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Reduction of Cu(II) and simultaneous production of acetate from inorganic carbon by *Serratia Marcescens* biofilms and plankton cells in microbial electrosynthesis systems

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1 **Abstract**

2 Simultaneous Cu(II) reduction (6.42 ± 0.02 mg/L/h), acetate production (1.13 ± 0.02 mg/L/h) from
3 inorganic carbon (i.e., CO₂ sequestration), and hydrogen evolution (0.0315 ± 0.0005 m³/m³/d) were
4 achieved in a *Serratia marcescens* Q1 catalyzed microbial electrosynthesis system (MES). The
5 biofilms released increasing amounts of extracellular polymeric substances (EPS) with a higher
6 compositional diversity and stronger Cu(II) complexation, compared to the plankton cells, at higher
7 Cu(II) concentrations (up to 80 mg/L) and circuital currents (cathodic potential of –900 mV vs.
8 standard hydrogen electrode (SHE)). Moreover, the biofilms reduced Cu(II) to Cu(0) more
9 effectively than the plankton cells. At Cu(II) concentrations below 80 mg/L, the dehydrogenase
10 activity in the biofilms was higher than in the plankton cells, and increased with circuital current,
11 which was converse to the lower activities of catalase (CAT), superoxide dismutase (SOD) and
12 antioxidative glutathione (GSH) in the biofilms than the plankton cells, although all these
13 physiological activities were positively correlated with the concentration of Cu(II). This is the first
14 study that evaluates the EPS constituents and the physiological activities of the biofilms and the
15 plankton cells in the MESs, that favors the production of acetate from CO₂ sequestration and the
16 simultaneous reduction of Cu(II) from organics-barren waters contaminated with heavy metals.

17
18 **Keywords:** microbial electrosynthesis system; Cu-tolerant biofilm and plankton cell; CO₂ fixation;
19 extracellular polymeric substances; physiological activity; variable cathode potential

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1. Introduction

Microbial electro-metallurgical processes combining bioelectrochemical systems with metallurgical processes, are emerging and sustainable technologies for the recovery of heavy metals, such as Cu(II), Cr(VI), Co(II) and Cd(II), from wastes and wastewaters (Nancharaiah et al., 2016; Dominguez-Benetton et al., 2018). These systems usually include biocathodes and bioanodes made by pure or mixed immobilized electrogenic bacteria cultures, fed by inorganic species (e.g., bicarbonate, HCO_3^-) in the cathodes and acetate in the anodes (Huang et al., 2015; 2018; Chen et al., 2016). Among the variety of heavy metals that can be recovered using microbial electro-metallurgical processes, the recovery of Cu(II) has attracted significant attention due to its frequent occurrence in acid mine drainage wastewaters (Ter Heijne et al., 2010; Zhang et al., 2015; Wu et al., 2016; Kiran et al., 2018). Likewise abiotic cathodes, biocathodes utilizing electrochemically active bacteria (EAB) have been shown to reduce Cu(II) to metallic Cu(0) and thus remove the heavy metal from the catholyte solution (Huang et al., 2015; 2018; Shen et al., 2017; Tao et al., 2017).

From another perspective, microbial electrosynthesis systems (MESs) are also emerging as potential systems for the synthesis of valuable products from waste streams. One notable example is the production of acetate from inorganic carbon such as bicarbonate, where the production rate of acetate is modulated artificially by the application of an electrical current that interferes with the microbial metabolism (Bajracharya et al., 2015; Venkata Mohan et al., 2016; Rojas et al., 2018). Therefore, the combination of microbial electro-metallurgical processes of Cu(II) recovery with MESs, in a single process, presents an attractive opportunity to achieve simultaneously the sequestration of CO_2 , its conversion to C1 chemicals (e.g., acetate) and the removal/recovery of Cu(II) from organics-barren waters contaminated with metals.

The production of C1 and other products in such combined process might be influenced by the

49 Cu(II) stressing conditions and modulated by the application of an external circuital current. Wide
50 groups of gram-negative bacteria, under the harmful effect of toxic substances, can adopt special
51 metabolic pathways as a protective mechanism, which may result in the release complex
52 heterogeneous extracellular polymeric substances (EPS) composed of variable degrees of saccharides
53 and proteins (Sheng et al., 2010; Cao et al., 2011; Zhou et al., 2017). In general, exoproteins in EPS
54 are more common than exopolysaccharides since, due to the higher content of negatively charged
55 amino acids, exoproteins can form electrostatic bonds with positively charged metal ions, whereas
56 higher carbohydrate in EPS released by *Shewanella* sp. offers protection against excessive
57 concentrations of Fe(II) and Mn(II) (Cao et al., 2011). Another important aspect is the bacterial
58 source of the EPS. In general, bacterial cells forming biofilms normally produce higher quantity of
59 EPS, as compared to plankton cells in suspension in conventional biological processes (Sheng et al.,
60 2010; Cao et al., 2011). Biofilms and plankton cells also play different roles in electron transfer
61 processes, with the former dominating direct electron transfer while the latter contributing to
62 extracellular electron transfer through mediators (Carmona-Martinez et al., 2013; TerAvest and
63 Angenent, 2014; Grobblor et al., 2018). Thus, several factors are needed to be evaluated to unravel
64 the roles played by the biofilms and the plankton cells in MESs in the removal of Cu(II) with
65 simultaneous sequestration of CO₂, including: (i) the evaluation of the EPS constituent species in
66 both biofilms and plankton cells; (ii) the determination of the binding constants of the these species
67 with the metal ions (Lakowicz, 2006); (iii) the evaluation of the modulation factor of an applied
68 cathodic potential (i.e., a circuital current) on the nature, composition and amount of EPS released by
69 both biofilms and plankton cells; (iv) the determination of the intracellular physiological activities of
70 the EAB biofilms and the plankton cells in the presence of Cu(II) and as a function of the circuital
71 current. The bacterial activities of antioxidative glutathione (GSH), catalase (CAT) and superoxide
72 dismutase (SOD) can function as important metabolic indicators for either copper sequestration or

for scavenging $O_2^{\cdot -}$ induced by Cu(II) (Nguyen et al., 2013) whereas the dehydrogenase activity usually indicates the biocatalytic ability for hydrogen evolution (Venkateswar Reddy et al., 2010).

In this study, efficient removal of Cu(II) and the simultaneous production of acetate from bicarbonate was investigated in a metallurgical MES using *Serratia marcescens* Q1 EAB isolated from a well-adapted biocathode utilized in a bioelectrochemical system to remove Cu(II) (Huang et al., 2015). The impact of cathode potential and Cu(II) concentration on the amount and composition of EPS released by both the biofilms and the plankton cells was investigated. This was quantified by the evaluation of the binding constant of Cu(II) with the EPS constituent species. In addition, the endogenous physiological activities of GSH, SOD, CAT and dehydrogenase in both the biofilms and the plankton cells were examined to determine their roles in Cu(II) removal and acetate production.

2. Materials and methods

2.1. Sampling and EAB isolation

EAB was isolated from the biocathodes of bioelectrochemical systems (Huang et al., 2015) fed with Cu(II) ions of 20 mg/L by streaking cathode scrapings onto agar plates containing an anaerobic medium amended with agar, Cu(II) (20 mg/L) and $NaHCO_3$ (10 mg/L), in an anaerobic glove box (Xinmiao, Shanghai). The composition of the anaerobic medium, the incubation conditions of the agar plates and the bacterial isolation procedure were as described previously (Shen et al., 2017; Tao et al., 2017; Hou et al., 2018).

2.2. Isolates identification

The bacterial DNA was extracted using the Ezup DNA kit (cat. No. SK8255, Sangon Biotech (Shanghai) Co. Ltd., China) according to the manufacturer's instructions. Primers 518F and 1540R were used to amplify the bacterial 16S rDNA. PCR solution components, amplification, purification,

clone procedure and sequencing were as previously described (Shen et al., 2017; Tao et al., 2017).

The 16S rDNA gene sequence was compared with those available in GenBank by using the BLAST program to identify the most similar 16S rDNA fragments.

2.3. EAB inoculation and reactor operation

The bacteria were harvested after 24 h incubation in an anaerobic and sterile medium (pH = 5.8), as previously described (Shen et al., 2017; Tao et al., 2017). The bacterial cells were then concentrated and washed 3 times with a sterile physiological saline solution. The catholyte composed of NH_4HCO_3 (0.08 g/L), KHCO_3 (0.10 g/L), KH_2PO_4 (0.012 g/L); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2.0 mg/L), vitamins 0.6 mL/L and mineral 0.6 mL/L and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, was firstly sparged with N_2 gas for 15 min and then slowly inoculated with the isolates (10^8 CFU/mL) in an anaerobic glovebox (YQX-II, Xinmiao, Shanghai) (Huang et al., 2010).

Typical Cu(II) concentration in acid mine drainage wastewaters may vary over a wide range, from 10 to 1000 mg/L (Bilal et al., 2013), which makes difficulty to select a narrow range of Cu(II) concentration to be used in this study. In contrast, typical Cu(II) concentrations in biocathodes of bioelectrochemical systems (1 – 20 mg/L) (Huang et al., 2015; Shen et al., 2017; Tao et al., 2017) and in conventional biological processes (40 – 150 mg/L) (De Alencar et al., 2017), are normally in the lower range, as a result of the potential toxicity exerted by Cu(II) to the bacteria. Therefore, unless otherwise stated, in this study the initial concentration of Cu(II) in the catholyte was varied as 60, 80 and 120 mg/L. The anolyte was a buffer solution composed of NaH_2PO_4 - NaHPO_4 . The initial pH and conductivity of the electrolyte solutions in both chambers were fixed values equal to 5.8 and 5.8 mS/cm, respectively (Huang et al., 2015). The catholyte pH equal to 5.8 was chosen considering several factors including that pHs less than 6.0 are common in practical acid mine drainage wastewaters, that pHs in the range 3.0 – 6.0 are used in conventional bacterial removal of Cu(II)

(Bilal et al., 2013), and that this pH value has previously been used for removal of Cu(II) in other bioelectrochemical systems (Huang et al., 2015; Shen et al., 2017; Tao et al., 2017). The pH of the catholyte was not controlled during the experiments and only increased to 6.0 - 6.3 at the end of each experiment.

Graphite felt ($1.0 \times 1.0 \times 1.0$ cm, 6 pieces) was used as the cathode due to its large porosity and good affinity with the EAB (Huang et al., 2013b; 2013c; Shen et al., 2017), whereas a carbon rod was used as the anode. The anodic and cathodic chambers were separated by a cation exchange membrane (Ultrex CMI-7000, Membranes International, Glen Rock, NJ) since preliminary results showed similar performance to the use of anion exchange membrane, particularly at such low Cu(II) concentrations and short operational times (see Supporting Information and Fig. S1), besides avoiding transfer of anions (eg. acetate and phosphate groups) (Shen et al., 2017; Tao et al., 2017). A constant cathodic potential of -900 mV or -600 mV versus standard hydrogen electrode (SHE) (VSP, BioLogic) were applied to the reactors and the circuital current was automatically monitored. The choice of these cathodic potentials were based on the reported range of cathodic potentials of $-1100 \sim -600$ mV (Mohanakrishna et al., 2016). The reactors were operated in fed-batch mode at room temperature (25 ± 3 °C) for 12 h, unless otherwise stated. Such time course was adequate to monitor the conversion of inorganic carbon (HCO_3^-) and the production of acetate (Fig. S2). Three duplicate reactors were used in all experiments.

Three reactors types were used as controls. The first was operated without the inoculum (abiotic control). The second was inoculated but operated under open circuit conditions (OCC), to examine acetate production and Cu(II) removal in the absence of a circuital current and to confirm that hydrogen production was only produced in the presence of a circuital current (CCC). The third was operated in the absence of Cu(II) to monitor the variation of the production rates of acetate, hydrogen, exoproteins and exopolysaccharides from the system.

2.4. EPS extraction, fluorescence spectroscopy and quenching titration

EPS extraction was performed at the end of each 12 h experiment. Dissolved EPS was extracted as previously reported (Kang et al., 2014). Briefly, the catholyte solution and the graphite felt cathode electrode were carefully sampled from each reactor. The electrode was then submerged into a physiological saline solution (10 mL) and sonicated on ice, three times, for 10 s intervals at an intensity of $1.0 \text{ W}\cdot\text{cm}^2$ and a frequency of 20 kHz (Branson Sonifier 250, Danbury, USA) to extract the biofilms. The electrode was removed from the solution, rinsed with an additional 3 mL of physiological saline solution and discarded. The biofilm solution formed and the catholyte solution sampled from the reactor were separately sonicated at an intensity of $2.7 \text{ W}\cdot\text{cm}^2$ and a frequency of 40 kHz (Branson Sonifier 250, Danbury, USA) on ice for 10 min and samples were immediately centrifuged at $11000 \times g$ for 20 min at 4 °C. The precipitates were separately collected and used to determine the endogenous physiological activities of the biofilms and of the plankton cells, as described in Section 2.5, whereas the supernatants were filtered through a 0.22 μm acetate cellulose membrane. Negligible amount of nucleic acid was observed in both filtrate solutions, implying that cell lysis did not take place during the extraction of EPS in both the biofilms and the plankton cells (Guo and Chang, 2017). The supernatants obtained were separately fixed to a final volume of 15 mL and operationally defined as biofilms loosely associated EPS (biofilm-EPS) and plankton cells loosely associated EPS (plankton-EPS), respectively (Sheng et al., 2010; Kang et al., 2014).

The biofilm-EPS and the plankton-EPS, together with the biofilm coated electrode and the associated plankton cells in the catholyte were analyzed by CV, as described in section 2.6.

In parallel, the exopolysaccharides in the biofilm-EPS and the plankton-EPS were measured by phenol-sulfuric acid method (Suzanne Nielsen, 2009) whereas exoproteins were quantified by the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a calibration standard.

Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy performed on F-7000 (Hitachi, Japan) equipped with 1.0 cm quartz cell and a thermostat bath was used to quantify and characterize the EPS. The EEM spectra were collected with subsequent scanning emission spectra by varying the excitation wavelengths from 200 nm to 450 nm in 10 nm increments, and the emission spectra from 250 nm to 500 nm at 0.5 nm increments. The width of the excitation/emission slit was set to 5.0 nm, and the scanning speed was set to 1200 nm/min for all the fluorescence measurements. The fluorometer's response to a milli-Q water was subtracted from the fluorescence spectra recorded for EPS. EEM data were processed using the software Sigma plot 10.0 (Systat, USA).

Prior to the EEM data analysis, the Raman and Rayleigh scatter was removed according to the protocol of Bahram et al. (2006). The EEMs were then normalized to the area under the Milli-Q water Raman peak (Ex = 350 nm) collected on the same day (Lawaetz and Stedmon, 2009), to produce corrected fluorescence intensities in Raman Units (R.U.). Five regions (Regions I, II, III, IV and V) in each EEM were based largely upon supporting literature, as summarized by Chen et al. (2003). Briefly, peaks at shorter excitation wavelengths (< 250 nm) and shorter emission wavelengths (< 350 nm) related to simple aromatic proteins such as tyrosine (Regions I and II). Peaks at intermediate excitation wavelengths (250 ~ 280 nm) and shorter emission wavelength (260 ~ 380 nm) related to soluble microbial byproduct-like material (Region IV). Peaks at longer excitation wavelengths (> 280 nm) and longer emission wavelengths (> 380 nm) related to humic acid-like organics (Region V). Peaks at shorter excitation wavelengths (< 250 nm) and longer emission wavelengths (> 350 nm) related to fulvic acid-like materials (Region III). The relative abundance of each component was then calculated as the integration beneath EEMs within each specific selected region, divided by the sum of the entire region.

The interaction mechanism between Cu(II) and EPS was determined by fluorescence quenching

titration. 1.0 mL EPS and different volumes of pre-determined Cu(II) solution were successively added into a 5.0 mL centrifuge tube. The final Cu(II) concentration in the mixed solution was varied from 0 to 160 mg/L. After each addition of Cu(II), the solution was fully mixed using a magnetic stirrer for 15 min and the fluorescence spectra were recorded. The equilibrium time of 15 min produced steady fluorescence intensity peaks. The EEM data analysis and region division were carried out as mentioned above.

The binding constants ($\log K_b$), revealing the interaction between the small Cu(II) and the macromolecules of EPS, were calculated from (Lakowicz, 2006):

$$\log[(F_0 - F)/F] = \log K_b + n \times \log[\text{Cu(II)}] \quad (1)$$

where F_0 and F are the fluorescence intensities measured in the absence and in the presence of Cu(II), respectively. K_b is the binding constant, n is the number of binding sites and [Cu(II)] is the Cu(II) concentration.

2.5. Endogenous physiological activities

The *S. marcescens* biofilms and the plankton cells were re-suspended in a physiological saline solution and ultrasonically disrupted three times at 20 kHz for 5 min, respectively (Sonics Vibra-CellTM). After centrifugation for 10 min at $10000 \times g$ at 4 °C, the supernatant obtained was either immediately assessed or frozen at –80 °C for subsequent analysis to determine the dehydrogenase, SOD, CAT and GSH activities. The dehydrogenase activity was estimated as previously described (Venkateswar Reddy et al., 2010). The methods for SOD and CAT activities were referred to the description by Branco et al. (2010). The GSH content was quantified as described by Rahman et al. (2006). Results expressed were based on per gram cell dry weight (mg/g DW). The procedure was repeated at least three times.

2.6. Cycle voltammetry analysis and characterization

The redox behaviors of the *S. marcescens* cathode, the catholyte with the plankton cells, the biofilm-EPS and the plankton-EPS were analyzed by cycle voltammetry (CV) (VSP, BioLogic). The potential was scanned between -1.0 and $+0.6$ V (vs. SHE) at a scan rate of 0.1 mV/s using a standard three-electrode arrangement including the biocathode as the working electrode, a platinum plate as the counter electrode, and a saturated calomel electrode (-0.241 V vs SHE) as the reference electrode (Huang et al., 2012).

The elemental composition of the copper adsorbed on the surface of the *S. marcescens* biofilms and the plankton cells was analyzed by X-ray photoelectron spectroscopy (XPS; Kratos AXIS Ultra DLD). For these determinations, the cathodes loaded with *S. marcescens* and the catholyte with plankton cells were operated at a Cu(II) of 80 mg/L for 7 cycles to allow the preparation of a sufficient amount of samples for XPS analysis.

The concentration of Cu(II) in catholyte was determined by atomic absorption spectroscopy (AAAnalyst 700, PerkinElmer). Removal of Cu(II) (mg/L/h) at the end of one batch cycle operation was calculated based on the decreased Cu(II) in the catholyte divided by operation time. Accordingly, the production of acetate (mg/L/h) in the reactors was quantified using a high performance liquid chromatograph (HPLC, Agilent 1100) and divided by operation time (Huang et al., 2014), and the production of hydrogen ($\text{m}^3/\text{m}^3/\text{d}$) was determined from the volume recovered from the headspace of the cathodic chamber, normalized by the volume of the catholyte (Huang et al., 2014; Chen et al., 2016). The biomass immobilized on the cathode surfaces and suspended in the catholyte was determined as previously described (Wei et al., 2010).

The coulombic efficiencies for Cu(II) reduction (CE_{Cu}), production of acetate (CE_{acetate}) and hydrogen evolution (CE_{H_2}) were calculated as the ratios between the measured charge transferred through the external circuit, and the charge recovered for Cu(II) reduction, acetate production and

hydrogen evolution, respectively, similarly to other studies (Zou and He, 2018; Jain and He, 2018).

One-way ANOVA in SPSS 19.0 was used to analyze the statistical variation of the data, and all of the data indicated significance levels of $p < 0.05$.

3. Results and Discussion

3.1. Bacteria isolation

One rod-shaped, gram-negative and major opportunistic to facultative strain was successfully isolated from a bacterial community amended with Cu(II). This strain is able to metabolize multifarious organic compounds and inorganic carbonate, allowing its switching between autotrophic and heterotrophic cultures. This Q1 strain matched (100%) the genetic sequence of *Serratia marcescens*, which belongs to the largest group of *Proteobacteria* bacteria, exhibiting rich metabolic diversity, and robust survival in heavy metal stressing environments (Ntougias et al., 2006). *S. marcescens* has been investigated for the removal of a wide range of heavy metals including Cr, Pb, Cd, Ni and Hg (Kannan et al., 2002; Cristani et al., 2012). This strain has also recently been observed to secrete urease during Cd(II) removal (Bhattacharya et al., 2018). This opportunistic *S. marcescens* Q1 thriving in autotrophic and heterotrophic cultures, was unsurprisingly similar to many other CO₂ fixation bacteria including *Metallosphaera yellowstonensis*, *Stenotrophomonas* sp. WH-11, *Ochrobactrum* sp. WH-13, *Castellaniella* sp. WH-14, and *Sinomicrobium oceani* WH-15 (Jennings et al., 2014; Hu et al., 2015).

3.2. Cu(II) removal and acetate production

At an initial Cu(II) of 80 mg/L, higher Cu(II) removal rate (6.42 ± 0.02 mg/L/h) (Fig. 1A), acetate production rate (1.13 ± 0.02 mg/L/h) (Fig. 1B), hydrogen evolution rate (0.0315 ± 0.0005 m³/m³/d) (Fig. 1C), circuital currents (Fig. 1D), CE_{acetate} and CE_{H_2} (Table S1) were achieved at a

cathode potential of –900 mV, rather than at –600 mV. These results were explained by the availability of more cathodic electrons at –900 mV than –600 mV for hydrogen evolution, acetate production and Cu(II) removal. The application of both potentials obtained invariably values higher than the abiotic and the OCC controls (Fig. 1A and B), asserting the ability of *S. marcescens* species for the simultaneous removal of Cu(II) and production of acetate from bicarbonate in MES. The removal of Cu(II) and the production of acetate invariably increased with the increase in initial Cu(II) concentration up to 80 mg/L, implying the stimulating role of copper stressing conditions in inducing the *S. marcescens* physiological metabolism to produce acetate. This effect was probably similar to the role played by copper-coated cathodes in enhancing the production of methane in bioelectrochemical, anaerobic digestion, sequencing batch reactors (Park et al., 2018). However, the production of acetate decreased at excessive Cu(II) concentration (120 mg/L) (Fig. 1B), whereas the removal of Cu(II) continued to increase (Fig. 1A), implying a change in regulating mechanism at such high Cu(II) stressing conditions.

Here Fig. 1

The robustness of *S. marcescens* sp. for reducing Cu(II) was quantified by the highest Cu(II) removal rate (6.42 mg/L/h at a Cu(II) of 80 mg/L) measured in this study, which was appreciably higher than the values (3.61 – 3.64 mg/L/h) reported with *Pseudomonas aeruginosa* JY5 or *Stenotrophomonas* sp. JY6 (Shen et al., 2017), or the values (1.0 – 1.1 mg/L/h) reported with mixed cultures (Huang et al., 2015; Wu et al., 2018) at Cu(II) of 5 – 20 mg/L (Table S2). The Cu(II) removal rate was also within the wider range (0.1 – 69.1 mg/L/h) reported using abiotic cathodes (Table S2). The corresponding production rate of acetate (1.13 mg/L/h at –900 mV) was also significantly higher than the values (0.28 – 0.30 mg/L/h) reported with *Geobacter sulfurreducens* at –600 mV (vs. SHE), and within the wider range (0.05 – 8.2 mg/L/h) reported with *Sporomusa ovata*, *Clostridium ljungdahlii*, *Acetoanaerobium* or *Moorella* at potentials from –0.7 to –0.4 V (vs. SHE) in

the absence of Cu(II) (Nevin et al., 2011; Bajracharya et al., 2015; Jourdin et al., 2016). The acetate production rate was also higher than the rates (0.07 – 0.36 mg/L/h) measured with pure cultures of *Stenotrophomonas* sp. WH-11, *Ochrobactrum* sp. WH-13, *Castellaniella* sp. WH-14, *Sinomicrobium oceani* WH-15 or *Metallosphaera yellowstonensis*, and the rates with mixed cultures (0.78 – 5.93 mg/L/h) in the absence of Cu(II) (Jennings et al., 2014; Hu et al., 2015; Mohanakrishna et al., 2016; 2018). However, it was significantly lower than the rate in a mixed culture (66 mg/L/h) in the absence of Cu(II) (Rojas et al., 2018). The production rate of hydrogen (0.032 m³/m³/d) was significantly lower than the 0.31 – 2.64 m³/m³/d by *G. sulfurreducens* or *Methanosarcina barkeri* at –700 – –600 mV (Nevin et al., 2011; Bajracharya et al., 2015; Jourdin et al., 2016), suggesting a weak performance of this Cu-tolerant *S. marcescens* as a hydrogen producer, although hydrogen is a well-known energy vector for acetate production from CO₂ (Bajracharya et al., 2015; Venkata Mohan et al., 2016). These results collectively demonstrated the robust conditions of the Cu(II)-tolerant *S. marcescens* in this MES for simultaneous Cu(II) removal and acetate production from CO₂, although hydrogen can only be considered an insignificant by-product.

3.3. CV analysis

CV analysis of the *S. marcescens* biofilms showed no oxidation-reduction peaks in the absence of Cu(II) (Fig. 2). However, one strong oxidation-reduction peak appeared on the *S. marcescens* biofilms after spiking with 60 mg/L Cu(II), shown as reductive onset potentials of 0.34 V (vs. SHE) at –900 mV (Fig. 2A and Table 1) and 0.32 V (vs. SHE) at –600 mV (Fig. 2E and Table 1). Both peaks were more positive than 0.27 V (vs. SHE) in the abiotic controls (Fig. S3 and Table 1) and 0.30 V (vs. SHE) under OCC, at the same Cu(II) concentration (Fig. 2I and Table 1).

The highest Cu(II) concentration (120 mg/L) at –900 mV achieved the most positive reductive onset potentials and reductive peak potentials, as well as the highest reductive peak currents (Fig. 2A

313 and Table 1), demonstrating the strong catalytic activity of *S. marcescens* biofilms toward Cu(II)
314 reduction.

315 The CV of plankton cells invariably exhibited similar trends but lower values than those
316 observed with the biofilms, under each different Cu(II) concentrations and cathode potentials, in
317 terms of reductive onset potential, reductive peak potential, and reductive peak current (Fig. 2B, F
318 and J). This evidence asserted a higher biofilm catalytic activity in comparison to the plankton cells
319 for Cu(II) reduction, under the adopted circuital current conditions. Similarly, under OCC the
320 biofilms exhibited more positive reductive onset potentials and higher reductive peak currents than
321 the plankton cells (Table 1), in agreement with the higher activity of biofilms over plankton cells for
322 efficient Cu(II) removal in conventional bioreactors (Liu et al., 2018).

323 In addition to the catalytic activity of *S. marcescens* towards simultaneous acetate production
324 under Cu(II) reduction, the CV performed on the EPS released by the biofilms under the metal stress
325 also exhibited an oxidation-reduction peak (Fig. 2C, G and K, Table 1), suggesting that the EPS may
326 also contribute to this cooperative catalytic process. The literature indicate that EPS released by other
327 bacteria under Au(III) or Se(IV) stress may favor the reduction to their metallic state (Kang et al.,
328 2014; 2017). More positive reductive onset potentials and reductive peak potentials, along with
329 higher reductive peak currents were observed in the biofilm-EPS than the plankton-EPS (Fig. 2D, H
330 and L, Table 1), confirming the higher catalytic activities of the biofilm-EPS over the plankton-EPS.
331 The oxidation-reduction peaks for all EPS increased in intensity when the Cu(II) concentration was
332 increased from 60 mg/L to 120 mg/L, indicating an enhancement of the electrochemical activity of
333 all EPS at higher Cu(II) loadings (Fig. 2C, D, G, H, K and L).

334 Here Fig. 2

335 Here Table 1

336 While the presence of an electron acceptor (Cu(II) or bicarbonate) was clearly beneficial for the

electron transfer between Cu(II) and the cathode here, the reason for this beneficial effect is not well established (Nancharaiah et al., 2016). Very recently, Rowe et al (2018) demonstrated that *Shewanella oneidensis* MR-1 immobilized on a cathode acquires energy under an aerobic environment lacking exogenous organic carbon sources for oxygen reduction. This respiratory process has important implications for understanding the physiological behavior of Cu(II)-tolerant *S. marcescens* in the present cathodic energy-limited environments, where the *S. marcescens* might have utilized cathodic electrons and proceeded the occurrence of the cellular conversion of the cathodic electrons to microbially synthesize acetate, despite that direct proofs for such mechanism is still needed.

3.4. Comparison of exoprotein and exopolysaccharide released by the *S. marcescens* biofilms and plankton cells

A higher amount of exoproteins (Fig. 3A) and exopolysaccharides (Fig. 3B) was released by the *S. marcescens* biofilms than the plankton cells at a same cathodic potential (−900 mV), all of which were higher than those measured at −600 mV and under OCC, implying a stimulating effect by the circuit current that promoted the release of EPS from the biofilms. Such stimulating effect has also been observed during the removal of nitrate in mixed cultures, in the absence of Cu(II), where the EPS production of the cathodic biofilms was significantly influenced by the nature of the carbon source and by the circuit current. The maximum EPS was achieved at an appropriate circuit current of 15 mA, rather than 5 mA or 25 mA, using starch as carbon source instead of glucose or HCO_3^- (Huang et al., 2013a). The different productions of exoproteins and exopolysaccharides by the biofilms and the plankton cells might indicate their different roles in extracellular electron transfer, similar to the behavior observed with *Shewanella* biofilms and plankton cells, in the absence of Cu(II) (Carmona-Martinez et al., 2013; TerAvest and Angenent, 2014; Grobblor et al., 2018). Under OCC,

the biofilms also released a higher amount of EPS than the plankton cells (Fig. 3A and B), which was consistent with the behavior of conventional mixed cultures, in the absence of Cu(II) (Sheng et al., 2010).

An increase in Cu(II) concentration up to 80 mg/L invariably enhanced the release of exoproteins (Fig. 3A) and exopolysaccharides (Fig. 3B), and a more negative –900 mV enlarged the tolerance of the *S. marcescens* biofilms to a higher Cu(II) of 120 mg/L, coincident with the release of more exopolysaccharides (Fig. 3B). Thus, the *S. marcescens* biofilms may exert a protective mechanism under increasing Cu(II) stressing conditions and enhanced circuit current, which results in the release of a higher amount of exopolysaccharides than exoproteins. Under OCC, higher Cu(II) concentration invariably diminished the amount of exoproteins released by the biofilms and plankton cells (Fig. 3A), reflecting the importance of circuit current for more exoproteins at higher Cu(II) concentration. This result was similar to the behavior of conventional *Pseudomonas* sp., *Escherichia coli* or anaerobic mixed cultures under metal stressing (Cd(II), Cu(II), Cr(VI), Ag(I) or Au(III)) conditions (Chien et al., 2013; Kang et al., 2014; 2017).

The application of a circuit current stimulated the *S. marcescens* to efficiently metabolize inorganic carbon (–600 V: 70%; –900 mV: 88%), compared to the OCC (21 – 31%) (Fig. 3C). The conversion of inorganic carbon was significantly higher than the values observed with mixed cultures or with an acetate producer *Clostridium ljungdahlii* (4 – 26% at –900 ~ – 700 mV vs. SHE) (Bajracharya et al., 2015). The specific metabolism of the *S. marcescens* towards the production of acetate in this metallurgical MES was further evidenced by the absence of reaction by-products in both the headspace (absence of methane) and in the catholyte (absence of ethanol or oxybutyrate) with or without Cu(II).

Here Fig. 3

3.5. Analysis of fluorescence excitation emission matrix

Three-dimensional EEM fluorescence spectroscopy analysis reported the EPS constituents (Fig. 4) and the associated relative abundances of the components (Fig. S4) released by the *S. marcescens* biofilms and by the plankton cells, in the presence/absence of Cu(II) and under different cathode potentials (circuital currents). The *S. marcescens* biofilms released a higher amount (1283 R.U., Fig. 4A) of protein (tryptophan)-like matters in series II (Ex / Em = 200 – 240 nm / 330 – 350 nm) than the plankton cells (954 R.U., Fig. 4F) in the presence of Cu(II) (80 mg/L) and at a cathode potential of –900 mV. These values were respectively higher than the protein (tryptophan)-like matters released in the absence of Cu(II) (biofilms: 1058 R.U.; plankton cells: 547 R.U.) (Fig. 4B and G). The absolute amounts of protein (tryptophan)-like matters decreased at a less negative cathode potential of –600 mV (biofilms: 1023 R.U.; plankton cells: 793 R.U.) (Fig. 4C and H), and the absence of Cu(II) further decreased these matters in both the biofilms (792 R.U.) and the plankton cells (584 R.U.) (Fig. 4D and I). Similarly, the release of these matters was further suppressed under OCC (biofilms: 610 R.U.; plankton cells: 572 R.U., Fig. 4E and J). These results demonstrated that Cu(II) induced an increase in the release of protein (tryptophan)-like matters, which was more significant in the biofilms, and that circuital current positively affected the release.

In contrast to the previous results, higher amounts of soluble microbial byproduct-like matters in series IV (Ex / Em = 250 – 280 nm / 260 – 380 nm) were released by the *S. marcescens* plankton cells (734 R.U.) (Fig. 4F) than the biofilms (643 R.U.) (Fig. 4A) at the same cathodic potential (–900 mV) and in the presence of Cu(II) (80 mg/L). In the absence of Cu(II), even higher amounts of these matters were released (plankton cells: 869 R.U.; biofilms: 743 R.U.) (Fig. 4B and G), compared to the negligible changes observed under OCC (plankton cells: 696 R.U.; biofilms: 765 R.U.) (Fig. 4E and J). The amount of soluble microbial byproduct-like matters negligibly changed at a less negative cathode potential of –600 mV (plankton cells: 712 R.U.; biofilms: 758 R.U.) (Fig. 4H and C) and in

the absence of Cu(II), the release increased (plankton cells: 889 R.U.; biofilms: 995 R.U.) (Fig. 4I and D). These results demonstrated that Cu(II) induced a decrease in the release of soluble microbial byproduct-like matters, which was more significant in the biofilms. *Shewanella* biofilms reportedly dominate the direct electron transfer, whereas the plankton cells give a minor contribution to extracellular electron transfer through mediators (Carmona-Martinez et al., 2013; TerAvest and Angenent, 2014; Grobblor et al., 2018). Considering the electrochemically active cytochromes in the EPS matrix associated with extracellular electron transfer processes (Xiao et al., 2017), the role played by the soluble microbial byproduct-like matters in series IV in extracellular electron transfer processes, under Cu(II) stressing conditions, cannot be excluded.

Exceptionally high amount of polycarboxylate-type humic acid (fulvic acid in ultraviolet region)-like matters in series III (Ex / Em = 200 – 240 nm / 380 – 500 nm) was released by both the biofilms (763 R.U.) and the plankton cells (651 R.U.), in the presence of Cu(II) and at –900 mV (Fig. 4D and I). The diversity of the components of the EPS released by the *S. marcescens* biofilms and the plankton cells under different Cu(II) stressing and circuital current conditions, may have contributed to the appreciable higher rates of acetate and hydrogen production, and Cu(II) removal in the metallurgical MES (Fig. 1A, B and C).

Here Fig. 4

A good linear relationship ($R^2 > 0.975$) was observed between $\log[(F_0-F)/F]$ and $\log[\text{Cu(II)}]$ (Eq. 1). The Cu(II) binding constants K_b associated with the biofilms and the plankton cells, always followed the same order of peak II > peak III > peak V > peak IV. The highest Cu(II) binding constant was associated with the *S. marcescens* biofilms (Table 2) at the more negative potential of –900 mV and in the presence of Cu(II), suggesting that the EPS produced by the biofilms rather than the plankton cells had stronger complexation capability for Cu(II), consistent with the higher catalytic activities recorded (Fig. 2) and the higher amount of EPS (Fig. 3) released by the biofilms.

In addition, higher values of the binding sites (n) were observed for the biofilms rather than for the plankton cells in all the four peaks. The biofilms also reported lower $\log K_b$ values than the plankton cells under OCC or in the absence of Cu(II), reflecting the stimulating effect of circuital current or of Cu(II) on the release of substances with higher binding capabilities for Cu(II).

Here Table 2

3.6. Endogenous physiological activities

The level of dehydrogenase activity in the biofilms (201.9 ± 20.5 mg/g DW) was found to be significantly higher than in the plankton cells (122.1 ± 6.6 mg/g DW) at a same potential of -600 mV and in the absence of Cu(II) (Fig. 5A), which corroborated with the higher capability that the biofilms possessed for receiving cathodic electrons for hydrogen evolution. The dehydrogenase activity increased on average by 32.3% at more negative potentials (-900 mV) and approached a maximum at a Cu(II) concentration of 80 mg/L (382.4 ± 4.2 mg/g DW), after which it decreased (Fig. 5A). Under OCC, the dehydrogenase activity in both the biofilms and in the plankton cells was significantly lower than in the presence of circuital current, and presented negligible differences, while the activity slightly decreased in the presence of Cu(II).

Compared to the OCC and at a Cu(II) concentration up to 80 mg/L, the presence of increasing levels of circuital current always diminished the activities of CAT (Fig. 5B), SOD (Fig. 5C) and GSH (Fig. 5D) in both the biofilms and the plankton cells, although the response positively correlated with the concentration of Cu(II). Lower CAT, SOD and GSH activities (Fig. 5B – D) in the biofilms than in the plankton cells and negligible changes recorded under OCC, demonstrated a more robust response of the biofilms than the plankton cells to the toxic effect of Cu(II), in the presence of circuital current.

Excessive levels of Cu(II) (-900 mV: 120 mg/L; -600 mV: 80 mg/L) decreased the

dehydrogenase activity in both the biofilms and in the plankton cells (Fig. 5A). At such high concentration, the CAT (Fig. 5B), SOD (Fig. 5C) and GSH (Fig. 5D) activities in the plankton cells was more affected than in the biofilms, although, in most of the cases these activities remained at levels higher than under OCC, particularly at a less negative potential (–600 mV).

The biofilms and the plankton cells exhibited variable endogenous physiological activities of dehydrogenase, CAT, SOD and GSH, along with simultaneous Cu(II) removal and acetate production, illustrating the diverse biochemical responses of the biofilms and the plankton cells to their functional Cu(II) removal and acetate production. The dependence of activities of dehydrogenase, CAT, SOD and GSH in the biofilms and in the plankton cells on Cu(II) concentration and circuital current, further demonstrated the close correlations between simultaneous Cu(II) removal and acetate production, and these physiological activities of the biofilms and the plankton cells in the MESs.

Here Fig. 5

3.7. XPS spectra analysis

XPS analysis of the *S. marcescens* biofilms collected from the cathodes (Fig. 6A – C) and of the plankton cells recovered from the catholyte (Fig. 6D – F), reported the exclusive presence of Cu(0) after the passage of circuital currents at –900 mV (Fig. 6A and D) or at –600 mV potentials (Fig. 6B and E; Table S3), while under OCC only Cu(II) was observed (Fig. 6C and F; Table S3). Therefore, the *S. marcescens* biofilms and the plankton cells reduced Cu(II) to Cu(0) only in the presence of a circuital current (Fig. 6A, B, D and E; Table S3), whereas only chemical adsorption of Cu(II) on the electrodes was observed under OCC (Fig. 6C and F; Table S3).

The amount of Cu(II) reduced to Cu(0) by *S. marcescens* was higher in the biofilms (–900 mV: $72.1 \pm 0.7\%$; –600 mV: $63.1 \pm 1.0\%$) than in the plankton cells (–900 mV: $54.1 \pm 0.6\%$; –600 mV: $42.6 \pm 1.6\%$) (Table S3), corroborating with the higher catalytic activity exerted by the *S. marcescens*

biofilms than the plankton cells for reduction of copper, supporting the results in Figs. 2 – 5.

Collectively, these results demonstrated the *S. marcescens* biofilms were more effective than the plankton cells at reducing Cu(II) to Cu(0), at higher circuital currents.

The valence state of the Cu(II) reduction products is influenced by the initial Cu(II) concentration, the dissolved oxygen concentration, the circuital current, and by the composition, concentration and functional groups of dissolved organics (Tao et al., 2011; Ninh Pham et al., 2012; Wu et al., 2016). The reduction of Cu(II) to Cu(0) in bioelectrochemical systems in the absence of oxygen has been reported in the range of Cu(II) initial concentrations (200 – 6400 mg/L) (Tao et al., 2011; Wu et al., 2016), whereas the reduction of Cu(II) to Cu(I) is favored at increasing concentration of natural organic matter (Suwannee River fulvic acid, Ninh Pham et al., 2012). The detection of Cu(0) only in this study (Fig. 6) and the absence of Cu(I) may be attributed to the low initial Cu(II) concentration used (80 mg/L), despite the uncertainty on the role played by the EPS functional groups. This result also reflects the advantage of this system for producing purer Cu(0) product using low Cu(II) concentrations, in agreement with other studies (Huang et al., 2018; Shen et al., 2017; Ter Heijne et al., 2010; Wu et al., 2015, 2016; Zhang et al., 2015).

Here Fig. 6

3.8. Wider significance and prospects

In this study we demonstrated the feasibility of the Cu(II)-tolerant *S. marcescens* Q1 for production of acetate from inorganic carbon with simultaneous Cu(II) removal/recovery in a metallurgical MES. The use of such MES with tailored *S. marcescens* Q1 may enable new prospects for the efficient conversion of CO₂, from a broad range of sources, to C1 feedstocks, with simultaneous heavy metals removal/recovery from contaminated waters, and thus stress the multiple functions of the Cu(II)-tolerant EAB in the treatment of Cu(II)-contaminated organics-barren wastewaters.

In terms of practical acid mine drainage wastewaters with organics-barren characters (Ter Heijne et al., 2010; Zhang et al., 2015; Wu et al., 2016), this *S. marcescens* catalyzed MESs using HCO_3^- as carbon source, providing a cost-effective and efficient approach for Cu(II) removal, without the requirement of adding external organics. The detection of Cu(0) only in this study and the absence of Cu(I) at a low initial Cu(II) concentration (80 mg/L) demonstrates the advantage of this system for producing purer Cu(0) product using low Cu(II) concentrations.

For the first time, we elucidated the roles of the biofilms and the plankton cells of *S. marcescens* in a metallurgical MES for the production of acetate from inorganic carbon with simultaneous Cu(II) removal/recovery. The release of varying amounts of EPS with a compositional diversity, provides a strategy for controlling the rate of production of acetate and the rate of Cu(II) removal. The amount and constituent species released by *S. marcescens*, in both the biofilms and in the plankton cells, and their physiological activities can be correlated to the applied cathodic potential and to the Cu(II) concentration. The quantitative binding constant in this work expounded the differences in Cu(II) binding features of the individual fluorescent constituents and functional groups of the EPS released. These results provide a more detailed theoretical basis to investigate at the molecular level the interaction mechanism of Cu(II) and other heavy metals with EPS released by *S. marcescens*, which is key for the application of this metallurgical MES for efficient control of the migration and transformation of Cu(II) in polluted sites.

Further studies should focus on the identification of the copper-related genetic regulatory networks in the *S. marcescens* Q1, which govern their function predictions, and to the optimal design of MESs and biocompatibility with the electrode materials, which can increase the system performance. The recovery of the metals (e.g., copper) from the electrodes may be carried out by periodically removing the biomass from the electrodes and using a clarifier (settling tank) to collect the solids. In addition, it is increasingly important to develop and characterize a continuous treatment

processes in order to improve the system efficiency and the large scale performance of such MESs (Venkata Mohan et al., 2016). The scale up of MES from laboratory to a commercially viable process, requires the rates of acetate production and copper reduction to be increased by one order of magnitude (May et al., 2016) and the interference from other water matrix components. Considering the rates of copper reduction achieved in such MESs, after scale-up, it should be possible to treat low-strength, copper-laden acid mine drainage wastewaters with simultaneous formation of acetate and hydrogen by-products. Practical implementation will depend on the long-term operation of the MESs and other characteristics of the wastewaters, as well as the cost of the system relative to conventional treatment processes.

Regarding energy consumption, at least 3.0 kWh is needed per kilogram of acetate production with over 85% of the energy used for acetate separation in conventional MESs, which is higher than that amount required for the industrial production of acetate via carbonylation of methanol (~1.65 kWh/kg acetate/acetic acid) (Zou and He, 2018; Jain and He, 2018). However, the use of green energy sources such as solar energy, as well as electricity from intermittent, stranded, or curtailed power, might be supplied (May et al., 2016), in addition to the CO₂ (eg. inorganic carbon) and Cu(II) from waste streams. The feedstock for this MES could be considered to be a waste product and its valorization would help address the need to store energy and carbon, as well as, to recover copper heavy metal. Further studies on these aspects are warranted.

4. Conclusions

Simultaneous Cu(II) reduction (6.42 ± 0.02 mg/L/h), acetate production (1.13 ± 0.02 mg/L/h) from inorganic carbon, and hydrogen evolution (0.0315 ± 0.0005 m³/m³/d) were achieved in a *Serratia marcescens* Q1 catalyzed MES. The physiological activities of GSH, SOD, CAT and dehydrogenase in the biofilms and the plankton cells were correlated to the rates of Cu(II) removal

and acetate production in the metallurgical MESs. Higher currents and higher Cu(II) concentrations induced the biofilms to release higher amounts of EPS with a higher degree of diversity, which exhibited stronger Cu(II) complexation than the plankton cells. Such understanding should enable a more precise manipulation of the metallurgical MES to favor the production of acetate from CO₂ sequestration and the removal of Cu(II) from organics-barren waters contaminated with heavy metals.

Conflicts of interest

There are no conflicts to declare.

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741 **Table 1** Parameters of CVs on the *S. marcescens* biofilms, plankton cells, biofilms associated EPS,
742 and plankton cells associated EPS at cathode potentials of –600 or –900 mV (vs. SHE), or in the
743 open circuit conditions (OCC) under Cu(II) concentrations of 60 mg/L, 80 mg/L, or 120 mg/L.

744 **Table 2** Comparison of binding constants of Cu(II)-EPS associated with the plankton cells or the
745 biofilms at cathode potentials of –900 mV or –600 mV, or under open circuit conditions (OCC)
746 in the presence or absence of Cu(II).

747 **Fig. 1** Comparison of Cu(II) removal (A), acetate production (B), hydrogen production (C), and
748 circuit current (D) in the *S. marcescens* catalyzed cathodes at various initial Cu(II)
749 concentrations and cathode potentials of –900 mV, 600 mV, or under open circuit conditions
750 (OCC).

751 **Fig. 2** Comparison of CVs of *S. marcescens* biofilm (A, C, E, G, I and K), plankton cells (B, D, F, H,
752 J and L), biofilm EPS only (C, G and K), and plankton EPS only (D, H and L) under potentials of
753 –900 mV (A, B, C and D), –600 mV (E, F, G and H) or open circuit conditions (OCC) (I, J, K
754 and L) at different concentrations of Cu(II) electrolytes.

755 **Fig. 3** Comparison of exoprotein (A) and exopolysaccharide (B) released by the *S. marcescens*
756 biofilms and plankton cells under various initial Cu(II) concentrations or cathode potentials.
757 Distribution of total carbon at cathode potentials of –900 mV or –600 mV, or under open circuit
758 conditions (OCC).

759 **Fig. 4** Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy of
760 extracellular polymer substances produced by the *S. marcescens* biofilms (A, B, C, D and E) or the
761 plankton cells (F, G, H, I and J) at cathode potentials of either –900 mV (vs. standard hydrogen
762 electrode (SHE)) (A, B, F and G) or –600 mV (vs. SHE) (C, D, H and I), or under open circuit
763 conditions (E and J) in the absence of Cu(II) (B, D, G and I) or presence of Cu(II) of 80 mg/L (A,
764 C, E, F, H and J).

765 **Fig. 5** Comparison of physiological activities of dehydrogenase (A), catalase (CAT) (B), superoxide
766 dismutase (SOD) (C), and glutathione transferases (GSH) (D) in the *S. marcescens* biofilms and
767 the plankton cells at potentials of –900 mV or –600 mV, or under open circuit conditions (OCC).

768 **Fig. 6** XPS analysis for Cu element on the *S. marcescens* biofilms (A, B and C) or the plankton cells
769 (D, E and F) at cathode potentials of –900 mV (A and D) or –600 mV (B and E), or under open
770 circuit conditions (OCC) (C and F).

Highlights

- Cu(II)-tolerant *Serratia marcescens* Q1 favors acetate production and Cu(II) removal;
- Circuital current and Cu(II) stress induce biofilms for more EPS than planktonic cells;
- EPS correlates with Cu(II) removal and acetate production;
- Physiological activities correlate with Cu(II) removal and acetate production;
- Biofilms exhibit higher catalytic activities toward the reduction of Cu(II) to Cu(0).

1 **Abstract**

2 Simultaneous Cu(II) reduction (6.42 ± 0.02 mg/L/h), acetate production (1.13 ± 0.02 mg/L/h) from
3 inorganic carbon (i.e., CO₂ sequestration), and hydrogen evolution (0.0315 ± 0.0005 m³/m³/d) were
4 achieved in a *Serratia marcescens* Q1 catalyzed microbial electrosynthesis system (MES). The
5 biofilms released increasing amounts of extracellular polymeric substances (EPS) with a higher
6 compositional diversity and stronger Cu(II) complexation, compared to the plankton cells, at higher
7 Cu(II) concentrations (up to 80 mg/L) and circuital currents (cathodic potential of –900 mV vs.
8 standard hydrogen electrode (SHE)). Moreover, the biofilms reduced Cu(II) to Cu(0) more
9 effectively than the plankton cells. At Cu(II) concentrations below 80 mg/L, the dehydrogenase
10 activity in the biofilms was higher than in the plankton cells, and increased with circuital current,
11 which was converse to the lower activities of catalase (CAT), superoxide dismutase (SOD) and
12 antioxidative glutathione (GSH) in the biofilms than the plankton cells, although all these
13 physiological activities were positively correlated with the concentration of Cu(II). This is the first
14 study that evaluates the EPS constituents and the physiological activities of the biofilms and the
15 plankton cells in the MESs, that favors the production of acetate from CO₂ sequestration and the
16 simultaneous reduction of Cu(II) from organics-barren waters contaminated with heavy metals.

17
18 **Keywords:** microbial electrosynthesis system; Cu-tolerant biofilm and plankton cell; CO₂ fixation;
19 extracellular polymeric substances; physiological activity; variable cathode potential

1. Introduction

Microbial electro-metallurgical processes combining bioelectrochemical systems with metallurgical processes, are emerging and sustainable technologies for the recovery of heavy metals, such as Cu(II), Cr(VI), Co(II) and Cd(II), from wastes and wastewaters (Nancharaiah et al., 2016; Dominguez-Benetton et al., 2018). These systems usually include biocathodes and bioanodes made by pure or mixed immobilized electrogenic bacteria cultures, fed by inorganic species (e.g., bicarbonate, HCO_3^-) in the cathodes and acetate in the anodes (Huang et al., 2015; 2018; Chen et al., 2016). Among the variety of heavy metals that can be recovered using microbial electro-metallurgical processes, the recovery of Cu(II) has attracted significant attention due to its frequent occurrence in acid mine drainage wastewaters (Ter Heijne et al., 2010; Zhang et al., 2015; Wu et al., 2016; Kiran et al., 2018). Likewise abiotic cathodes, biocathodes utilizing electrochemically active bacteria (EAB) have been shown to reduce Cu(II) to metallic Cu(0) and thus remove the heavy metal from the catholyte solution (Huang et al., 2015; 2018; Shen et al., 2017; Tao et al., 2017).

From another perspective, microbial electrosynthesis systems (MESs) are also emerging as potential systems for the synthesis of valuable products from waste streams. One notable example is the production of acetate from inorganic carbon such as bicarbonate, where the production rate of acetate is modulated artificially by the application of an electrical current that interferes with the microbial metabolism (Bajracharya et al., 2015; Venkata Mohan et al., 2016; Rojas et al., 2018). Therefore, the combination of microbial electro-metallurgical processes of Cu(II) recovery with MESs, in a single process, presents an attractive opportunity to achieve simultaneously the sequestration of CO_2 , its conversion to C1 chemicals (e.g., acetate) and the removal/recovery of Cu(II) from organics-barren waters contaminated with metals.

The production of C1 and other products in such combined process might be influenced by the

49 Cu(II) stressing conditions and modulated by the application of an external circuital current. Wide
50 groups of gram-negative bacteria, under the harmful effect of toxic substances, can adopt special
51 metabolic pathways as a protective mechanism, which may result in the release complex
52 heterogeneous extracellular polymeric substances (EPS) composed of variable degrees of saccharides
53 and proteins (Sheng et al., 2010; Cao et al., 2011; Zhou et al., 2017). In general, exoproteins in EPS
54 are more common than exopolysaccharides since, due to the higher content of negatively charged
55 amino acids, exoproteins can form electrostatic bonds with positively charged metal ions, whereas
56 higher carbohydrate in EPS released by *Shewanella* sp. offers protection against excessive
57 concentrations of Fe(II) and Mn(II) (Cao et al., 2011). Another important aspect is the bacterial
58 source of the EPS. In general, bacterial cells forming biofilms normally produce higher quantity of
59 EPS, as compared to plankton cells in suspension in conventional biological processes (Sheng et al.,
60 2010; Cao et al., 2011). Biofilms and plankton cells also play different roles in electron transfer
61 processes, with the former dominating direct electron transfer while the latter contributing to
62 extracellular electron transfer through mediators (Carmona-Martinez et al., 2013; TerAvest and
63 Angenent, 2014; Grobblor et al., 2018). Thus, several factors are needed to be evaluated to unravel
64 the roles played by the biofilms and the plankton cells in MESs in the removal of Cu(II) with
65 simultaneous sequestration of CO₂, including: (i) the evaluation of the EPS constituent species in
66 both biofilms and plankton cells; (ii) the determination of the binding constants of the these species
67 with the metal ions (Lakowicz, 2006); (iii) the evaluation of the modulation factor of an applied
68 cathodic potential (i.e., a circuital current) on the nature, composition and amount of EPS released by
69 both biofilms and plankton cells; (iv) the determination of the intracellular physiological activities of
70 the EAB biofilms and the plankton cells in the presence of Cu(II) and as a function of the circuital
71 current. The bacterial activities of antioxidative glutathione (GSH), catalase (CAT) and superoxide
72 dismutase (SOD) can function as important metabolic indicators for either copper sequestration or

for scavenging $O_2^{\cdot-}$ induced by Cu(II) (Nguyen et al., 2013) whereas the dehydrogenase activity usually indicates the biocatalytic ability for hydrogen evolution (Venkateswar Reddy et al., 2010).

In this study, efficient removal of Cu(II) and the simultaneous production of acetate from bicarbonate was investigated in a metallurgical MES using *Serratia marcescens* Q1 EAB isolated from a well-adapted biocathode utilized in a bioelectrochemical system to remove Cu(II) (Huang et al., 2015). The impact of cathode potential and Cu(II) concentration on the amount and composition of EPS released by both the biofilms and the plankton cells was investigated. This was quantified by the evaluation of the binding constant of Cu(II) with the EPS constituent species. In addition, the endogenous physiological activities of GSH, SOD, CAT and dehydrogenase in both the biofilms and the plankton cells were examined to determine their roles in Cu(II) removal and acetate production.

2. Materials and methods

2.1. Sampling and EAB isolation

EAB was isolated from the biocathodes of bioelectrochemical systems (Huang et al., 2015) fed with Cu(II) ions of 20 mg/L by streaking cathode scrapings onto agar plates containing an anaerobic medium amended with agar, Cu(II) (20 mg/L) and $NaHCO_3$ (10 mg/L), in an anaerobic glove box (Xinmiao, Shanghai). The composition of the anaerobic medium, the incubation conditions of the agar plates and the bacterial isolation procedure were as described previously (Shen et al., 2017; Tao et al., 2017; Hou et al., 2018).

2.2. Isolates identification

The bacterial DNA was extracted using the Ezup DNA kit (cat. No. SK8255, Sangon Biotech (Shanghai) Co. Ltd., China) according to the manufacturer's instructions. Primers 518F and 1540R were used to amplify the bacterial 16S rDNA. PCR solution components, amplification, purification,

clone procedure and sequencing were as previously described (Shen et al., 2017; Tao et al., 2017).

The 16S rDNA gene sequence was compared with those available in GenBank by using the BLAST program to identify the most similar 16S rDNA fragments.

2.3. EAB inoculation and reactor operation

The bacteria were harvested after 24 h incubation in an anaerobic and sterile medium (pH = 5.8), as previously described (Shen et al., 2017; Tao et al., 2017). The bacterial cells were then concentrated and washed 3 times with a sterile physiological saline solution. The catholyte composed of NH_4HCO_3 (0.08 g/L), KHCO_3 (0.10 g/L), KH_2PO_4 (0.012 g/L); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2.0 mg/L), vitamins 0.6 mL/L and mineral 0.6 mL/L and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, was firstly sparged with N_2 gas for 15 min and then slowly inoculated with the isolates (10^8 CFU/mL) in an anaerobic glovebox (YQX-II, Xinmiao, Shanghai) (Huang et al., 2010).

Typical Cu(II) concentration in acid mine drainage wastewaters may vary over a wide range, from 10 to 1000 mg/L (Bilal et al., 2013), which makes difficulty to select a narrow range of Cu(II) concentration to be used in this study. In contrast, typical Cu(II) concentrations in biocathodes of bioelectrochemical systems (1 – 20 mg/L) (Huang et al., 2015; Shen et al., 2017; Tao et al., 2017) and in conventional biological processes (40 – 150 mg/L) (De Alencar et al., 2017), are normally in the lower range, as a result of the potential toxicity exerted by Cu(II) to the bacteria. Therefore, unless otherwise stated, in this study the initial concentration of Cu(II) in the catholyte was varied as 60, 80 and 120 mg/L. The anolyte was a buffer solution composed of NaH_2PO_4 - NaHPO_4 . The initial pH and conductivity of the electrolyte solutions in both chambers were fixed values equal to 5.8 and 5.8 mS/cm, respectively (Huang et al., 2015). The catholyte pH equal to 5.8 was chosen considering several factors including that pHs less than 6.0 are common in practical acid mine drainage wastewaters, that pHs in the range 3.0 – 6.0 are used in conventional bacterial removal of Cu(II)

(Bilal et al., 2013), and that this pH value has previously been used for removal of Cu(II) in other bioelectrochemical systems (Huang et al., 2015; Shen et al., 2017; Tao et al., 2017). The pH of the catholyte was not controlled during the experiments and only increased to 6.0 - 6.3 at the end of each experiment.

Graphite felt ($1.0 \times 1.0 \times 1.0$ cm, 6 pieces) was used as the cathode due to its large porosity and good affinity with the EAB (Huang et al., 2013b; 2013c; Shen et al., 2017), whereas a carbon rod was used as the anode. The anodic and cathodic chambers were separated by a cation exchange membrane (Ultrex CMI-7000, Membranes International, Glen Rock, NJ) since preliminary results showed similar performance to the use of anion exchange membrane, particularly at such low Cu(II) concentrations and short operational times (see Supporting Information and Fig. S1), besides avoiding transfer of anions (eg. acetate and phosphate groups) (Shen et al., 2017; Tao et al., 2017). A constant cathodic potential of -900 mV or -600 mV versus standard hydrogen electrode (SHE) (VSP, BioLogic) were applied to the reactors and the circuital current was automatically monitored. The choice of these cathodic potentials were based on the reported range of cathodic potentials of $-1100 \sim -600$ mV (Mohanakrishna et al., 2016). The reactors were operated in fed-batch mode at room temperature (25 ± 3 °C) for 12 h, unless otherwise stated. Such time course was adequate to monitor the conversion of inorganic carbon (HCO_3^-) and the production of acetate (Fig. S2). Three duplicate reactors were used in all experiments.

Three reactors types were used as controls. The first was operated without the inoculum (abiotic control). The second was inoculated but operated under open circuit conditions (OCC), to examine acetate production and Cu(II) removal in the absence of a circuital current and to confirm that hydrogen production was only produced in the presence of a circuital current (CCC). The third was operated in the absence of Cu(II) to monitor the variation of the production rates of acetate, hydrogen, exoproteins and exopolysaccharides from the system.

2.4. EPS extraction, fluorescence spectroscopy and quenching titration

EPS extraction was performed at the end of each 12 h experiment. Dissolved EPS was extracted as previously reported (Kang et al., 2014). Briefly, the catholyte solution and the graphite felt cathode electrode were carefully sampled from each reactor. The electrode was then submerged into a physiological saline solution (10 mL) and sonicated on ice, three times, for 10 s intervals at an intensity of $1.0 \text{ W}\cdot\text{cm}^2$ and a frequency of 20 kHz (Branson Sonifier 250, Danbury, USA) to extract the biofilms. The electrode was removed from the solution, rinsed with an additional 3 mL of physiological saline solution and discarded. The biofilm solution formed and the catholyte solution sampled from the reactor were separately sonicated at an intensity of $2.7 \text{ W}\cdot\text{cm}^2$ and a frequency of 40 kHz (Branson Sonifier 250, Danbury, USA) on ice for 10 min and samples were immediately centrifuged at $11000 \times g$ for 20 min at 4 °C. The precipitates were separately collected and used to determine the endogenous physiological activities of the biofilms and of the plankton cells, as described in Section 2.5, whereas the supernatants were filtered through a 0.22 μm acetate cellulose membrane. Negligible amount of nucleic acid was observed in both filtrate solutions, implying that cell lysis did not take place during the extraction of EPS in both the biofilms and the plankton cells (Guo and Chang, 2017). The supernatants obtained were separately fixed to a final volume of 15 mL and operationally defined as biofilms loosely associated EPS (biofilm-EPS) and plankton cells loosely associated EPS (plankton-EPS), respectively (Sheng et al., 2010; Kang et al., 2014).

The biofilm-EPS and the plankton-EPS, together with the biofilm coated electrode and the associated plankton cells in the catholyte were analyzed by CV, as described in section 2.6.

In parallel, the exopolysaccharides in the biofilm-EPS and the plankton-EPS were measured by phenol-sulfuric acid method (Suzanne Nielsen, 2009) whereas exoproteins were quantified by the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a calibration standard.

169 Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy performed on
170 F-7000 (Hitachi, Japan) equipped with 1.0 cm quartz cell and a thermostat bath was used to quantify
171 and characterize the EPS. The EEM spectra were collected with subsequent scanning emission
172 spectra by varying the excitation wavelengths from 200 nm to 450 nm in 10 nm increments, and the
173 emission spectra from 250 nm to 500 nm at 0.5 nm increments. The width of the excitation/emission
174 slit was set to 5.0 nm, and the scanning speed was set to 1200 nm/min for all the fluorescence
175 measurements. The fluorometer's response to a milli-Q water was subtracted from the fluorescence
176 spectra recorded for EPS. EEM data were processed using the software Sigma plot 10.0 (Systat,
177 USA).

178 Prior to the EEM data analysis, the Raman and Rayleigh scatter was removed according to the
179 protocol of Bahram et al. (2006). The EEMs were then normalized to the area under the Milli-Q
180 water Raman peak (Ex = 350 nm) collected on the same day (Lawaetz and Stedmon, 2009), to
181 produce corrected fluorescence intensities in Raman Units (R.U.). Five regions (Regions I, II, III, IV
182 and V) in each EEM were based largely upon supporting literature, as summarized by Chen et al.
183 (2003). Briefly, peaks at shorter excitation wavelengths (< 250 nm) and shorter emission
184 wavelengths (< 350 nm) related to simple aromatic proteins such as tyrosine (Regions I and II).
185 Peaks at intermediate excitation wavelengths (250 ~ 280 nm) and shorter emission wavelength (260
186 ~ 380 nm) related to soluble microbial byproduct-like material (Region IV). Peaks at longer
187 excitation wavelengths (> 280 nm) and longer emission wavelengths (> 380 nm) related to humic
188 acid-like organics (Region V). Peaks at shorter excitation wavelengths (< 250 nm) and longer
189 emission wavelengths (> 350 nm) related to fulvic acid-like materials (Region III). The relative
190 abundance of each component was then calculated as the integration beneath EEMs within each
191 specific selected region, divided by the sum of the entire region.

192 The interaction mechanism between Cu(II) and EPS was determined by fluorescence quenching

titration. 1.0 mL EPS and different volumes of pre-determined Cu(II) solution were successively added into a 5.0 mL centrifuge tube. The final Cu(II) concentration in the mixed solution was varied from 0 to 160 mg/L. After each addition of Cu(II), the solution was fully mixed using a magnetic stirrer for 15 min and the fluorescence spectra were recorded. The equilibrium time of 15 min produced steady fluorescence intensity peaks. The EEM data analysis and region division were carried out as mentioned above.

The binding constants ($\log K_b$), revealing the interaction between the small Cu(II) and the macromolecules of EPS, were calculated from (Lakowicz, 2006):

$$\log[(F_0 - F)/F] = \log K_b + n \times \log[\text{Cu(II)}] \quad (1)$$

where F_0 and F are the fluorescence intensities measured in the absence and in the presence of Cu(II), respectively. K_b is the binding constant, n is the number of binding sites and $[\text{Cu(II)}]$ is the Cu(II) concentration.

2.5. Endogenous physiological activities

The *S. marcescens* biofilms and the plankton cells were re-suspended in a physiological saline solution and ultrasonically disrupted three times at 20 kHz for 5 min, respectively (Sonics Vibra-CellTM). After centrifugation for 10 min at $10000 \times g$ at 4 °C, the supernatant obtained was either immediately assessed or frozen at –80 °C for subsequent analysis to determine the dehydrogenase, SOD, CAT and GSH activities. The dehydrogenase activity was estimated as previously described (Venkateswar Reddy et al., 2010). The methods for SOD and CAT activities were referred to the description by Branco et al. (2010). The GSH content was quantified as described by Rahman et al. (2006). Results expressed were based on per gram cell dry weight (mg/g DW). The procedure was repeated at least three times.

2.6. Cycle voltammetry analysis and characterization

The redox behaviors of the *S. marcescens* cathode, the catholyte with the plankton cells, the biofilm-EPS and the plankton-EPS were analyzed by cycle voltammetry (CV) (VSP, BioLogic). The potential was scanned between -1.0 and $+0.6$ V (vs. SHE) at a scan rate of 0.1 mV/s using a standard three-electrode arrangement including the biocathode as the working electrode, a platinum plate as the counter electrode, and a saturated calomel electrode (-0.241 V vs SHE) as the reference electrode (Huang et al., 2012).

The elemental composition of the copper adsorbed on the surface of the *S. marcescens* biofilms and the plankton cells was analyzed by X-ray photoelectron spectroscopy (XPS; Kratos AXIS Ultra DLD). For these determinations, the cathodes loaded with *S. marcescens* and the catholyte with plankton cells were operated at a Cu(II) of 80 mg/L for 7 cycles to allow the preparation of a sufficient amount of samples for XPS analysis.

The concentration of Cu(II) in catholyte was determined by atomic absorption spectroscopy (AAAnalyst 700, PerkinElmer). Removal of Cu(II) (mg/L/h) at the end of one batch cycle operation was calculated based on the decreased Cu(II) in the catholyte divided by operation time. Accordingly, the production of acetate (mg/L/h) in the reactors was quantified using a high performance liquid chromatograph (HPLC, Agilent 1100) and divided by operation time (Huang et al., 2014), and the production of hydrogen ($\text{m}^3/\text{m}^3/\text{d}$) was determined from the volume recovered from the headspace of the cathodic chamber, normalized by the volume of the catholyte (Huang et al., 2014; Chen et al., 2016). The biomass immobilized on the cathode surfaces and suspended in the catholyte was determined as previously described (Wei et al., 2010).

The coulombic efficiencies for Cu(II) reduction (CE_{Cu}), production of acetate (CE_{acetate}) and hydrogen evolution (CE_{H_2}) were calculated as the ratios between the measured charge transferred through the external circuit, and the charge recovered for Cu(II) reduction, acetate production and

hydrogen evolution, respectively, similarly to other studies (Zou and He, 2018; Jain and He, 2018).

One-way ANOVA in SPSS 19.0 was used to analyze the statistical variation of the data, and all of the data indicated significance levels of $p < 0.05$.

3. Results and Discussion

3.1. Bacteria isolation

One rod-shaped, gram-negative and major opportunistic to facultative strain was successfully isolated from a bacterial community amended with Cu(II). This strain is able to metabolize multifarious organic compounds and inorganic carbonate, allowing its switching between autotrophic and heterotrophic cultures. This Q1 strain matched (100%) the genetic sequence of *Serratia marcescens*, which belongs to the largest group of *Proteobacteria* bacteria, exhibiting rich metabolic diversity, and robust survival in heavy metal stressing environments (Ntougias et al., 2006). *S. marcescens* has been investigated for the removal of a wide range of heavy metals including Cr, Pb, Cd, Ni and Hg (Kannan et al., 2002; Cristani et al., 2012). This strain has also recently been observed to secrete urease during Cd(II) removal (Bhattacharya et al., 2018). This opportunistic *S. marcescens* Q1 thriving in autotrophic and heterotrophic cultures, was unsurprisingly similar to many other CO₂ fixation bacteria including *Metallosphaera yellowstonensis*, *Stenotrophomonas* sp. WH-11, *Ochrobactrum* sp. WH-13, *Castellaniella* sp. WH-14, and *Sinomicrobium oceani* WH-15 (Jennings et al., 2014; Hu et al., 2015).

3.2. Cu(II) removal and acetate production

At an initial Cu(II) of 80 mg/L, higher Cu(II) removal rate (6.42 ± 0.02 mg/L/h) (Fig. 1A), acetate production rate (1.13 ± 0.02 mg/L/h) (Fig. 1B), hydrogen evolution rate (0.0315 ± 0.0005 m³/m³/d) (Fig. 1C), circuital currents (Fig. 1D), CE_{acetate} and CE_{H_2} (Table S1) were achieved at a

cathode potential of –900 mV, rather than at –600 mV. These results were explained by the availability of more cathodic electrons at –900 mV than –600 mV for hydrogen evolution, acetate production and Cu(II) removal. The application of both potentials obtained invariably values higher than the abiotic and the OCC controls (Fig. 1A and B), asserting the ability of *S. marcescens* species for the simultaneous removal of Cu(II) and production of acetate from bicarbonate in MES. The removal of Cu(II) and the production of acetate invariably increased with the increase in initial Cu(II) concentration up to 80 mg/L, implying the stimulating role of copper stressing conditions in inducing the *S. marcescens* physiological metabolism to produce acetate. This effect was probably similar to the role played by copper-coated cathodes in enhancing the production of methane in bioelectrochemical, anaerobic digestion, sequencing batch reactors (Park et al., 2018). However, the production of acetate decreased at excessive Cu(II) concentration (120 mg/L) (Fig. 1B), whereas the removal of Cu(II) continued to increase (Fig. 1A), implying a change in regulating mechanism at such high Cu(II) stressing conditions.

Here Fig. 1

The robustness of *S. marcescens* sp. for reducing Cu(II) was quantified by the highest Cu(II) removal rate (6.42 mg/L/h at a Cu(II) of 80 mg/L) measured in this study, which was appreciably higher than the values (3.61 – 3.64 mg/L/h) reported with *Pseudomonas aeruginosa* JY5 or *Stenotrophomonas* sp. JY6 (Shen et al., 2017), or the values (1.0 – 1.1 mg/L/h) reported with mixed cultures (Huang et al., 2015; Wu et al., 2018) at Cu(II) of 5 – 20 mg/L (Table S2). The Cu(II) removal rate was also within the wider range (0.1 – 69.1 mg/L/h) reported using abiotic cathodes (Table S2). The corresponding production rate of acetate (1.13 mg/L/h at –900 mV) was also significantly higher than the values (0.28 – 0.30 mg/L/h) reported with *Geobacter sulfurreducens* at –600 mV (vs. SHE), and within the wider range (0.05 – 8.2 mg/L/h) reported with *Sporomusa ovata*, *Clostridium ljungdahlii*, *Acetoanaerobium* or *Moorella* at potentials from –0.7 to –0.4 V (vs. SHE) in

the absence of Cu(II) (Nevin et al., 2011; Bajracharya et al., 2015; Jourdin et al., 2016). The acetate production rate was also higher than the rates (0.07 – 0.36 mg/L/h) measured with pure cultures of *Stenotrophomonas* sp. WH-11, *Ochrobactrum* sp. WH-13, *Castellaniella* sp. WH-14, *Sinomicrobium oceani* WH-15 or *Metallosphaera yellowstonensis*, and the rates with mixed cultures (0.78 – 5.93 mg/L/h) in the absence of Cu(II) (Jennings et al., 2014; Hu et al., 2015; Mohanakrishna et al., 2016; 2018). However, it was significantly lower than the rate in a mixed culture (66 mg/L/h) in the absence of Cu(II) (Rojas et al., 2018). The production rate of hydrogen (0.032 m³/m³/d) was significantly lower than the 0.31 – 2.64 m³/m³/d by *G. sulfurreducens* or *Methanosarcina barkeri* at –700 – –600 mV (Nevin et al., 2011; Bajracharya et al., 2015; Jourdin et al., 2016), suggesting a weak performance of this Cu-tolerant *S. marcescens* as a hydrogen producer, although hydrogen is a well-known energy vector for acetate production from CO₂ (Bajracharya et al., 2015; Venkata Mohan et al., 2016). These results collectively demonstrated the robust conditions of the Cu(II)-tolerant *S. marcescens* in this MES for simultaneous Cu(II) removal and acetate production from CO₂, although hydrogen can only be considered an insignificant by-product.

3.3. CV analysis

CV analysis of the *S. marcescens* biofilms showed no oxidation-reduction peaks in the absence of Cu(II) (Fig. 2). However, one strong oxidation-reduction peak appeared on the *S. marcescens* biofilms after spiking with 60 mg/L Cu(II), shown as reductive onset potentials of 0.34 V (vs. SHE) at –900 mV (Fig. 2A and Table 1) and 0.32 V (vs. SHE) at –600 mV (Fig. 2E and Table 1). Both peaks were more positive than 0.27 V (vs. SHE) in the abiotic controls (Fig. S3 and Table 1) and 0.30 V (vs. SHE) under OCC, at the same Cu(II) concentration (Fig. 2I and Table 1).

The highest Cu(II) concentration (120 mg/L) at –900 mV achieved the most positive reductive onset potentials and reductive peak potentials, as well as the highest reductive peak currents (Fig. 2A

313 and Table 1), demonstrating the strong catalytic activity of *S. marcescens* biofilms toward Cu(II)
314 reduction.

315 The CV of plankton cells invariably exhibited similar trends but lower values than those
316 observed with the biofilms, under each different Cu(II) concentrations and cathode potentials, in
317 terms of reductive onset potential, reductive peak potential, and reductive peak current (Fig. 2B, F
318 and J). This evidence asserted a higher biofilm catalytic activity in comparison to the plankton cells
319 for Cu(II) reduction, under the adopted circuital current conditions. Similarly, under OCC the
320 biofilms exhibited more positive reductive onset potentials and higher reductive peak currents than
321 the plankton cells (Table 1), in agreement with the higher activity of biofilms over plankton cells for
322 efficient Cu(II) removal in conventional bioreactors (Liu et al., 2018).

323 In addition to the catalytic activity of *S. marcescens* towards simultaneous acetate production
324 under Cu(II) reduction, the CV performed on the EPS released by the biofilms under the metal stress
325 also exhibited an oxidation-reduction peak (Fig. 2C, G and K, Table 1), suggesting that the EPS may
326 also contribute to this cooperative catalytic process. The literature indicate that EPS released by other
327 bacteria under Au(III) or Se(IV) stress may favor the reduction to their metallic state (Kang et al.,
328 2014; 2017). More positive reductive onset potentials and reductive peak potentials, along with
329 higher reductive peak currents were observed in the biofilm-EPS than the plankton-EPS (Fig. 2D, H
330 and L, Table 1), confirming the higher catalytic activities of the biofilm-EPS over the plankton-EPS.
331 The oxidation-reduction peaks for all EPS increased in intensity when the Cu(II) concentration was
332 increased from 60 mg/L to 120 mg/L, indicating an enhancement of the electrochemical activity of
333 all EPS at higher Cu(II) loadings (Fig. 2C, D, G, H, K and L).

334 Here Fig. 2

335 Here Table 1

336 While the presence of an electron acceptor (Cu(II) or bicarbonate) was clearly beneficial for the

electron transfer between Cu(II) and the cathode here, the reason for this beneficial effect is not well established (Nancharaiah et al., 2016). Very recently, Rowe et al (2018) demonstrated that *Shewanella oneidensis* MR-1 immobilized on a cathode acquires energy under an aerobic environment lacking exogenous organic carbon sources for oxygen reduction. This respiratory process has important implications for understanding the physiological behavior of Cu(II)-tolerant *S. marcescens* in the present cathodic energy-limited environments, where the *S. marcescens* might have utilized cathodic electrons and proceeded the occurrence of the cellular conversion of the cathodic electrons to microbially synthesize acetate, despite that direct proofs for such mechanism is still needed.

3.4. Comparison of exoprotein and exopolysaccharide released by the *S. marcescens* biofilms and plankton cells

A higher amount of exoproteins (Fig. 3A) and exopolysaccharides (Fig. 3B) was released by the *S. marcescens* biofilms than the plankton cells at a same cathodic potential (−900 mV), all of which were higher than those measured at −600 mV and under OCC, implying a stimulating effect by the circuital current that promoted the release of EPS from the biofilms. Such stimulating effect has also been observed during the removal of nitrate in mixed cultures, in the absence of Cu(II), where the EPS production of the cathodic biofilms was significantly influenced by the nature of the carbon source and by the circuital current. The maximum EPS was achieved at an appropriate circuital current of 15 mA, rather than 5 mA or 25 mA, using starch as carbon source instead of glucose or HCO_3^- (Huang et al., 2013a). The different productions of exoproteins and exopolysaccharides by the biofilms and the plankton cells might indicate their different roles in extracellular electron transfer, similar to the behavior observed with *Shewanella* biofilms and plankton cells, in the absence of Cu(II) (Carmona-Martinez et al., 2013; TerAvest and Angenent, 2014; Grobblor et al., 2018). Under OCC,

the biofilms also released a higher amount of EPS than the plankton cells (Fig. 3A and B), which was consistent with the behavior of conventional mixed cultures, in the absence of Cu(II) (Sheng et al., 2010).

An increase in Cu(II) concentration up to 80 mg/L invariably enhanced the release of exoproteins (Fig. 3A) and exopolysaccharides (Fig. 3B), and a more negative –900 mV enlarged the tolerance of the *S. marcescens* biofilms to a higher Cu(II) of 120 mg/L, coincident with the release of more exopolysaccharides (Fig. 3B). Thus, the *S. marcescens* biofilms may exert a protective mechanism under increasing Cu(II) stressing conditions and enhanced circuit current, which results in the release of a higher amount of exopolysaccharides than exoproteins. Under OCC, higher Cu(II) concentration invariably diminished the amount of exoproteins released by the biofilms and plankton cells (Fig. 3A), reflecting the importance of circuit current for more exoproteins at higher Cu(II) concentration. This result was similar to the behavior of conventional *Pseudomonas* sp., *Escherichia coli* or anaerobic mixed cultures under metal stressing (Cd(II), Cu(II), Cr(VI), Ag(I) or Au(III)) conditions (Chien et al., 2013; Kang et al., 2014; 2017).

The application of a circuit current stimulated the *S. marcescens* to efficiently metabolize inorganic carbon (–600 V: 70%; –900 mV: 88%), compared to the OCC (21 – 31%) (Fig. 3C). The conversion of inorganic carbon was significantly higher than the values observed with mixed cultures or with an acetate producer *Clostridium ljungdahlii* (4 – 26% at –900 ~ –700 mV vs. SHE) (Bajracharya et al., 2015). The specific metabolism of the *S. marcescens* towards the production of acetate in this metallurgical MES was further evidenced by the absence of reaction by-products in both the headspace (absence of methane) and in the catholyte (absence of ethanol or oxybutyrate) with or without Cu(II).

Here Fig. 3

3.5. Analysis of fluorescence excitation emission matrix

Three-dimensional EEM fluorescence spectroscopy analysis reported the EPS constituents (Fig. 4) and the associated relative abundances of the components (Fig. S4) released by the *S. marcescens* biofilms and by the plankton cells, in the presence/absence of Cu(II) and under different cathode potentials (circuital currents). The *S. marcescens* biofilms released a higher amount (1283 R.U., Fig. 4A) of protein (tryptophan)-like matters in series II (Ex / Em = 200 – 240 nm / 330 – 350 nm) than the plankton cells (954 R.U., Fig. 4F) in the presence of Cu(II) (80 mg/L) and at a cathode potential of –900 mV. These values were respectively higher than the protein (tryptophan)-like matters released in the absence of Cu(II) (biofilms: 1058 R.U.; plankton cells: 547 R.U.) (Fig. 4B and G). The absolute amounts of protein (tryptophan)-like matters decreased at a less negative cathode potential of –600 mV (biofilms: 1023 R.U.; plankton cells: 793 R.U.) (Fig. 4C and H), and the absence of Cu(II) further decreased these matters in both the biofilms (792 R.U.) and the plankton cells (584 R.U.) (Fig. 4D and I). Similarly, the release of these matters was further suppressed under OCC (biofilms: 610 R.U.; plankton cells: 572 R.U., Fig. 4E and J). These results demonstrated that Cu(II) induced an increase in the release of protein (tryptophan)-like matters, which was more significant in the biofilms, and that circuital current positively affected the release.

In contrast to the previous results, higher amounts of soluble microbial byproduct-like matters in series IV (Ex / Em = 250 – 280 nm / 260 – 380 nm) were released by the *S. marcescens* plankton cells (734 R.U.) (Fig. 4F) than the biofilms (643 R.U.) (Fig. 4A) at the same cathodic potential (–900 mV) and in the presence of Cu(II) (80 mg/L). In the absence of Cu(II), even higher amounts of these matters were released (plankton cells: 869 R.U.; biofilms: 743 R.U.) (Fig. 4B and G), compared to the negligible changes observed under OCC (plankton cells: 696 R.U.; biofilms: 765 R.U.) (Fig. 4E and J). The amount of soluble microbial byproduct-like matters negligibly changed at a less negative cathode potential of –600 mV (plankton cells: 712 R.U.; biofilms: 758 R.U.) (Fig. 4H and C) and in

the absence of Cu(II), the release increased (plankton cells: 889 R.U.; biofilms: 995 R.U.) (Fig. 4I and D). These results demonstrated that Cu(II) induced a decrease in the release of soluble microbial byproduct-like matters, which was more significant in the biofilms. *Shewanella* biofilms reportedly dominate the direct electron transfer, whereas the plankton cells give a minor contribution to extracellular electron transfer through mediators (Carmona-Martinez et al., 2013; TerAvest and Angenent, 2014; Grobblor et al., 2018). Considering the electrochemically active cytochromes in the EPS matrix associated with extracellular electron transfer processes (Xiao et al., 2017), the role played by the soluble microbial byproduct-like matters in series IV in extracellular electron transfer processes, under Cu(II) stressing conditions, cannot be excluded.

Exceptionally high amount of polycarboxylate-type humic acid (fulvic acid in ultraviolet region)-like matters in series III (Ex / Em = 200 – 240 nm / 380 – 500 nm) was released by both the biofilms (763 R.U.) and the plankton cells (651 R.U.), in the presence of Cu(II) and at –900 mV (Fig. 4D and I). The diversity of the components of the EPS released by the *S. marcescens* biofilms and the plankton cells under different Cu(II) stressing and circuital current conditions, may have contributed to the appreciable higher rates of acetate and hydrogen production, and Cu(II) removal in the metallurgical MES (Fig. 1A, B and C).

Here Fig. 4

A good linear relationship ($R^2 > 0.975$) was observed between $\log[(F_0-F)/F]$ and $\log[\text{Cu(II)}]$ (Eq. 1). The Cu(II) binding constants K_b associated with the biofilms and the plankton cells, always followed the same order of peak II > peak III > peak V > peak IV. The highest Cu(II) binding constant was associated with the *S. marcescens* biofilms (Table 2) at the more negative potential of –900 mV and in the presence of Cu(II), suggesting that the EPS produced by the biofilms rather than the plankton cells had stronger complexation capability for Cu(II), consistent with the higher catalytic activities recorded (Fig. 2) and the higher amount of EPS (Fig. 3) released by the biofilms.

In addition, higher values of the binding sites (n) were observed for the biofilms rather than for the plankton cells in all the four peaks. The biofilms also reported lower $\log K_b$ values than the plankton cells under OCC or in the absence of Cu(II), reflecting the stimulating effect of circuital current or of Cu(II) on the release of substances with higher binding capabilities for Cu(II).

Here Table 2

3.6. Endogenous physiological activities

The level of dehydrogenase activity in the biofilms (201.9 ± 20.5 mg/g DW) was found to be significantly higher than in the plankton cells (122.1 ± 6.6 mg/g DW) at a same potential of -600 mV and in the absence of Cu(II) (Fig. 5A), which corroborated with the higher capability that the biofilms possessed for receiving cathodic electrons for hydrogen evolution. The dehydrogenase activity increased on average by 32.3% at more negative potentials (-900 mV) and approached a maximum at a Cu(II) concentration of 80 mg/L (382.4 ± 4.2 mg/g DW), after which it decreased (Fig. 5A). Under OCC, the dehydrogenase activity in both the biofilms and in the plankton cells was significantly lower than in the presence of circuital current, and presented negligible differences, while the activity slightly decreased in the presence of Cu(II).

Compared to the OCC and at a Cu(II) concentration up to 80 mg/L, the presence of increasing levels of circuital current always diminished the activities of CAT (Fig. 5B), SOD (Fig. 5C) and GSH (Fig. 5D) in both the biofilms and the plankton cells, although the response positively correlated with the concentration of Cu(II). Lower CAT, SOD and GSH activities (Fig. 5B – D) in the biofilms than in the plankton cells and negligible changes recorded under OCC, demonstrated a more robust response of the biofilms than the plankton cells to the toxic effect of Cu(II), in the presence of circuital current.

Excessive levels of Cu(II) (-900 mV: 120 mg/L; -600 mV: 80 mg/L) decreased the

dehydrogenase activity in both the biofilms and in the plankton cells (Fig. 5A). At such high concentration, the CAT (Fig. 5B), SOD (Fig. 5C) and GSH (Fig. 5D) activities in the plankton cells was more affected than in the biofilms, although, in most of the cases these activities remained at levels higher than under OCC, particularly at a less negative potential (–600 mV).

The biofilms and the plankton cells exhibited variable endogenous physiological activities of dehydrogenase, CAT, SOD and GSH, along with simultaneous Cu(II) removal and acetate production, illustrating the diverse biochemical responses of the biofilms and the plankton cells to their functional Cu(II) removal and acetate production. The dependence of activities of dehydrogenase, CAT, SOD and GSH in the biofilms and in the plankton cells on Cu(II) concentration and circuital current, further demonstrated the close correlations between simultaneous Cu(II) removal and acetate production, and these physiological activities of the biofilms and the plankton cells in the MESs.

Here Fig. 5

3.7. XPS spectra analysis

XPS analysis of the *S. marcescens* biofilms collected from the cathodes (Fig. 6A – C) and of the plankton cells recovered from the catholyte (Fig. 6D – F), reported the exclusive presence of Cu(0) after the passage of circuital currents at –900 mV (Fig. 6A and D) or at –600 mV potentials (Fig. 6B and E; Table S3), while under OCC only Cu(II) was observed (Fig. 6C and F; Table S3). Therefore, the *S. marcescens* biofilms and the plankton cells reduced Cu(II) to Cu(0) only in the presence of a circuital current (Fig. 6A, B, D and E; Table S3), whereas only chemical adsorption of Cu(II) on the electrodes was observed under OCC (Fig. 6C and F; Table S3).

The amount of Cu(II) reduced to Cu(0) by *S. marcescens* was higher in the biofilms (–900 mV: $72.1 \pm 0.7\%$; –600 mV: $63.1 \pm 1.0\%$) than in the plankton cells (–900 mV: $54.1 \pm 0.6\%$; –600 mV: $42.6 \pm 1.6\%$) (Table S3), corroborating with the higher catalytic activity exerted by the *S. marcescens*

biofilms than the plankton cells for reduction of copper, supporting the results in Figs. 2 – 5.

Collectively, these results demonstrated the *S. marcescens* biofilms were more effective than the plankton cells at reducing Cu(II) to Cu(0), at higher circuital currents.

The valence state of the Cu(II) reduction products is influenced by the initial Cu(II) concentration, the dissolved oxygen concentration, the circuital current, and by the composition, concentration and functional groups of dissolved organics (Tao et al., 2011; Ninh Pham et al., 2012; Wu et al., 2016). The reduction of Cu(II) to Cu(0) in bioelectrochemical systems in the absence of oxygen has been reported in the range of Cu(II) initial concentrations (200 – 6400 mg/L) (Tao et al., 2011; Wu et al., 2016), whereas the reduction of Cu(II) to Cu(I) is favored at increasing concentration of natural organic matter (Suwannee River fulvic acid, Ninh Pham et al., 2012). The detection of Cu(0) only in this study (Fig. 6) and the absence of Cu(I) may be attributed to the low initial Cu(II) concentration used (80 mg/L), despite the uncertainty on the role played by the EPS functional groups. This result also reflects the advantage of this system for producing purer Cu(0) product using low Cu(II) concentrations, in agreement with other studies (Huang et al., 2018; Shen et al., 2017; Ter Heijne et al., 2010; Wu et al., 2015, 2016; Zhang et al., 2015).

Here Fig. 6

3.8. Wider significance and prospects

In this study we demonstrated the feasibility of the Cu(II)-tolerant *S. marcescens* Q1 for production of acetate from inorganic carbon with simultaneous Cu(II) removal/recovery in a metallurgical MES. The use of such MES with tailored *S. marcescens* Q1 may enable new prospects for the efficient conversion of CO₂, from a broad range of sources, to C1 feedstocks, with simultaneous heavy metals removal/recovery from contaminated waters, and thus stress the multiple functions of the Cu(II)-tolerant EAB in the treatment of Cu(II)-contaminated organics-barren wastewaters.

In terms of practical acid mine drainage wastewaters with organics-barren characters (Ter Heijne et al., 2010; Zhang et al., 2015; Wu et al., 2016), this *S. marcescens* catalyzed MESs using HCO_3^- as carbon source, providing a cost-effective and efficient approach for Cu(II) removal, without the requirement of adding external organics. The detection of Cu(0) only in this study and the absence of Cu(I) at a low initial Cu(II) concentration (80 mg/L) demonstrates the advantage of this system for producing purer Cu(0) product using low Cu(II) concentrations.

For the first time, we elucidated the roles of the biofilms and the plankton cells of *S. marcescens* in a metallurgical MES for the production of acetate from inorganic carbon with simultaneous Cu(II) removal/recovery. The release of varying amounts of EPS with a compositional diversity, provides a strategy for controlling the rate of production of acetate and the rate of Cu(II) removal. The amount and constituent species released by *S. marcescens*, in both the biofilms and in the plankton cells, and their physiological activities can be correlated to the applied cathodic potential and to the Cu(II) concentration. The quantitative binding constant in this work expounded the differences in Cu(II) binding features of the individual fluorescent constituents and functional groups of the EPS released. These results provide a more detailed theoretical basis to investigate at the molecular level the interaction mechanism of Cu(II) and other heavy metals with EPS released by *S. marcescens*, which is key for the application of this metallurgical MES for efficient control of the migration and transformation of Cu(II) in polluted sites.

Further studies should focus on the identification of the copper-related genetic regulatory networks in the *S. marcescens* Q1, which govern their function predictions, and to the optimal design of MESs and biocompatibility with the electrode materials, which can increase the system performance. The recovery of the metals (e.g., copper) from the electrodes may be carried out by periodically removing the biomass from the electrodes and using a clarifier (settling tank) to collect the solids. In addition, it is increasingly important to develop and characterize a continuous treatment

processes in order to improve the system efficiency and the large scale performance of such MESs (Venkata Mohan et al., 2016). The scale up of MES from laboratory to a commercially viable process, requires the rates of acetate production and copper reduction to be increased by one order of magnitude (May et al., 2016) and the interference from other water matrix components. Considering the rates of copper reduction achieved in such MESs, after scale-up, it should be possible to treat low-strength, copper-laden acid mine drainage wastewaters with simultaneous formation of acetate and hydrogen by-products. Practical implementation will depend on the long-term operation of the MESs and other characteristics of the wastewaters, as well as the cost of the system relative to conventional treatment processes.

Regarding energy consumption, at least 3.0 kWh is needed per kilogram of acetate production with over 85% of the energy used for acetate separation in conventional MESs, which is higher than that amount required for the industrial production of acetate via carbonylation of methanol (~1.65 kWh/kg acetate/acetic acid) (Zou and He, 2018; Jain and He, 2018). However, the use of green energy sources such as solar energy, as well as electricity from intermittent, stranded, or curtailed power, might be supplied (May et al., 2016), in addition to the CO₂ (eg. inorganic carbon) and Cu(II) from waste streams. The feedstock for this MES could be considered to be a waste product and its valorization would help address the need to store energy and carbon, as well as, to recover copper heavy metal. Further studies on these aspects are warranted.

4. Conclusions

Simultaneous Cu(II) reduction (6.42 ± 0.02 mg/L/h), acetate production (1.13 ± 0.02 mg/L/h) from inorganic carbon, and hydrogen evolution (0.0315 ± 0.0005 m³/m³/d) were achieved in a *Serratia marcescens* Q1 catalyzed MES. The physiological activities of GSH, SOD, CAT and dehydrogenase in the biofilms and the plankton cells were correlated to the rates of Cu(II) removal

and acetate production in the metallurgical MESs. Higher currents and higher Cu(II) concentrations induced the biofilms to release higher amounts of EPS with a higher degree of diversity, which exhibited stronger Cu(II) complexation than the plankton cells. Such understanding should enable a more precise manipulation of the metallurgical MES to favor the production of acetate from CO₂ sequestration and the removal of Cu(II) from organics-barren waters contaminated with heavy metals.

Conflicts of interest

There are no conflicts to declare.

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741

742 **Table 1** Parameters of CVs on the *S. marcescens* biofilms, plankton cells, biofilms associated EPS,
743 and plankton cells associated EPS at cathode potentials of –600 or –900 mV (vs. SHE), or in the
744 open circuital conditions (OCC) under Cu(II) concentrations of 60 mg/L, 80 mg/L, or 120 mg/L.

745 **Table 2** Comparison of binding constants of Cu(II)-EPS associated with the plankton cells or the
746 biofilms at cathode potentials of –900 mV or –600 mV, or under open circuital conditions (OCC)
747 in the presence or absence of Cu(II).

748 **Fig. 1** Comparison of Cu(II) removal (A), acetate production (B), hydrogen production (C), and
749 circuital current (D) in the *S. marcescens* catalyzed cathodes at various initial Cu(II)
750 concentrations and cathode potentials of –900 mV, 600 mV, or under open circuital conditions
751 (OCC).

752 **Fig. 2** Comparison of CVs of *S. marcescens* biofilm (A, C, E, G, I and K), plankton cells (B, D, F, H,
753 J and L), biofilm EPS only (C, G and K), and plankton EPS only (D, H and L) under potentials of
754 –900 mV (A, B, C and D), –600 mV (E, F, G and H) or open circuital conditions (OCC) (I, J, K
755 and L) at different concentrations of Cu(II) electrolytes.

756 **Fig. 3** Comparison of exoprotein (A) and exopolysaccharide (B) released by the *S. marcescens*
757 biofilms and plankton cells under various initial Cu(II) concentrations or cathode potentials.
758 Distribution of total carbon at cathode potentials of –900 mV or –600 mV, or under open circuital
759 conditions (OCC).

760 **Fig. 4** Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy of
761 extracellular polymer substances produced by the *S. marcescens* biofilms (A, B, C, D and E) or the
762 plankton cells (F, G, H, I and J) at cathode potentials of either –900 mV (vs. standard hydrogen
763 electrode (SHE)) (A, B, F and G) or –600 mV (vs. SHE) (C, D, H and I), or under open circuital
764 conditions (E and J) in the absence of Cu(II) (B, D, G and I) or presence of Cu(II) of 80 mg/L (A,
765 C, E, F, H and J).

766 **Fig. 5** Comparison of physiological activities of dehydrogenase (A), catalase (CAT) (B), superoxide
767 dismutase (SOD) (C), and glutathione transferases (GSH) (D) in the *S. marcescens* biofilms and
768 the plankton cells at potentials of –900 mV or –600 mV, or under open circuital conditions (OCC).

769 **Fig. 6** XPS analysis for Cu element on the *S. marcescens* biofilms (A, B and C) or the plankton cells
770 (D, E and F) at cathode potentials of –900 mV (A and D) or –600 mV (B and E), or under open
771 circuital conditions (OCC) (C and F).

Table 1
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Table 1 Parameters of CVs on the *S. marcescens* biofilms, planktonic cells, biofilms associated EPS, and planktonic cells associated EPS at cathode potentials of –600 or –900 mV (vs. SHE), or in the open circuital conditions (OCC) under Cu(II) concentrations of 60 mg/L, 80 mg/L, or 120 mg/L.

		–900 mV			–600 mV			OCC			Abiotic controls		
		60	80	120	60	80	120	60	80	120	60	80	120
Biofilm	Reductive onset potential/V (vs. SHE)	0.34	0.35	0.37	0.32	0.33	0.35	0.30	0.32	0.34	---	---	---
	Reductive peak potential/V (vs. SHE)	0.04	0.05	0.07	0.02	0.02	0.04	–0.01	0.02	0.02	---	---	---
	Reductive peak current/mA	–0.52	–0.55	–0.61	–0.30	–0.32	–0.33	–0.28	–0.30	–0.31	---	---	---
Planktonic cells	Reductive onset potential/V (vs. SHE)	0.30	0.31	0.33	0.29	0.30	0.32	0.28	0.29	0.31	---	---	---
	Reductive peak potential/V (vs. SHE)	0.02	0.03	0.04	0.01	0.01	0.03	–0.01	0.00	0.03	---	---	---
	Reductive peak current/mA	–0.39	–0.41	–0.46	–0.23	–0.24	–0.25	–0.21	–0.23	–0.24	---	---	---
Biofilm-EPS	Reductive onset potential/V (vs. SHE)	0.31	0.32	0.34	0.30	0.31	0.33	0.28	0.30	0.31	---	---	---
	Reductive peak potential/V (vs. SHE)	0.03	0.04	0.05	0.02	0.02	0.03	–0.01	0.01	0.03	---	---	---
	Reductive peak current/mA	–0.37	–0.39	–0.49	–0.24	–0.26	–0.27	–0.19	–0.21	–0.22	---	---	---
Planktonic-EPS	Reductive onset potential/V (vs. SHE)	0.30	0.31	0.32	0.29	0.30	0.31	0.28	0.29	0.30	---	---	---
	Reductive peak potential/V (vs. SHE)	0.01	0.02	0.03	0.00	0.00	0.03	–0.02	0.00	0.03	---	---	---
	Reductive peak current/mA	–0.36	–0.37	–0.43	–0.18	–0.19	–0.20	–0.18	–0.20	–0.21	---	---	---
Abiotic controls	Reductive onset potential/V (vs. SHE)	---	---	---	---	---	---	---	---	---	0.27	0.27	0.29
	Reductive peak potential/V (vs. SHE)	---	---	---	---	---	---	---	---	---	–0.02	–0.01	0.03
	Reductive peak current/mA	---	---	---	---	---	---	---	---	---	–0.29	–0.30	–0.30

Table 2
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Table 2 Comparison of binding constants of Cu(II)-EPS associated with the plankton cells or the biofilms at cathode potentials of –900 mV or –600 mV, or under open circuital conditions (OCC) in the presence or absence of Cu(II).

Cathode potential (vs. SHE, mV)	Cu(II) concentration (mg/L)	Plankton cells				Biofilms		
		Peak	log K_b	n	R^2	log K_b	n	R^2
–900	0	II	4.008 ± 0.042	1.465 ± 0.011	0.989	4.263 ± 0.017	1.537 ± 0.004	0.993
		III	3.908 ± 0.021	1.442 ± 0.005	0.991	3.973 ± 0.025	1.456 ± 0.007	0.992
		IV	3.237 ± 0.013	1.280 ± 0.004	0.997	3.265 ± 0.010	1.284 ± 0.003	0.993
		V(a)	---	---	---	3.300 ± 0.014	1.292 ± 0.004	0.991
		V(b)	3.360 ± 0.015	1.305 ± 0.004	0.992	3.593 ± 0.016	1.363 ± 0.004	0.989
	80	II	4.223 ± 0.038	1.523 ± 0.011	0.990	4.675 ± 0.015	1.578 ± 0.016	0.994
		III	4.140 ± 0.012	1.505 ± 0.003	0.994	4.506 ± 0.049	1.606 ± 0.013	0.996
		IV	3.289 ± 0.010	1.292 ± 0.002	0.991	3.353 ± 0.098	1.301 ± 0.030	0.993
		V(a)	---	---	---	3.910 ± 0.024	1.444 ± 0.006	0.993
		V(b)	3.401 ± 0.010	1.316 ± 0.002	0.998	3.540 ± 0.009	1.349 ± 0.002	0.991
–600	0	II	3.708 ± 0.011	1.393 ± 0.002	0.993	3.798 ± 0.013	1.417 ± 0.004	0.997
		III	---	---	---	---	---	---
		IV	3.241 ± 0.009	1.281 ± 0.002	0.995	3.243 ± 0.009	1.284 ± 0.002	0.994
		V(a)	---	---	---	3.193 ± 0.005	3.193 ± 0.005	0.994
		V(b)	3.324 ± 0.010	1.296 ± 0.003	0.993	3.392 ± 0.009	1.313 ± 0.002	0.991
	80	II	3.871 ± 0.014	1.433 ± 0.004	0.994	4.027 ± 0.014	1.478 ± 0.004	0.996
		III	3.526 ± 0.008	1.343 ± 0.002	0.991	3.891 ± 0.015	1.439 ± 0.004	0.989
		IV	3.157 ± 0.007	1.261 ± 0.002	0.997	3.279 ± 0.008	1.291 ± 0.002	0.991
		V(a)	---	---	---	3.627 ± 0.009	1.373 ± 0.002	0.995
		V(b)	3.328 ± 0.010	1.303 ± 0.002	0.993	3.446 ± 0.009	1.325 ± 0.002	0.993
OCC	80	II	3.141 ± 0.009	1.258 ± 0.002	0.991	3.289 ± 0.008	1.293 ± 0.002	0.995
		III	---	---	---	---	---	---
		IV	3.109 ± 0.007	1.252 ± 0.001	0.996	3.182 ± 0.009	1.267 ± 0.002	0.992
		V(a)	---	---	---	3.165 ± 0.004	1.257 ± 0.001	0.994
		V(b)	3.066 ± 0.005	1.243 ± 0.001	0.989	3.099 ± 0.005	1.247 ± 0.001	0.991

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