0$ (data point from the Langmuir curve shown in Fig. 5). The differences between the theoretical and experimental uptake rates may be attributed to the curve-fit errors of the model and analytical errors in the biomass weight calculation. The experimental breakthrough curve for BGAC column indicated that the C/C_0 does not reach a value of 1, which shows a prolonged metal sorption over time, when compared to the GAC column due to actively growing microbial community.

4. Conclusion

The microbial community isolated from a heavy-metal-contaminated site in São Paulo, exhibited a two to three-fold higher uptake capacity for copper ions compared to previous studies with pure cultures. This is the first study utilizing a complex microbial community obtained from the environment for the removal of copper from water using a BGAC fixed bed column. Batch sorption studies with planktonic cells were developed first to understand the biomass-metal sorption process, and we found that equilibrium was achieved within 100 min with cells in exponential phase at pH value of 4. The biomass maximum sorption capacity for copper was determined to be 450.0 mg/g-dry-cells and the metal binding was attributed mainly to the carboxyl and hydroxyl functional groups present in the biomass. Prior to the development of the BGAC, we analyzed the biofilm formation and detachment in a metal-rich environment, in which 89% of the cells remained alive after the biosorption. The BGAC column showed a longer saturation time than the control; and retained 45% of the initial copper mass added to the column as opposed to the GAC column that removed only 17%. This study indicates that

microbial communities isolated from heavily contaminated environmental sites have a strong potential as metal biosorbents for water treatment processes, and that environmental consortia can offer a way to enhance current biological filter systems in water and wastewater treatment plants in developing countries.

Acknowledgments

This project was supported by the Houston-Louis Stokes Alliance for Minority Participation Scholars Program and the Rotary Foundation. The authors are thankful for the assistance of Dr. Heloisa Ramos Barbosa, Karina Yoshimoto, and Fabio Campos. We are also thankful for Maria Inês Zanoli Sato from CETESB that assisted us on the sediment and water collection in the contaminated dam.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.05.043.

REFERENCES

Baig, K.S., Doan, H.D., Wu, J., 2009. Multicomponent isotherms for biosorption of Ni^{2+} and Zn^{2+}. Desalination 249 (1), 429–439.
Bessbousse, H., Rhlalou, T., Verchère, J.F., Lebrun, L., 2008. Removal of heavy metal ions from aqueous solutions by filtration with a novel complexing membrane containing poly(ethyleneimine) in a poly(vinyl alcohol) matrix. J. Membr. Sci. 307 (2), 249–259.
Blais, J.F., Djedidi, Z., Cheikh, R.B., Tyagi, R.D., Mercier, G., 2008. Metals precipitation from effluents: review. Practice periodical of hazardous. Toxic. Radioact. Waste Manag. 12 (3), 135–149.
Bowman, G.T., Delfino, J.J., 1982. Determination of total kjeldahl nitrogen and total Phosphorus in surface waters and wastewaters. J. (Water Pollut. Control Fed. 54 (9), 1324–1330.
Cayllahua, J.E.B., Torem, M.L., 2010. Biosorption of aluminum ions onto *Rhodococcus opacus* from wastewaters. Chem. Eng. J. 161 (1–2), 1–8.
Chang, J.-S., Law, R., Chang, C.-C., 1997. Biosorption of lead, copper and cadmium by biomass of Pseudomonas aeruginosa PU21. Water Res. 31 (7), 1651–1658.
Chen, B.-Y., Utgikar, V.P., Harmon, S.M., Tabak, H.H., Bishop, D.F., Govind, R., 2000. Studies on biosorption of zinc(II) and copper(II) on *Desulfovibrio desulfuricans*. Int. Biodeterior. Biodegradation 46 (1), 11–18.
Chen, Q., Luo, Z., Hills, C., Xue, G., Tyrer, M., 2009. Precipitation of heavy metals from wastewater using simulated flue gas: sequent additions of fly ash, lime and carbon dioxide. Water Res. 43 (10), 2605–2614.
Chu, K.H., 2004. Improved fixed bed models for metal biosorption. Chem. Eng. J. 97 (2–3), 233–239.
Coates, J., 2000. In: Practical Approach, A., Meyers, R.A. (Eds.), Interpretation of Infrared Spectra. John Wiley & Sons Ltd, Chichester, pp. 10815–10837.
Cresson, R., Escudié, R., Steyer, J.-P., Delgenès, J.-P., Bernet, N., 2008. Competition between planktonic and fixed microorganisms during the start-up of methanogenic biofilm reactors. Water Res. 42 (3), 792–800.
Davis, T.A., Volesky, B., Mucci, A., 2003. A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. 37 (18), 4311–4330.
Haseborg, E.t., Zamora, T.M., Fröhlich, J., Frimmel, F.H., 2010. Nitrifying microorganisms in fixed-bed biofilm reactors fed with different nitrite and ammonia concentrations. Bioresour. Technol. 101 (6), 1701–1706.
Hentzer, M., Eberl, L., Nielsen, J., Givskov, M., 2003. Quorum sensing : a novel target for the treatment of biofilm infections. BioDrugs 17 (4), 241–250.
Ho, Y.S., McKay, G., 1999. Pseudo-second order model for sorption processes. Process Biochem. 34 (5), 451–465.
Kratochvil, D., Volesky, B., 1998. Advances in the biosorption of heavy metals. Trends Biotechnol. 16 (7), 291–300.
Kumar, K.V., Sivanesan, S., 2006. Pseudo second order kinetics and pseudo isotherms for malachite green onto activated carbon: comparison of linear and non-linear regression methods. J. Hazard. Mater. 136 (3), 721–726.
Kunhikrishnan, A., Bolan, N.S., Müller, K., Laurenson, S., Naidu, R., Kim, W.-I., 2012. In: Donald, L.S. (Ed.), Advances in Agronomy. Academic Press, pp. 215–297.
Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Res. 36 (11), 2711–2720.
Lewis, A.E., 2010. Review of metal sulphide precipitation. Hydrometallurgy 104 (2), 222–234.
Lezcano, J.M., Gonzalez, F., Ballester, A., Blazquez, M.L., Munoz, J.A., Garcia-Balboa, C., 2010. Biosorption of Cd(II), Cu(II), Ni(II), Pb(II) and Zn(II) using different residual biomass. Chem. Ecol. 26 (1), 1–17.
Liu, H.-L., Chen, B.-Y., Lan, Y.-W., Cheng, Y.-C., 2004. Biosorption of Zn(II) and Cu(II) by the indigenous *Thiobacillus thiooxidans*. Chem. Eng. J. 97 (2–3), 195–201.
Lu, W.-B., Shi, J.-J., Wang, C.-H., Chang, J.-S., 2006. Biosorption of lead, copper and cadmium by an indigenous isolate *Enterobacter* sp. J1 possessing high heavy-metal resistance. J. Hazard. Mater. 134 (1–3), 80–86.
Mejias Carpio, I.E., Santos, C.M., Wei, X., Rodrigues, D.F., 2012. Toxicity of a polymer-graphene oxide composite against bacterial planktonic cells, biofilms, and mammalian cells. Nanoscale 4 (15), 4746–4756.
Mohamad, O., Hatab, S., Liu, Z., Li, Z., Kong, Z., Wei, G., 2012. Biosorption and bioaccumulation of Cu^{2+} from aqueous solution using living *M. amorphae* isolated from Mine Tailings. Mine Water Environ. 31 (4), 312–319.
Monchy, S., Benotmane, M.A., Janssen, P., Vallaeys, T., Taghavi, S., van der Lelie, D., Mergeay, M., 2007. Plasmids pMOL28 and pMOL30 of *Cupriavidus metallidurans* are specialized in the maximal viable response to heavy metals. J. Bacteriol. 189 (20), 7417–7425.
Monteiro, C., Castro, P., Malcata, F., 2011. Capacity of simultaneous removal of zinc and cadmium from contaminated media, by two microalgae isolated from a polluted site. Environ. Chem. Lett. 9 (4), 511–517.
Moon, E.M., Peacock, C.L., 2011. Adsorption of Cu(II) to Bacillus subtilis: a pH-dependent EXAFS and thermodynamic modelling study. Geochim. Et. Cosmochim. Acta 75 (21), 6705–6719.
Morillo Pérez, J.A., García-ribera, R., Quesada, T., Aguilera, M., Ramos-cormenzana, A., Monteoliva-sánchez, M., 2008. Biosorption of heavy metals by the exopolysaccharide produced by *Paenibacillus jamilae*. World J. Microbiol. Biotechnol. 24 (11), 2699–2704.
Mudhoo, A., Garg, V.K., Wang, S.B., 2012. Removal of heavy metals by biosorption. Environ. Chem. Lett. 10 (2), 109–117.

Nakajima, A., Ueda, Y., 2008. Relationship between copper biosorption and microbial inhibition of hydroxyl radical formation in a copper(II)-hydrogen peroxide system. World J. Microbiol. Biotechnol. 24 (8), 1253–1257.
Oh, S.E., Hassan, S.H.A., Joo, J.H., 2009. Biosorption of heavy metals by lyophilized cells of Pseudomonas stutzeri. World J. Microbiol. Biotechnol. 25 (10), 1771–1778.
Ozdemir, G., Ozturk, T., Ceyhan, N., Isler, R., Cosar, T., 2003. Heavy metal biosorption by biomass of *Ochrobactrum anthropi* producing exopolysaccharide in activated sludge. Bioresour. Technol. 90 (1), 71–74.
Pal, A., Paul, A.K., 2008. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian J. Microbiol. 48 (1), 49–64.
Peters, R.W., 1999. Chelant extraction of heavy metals from contaminated soils. J. Hazard Mater. 66 (1–2), 151–210.
Pistorius, A.M., DeGrip, W.J., Egorova-Zachernyuk, T.A., 2009. Monitoring of biomass composition from microbiological sources by means of FT-IR spectroscopy. Biotechnol. Bioeng. 103 (1), 123–129.
Rodrigues, D.F., 2011. Biofilters: a solution for heavy metals removal from water. J. Bioremed. Biodegr. 2, e101.
Rodrigues, D.F., Jaisi, D.P., Elimelech, M., 2012. Toxicity of functionalized single-walled carbon nanotubes on soil microbial communities: implications for nutrient cycling in soil. Environ. Sci. Technol. 47 (1), 625–633.
Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D.S., Alston, M., Stringer, M.F., Betts, R.P., Baranyi, J., Peck, M.W., Hinton, J.C.D., 2012. Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. J. Bacteriol. 194 (3), 686–701.
Romera, E., Gonzalez, F., Ballester, A., Blazquez, M.L., Munoz, J.A., 2008. Biosorption of heavy metals by *Fucus spiralis*. Bioresour. Technol. 99 (11), 4684–4693.
Salehizadeh, H., Shojaosadati, S.A., 2003. Removal of metal ions from aqueous solution by polysaccharide produced from Bacillus firmus. Water Res. 37 (17), 4231–4235.
Schmitt, J., Flemming, H.-C., 1998. FTIR-spectroscopy in microbial and material analysis. Int. Biodeterior. Biodegrad. 41 (1), 1–11.
Smith, S.C., Ahmed, F., Gutierrez, K.M., Rodrigues, D.F., 2014. A comparative study of lysozyme adsorption with graphene, graphene oxide, and single-Walled carbon nanotubes: potential environmental applications. Chem. Eng. J. 240, 147–154.
Stewart, P.S., Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358 (9276), 135–138.
Takenaka, Y., Saito, T., Nagasaki, S., Tanaka, S., Kozai, N., Ohnuki, T., 2007. Metal sorption to *Pseudomonas fluorescens*: influence of pH, ionic strength and metal concentrations. Geomicrobiol. J. 24 (3–4), 205–210.
Teitzel, G.M., Parsek, M.R., 2003. Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa. Appl. Environ. Microbiol. 69 (4), 2313–2320.
USEPA, 1992. Method 3010A: Acid Digestion of Aqueous Samples and Extracts for Total Metals for Analysis by FLAA or ICP Spectroscopy.
USEPA, 1996. In: EPA, U (Ed.), Method 3050B: Acid Digestion of Sediments, Sludges, and Soils.
Volesky, B., 2007. Biosorption and me. Water Res. 41 (18), 4017–4029.
Wang, J., Chen, C., 2009. Biosorbents for heavy metals removal and their future. Biotechnol. Adv. 27 (2), 195–226.
Warren, L.A., Haack, E.A., 2001. Biogeochemical controls on metal behaviour in freshwater environments. Earth Sci. Rev. 54 (4), 261–320.