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Changes in microbial composition during the removal of copper and zinc in a bioreactor with a limestone pre-column system

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DEDICATORIA

A mis padres.

A Antonio y a mi hija Antonia,

por serlo todo y por serlo siempre.

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RESUMEN

La bioprecipitación de metales es un proceso pasivo de biorremediación impulsado por bacterias sulfato reductoras, BSR, que viven en ambientes anaerobios y que típicamente forman consorcios con otras comunidades microbianas. La degradación o la inmovilización biológica de contaminantes en sistemas de tratamiento de aguas residuales requiere el estudio de las interacciones entre los microorganismos involucrados; la bioprecipitación no es una excepción. Esta investigación estudia los cambios de composición microbiana durante la eliminación de cobre y zinc de un drenaje ácido de roca sintético en un biorreactor reductor de sulfato con una pre-columna de piedra caliza, utilizando acetato como fuente de carbono y como donador de electrones.

Las actividades de investigación incluyeron: Operación y monitoreo del biorreactor, en tres períodos, durante un año. Validación del método analítico y construcción de curvas estándar de PCR en tiempo real, qPCR, para la enumeración de BSR, basadas en la amplificación de genes *dsrA* y *apsA*. Y, análisis moleculares de muestras de lodo en tres etapas de operación del biorreactor, mediante qPCR y Metagenómica.

Fueron observadas altas eficiencias de eliminación de Cu (II) y Zn (II), superiores al 99%, en el biorreactor y el consumo de acetato excedió el 70% de la demanda química de oxígeno inicial. El método qPCR fue validado exitosamente para muestras ambientales e industriales, utilizando el set de primers DSR1F y RH3-dsr-R. Durante el funcionamiento del biorreactor, las BSR mostraron una menor concentración en los primeros días de operación, aproximadamente 1E+05 células mL^{-1}, aumentando a partir del día 150, aproximadamente a 1E+06 células mL^{-1}, y estabilizándose en ese valor hasta el final de la operación.

De acuerdo con el análisis de diversidad microbiana del lodo del biorreactor reductor de sulfato, los microorganismos más abundantes son arqueas metanógenas afiliadas al género *Methanosarcina*, sin embargo no hubo producción de metano. Las SRB identificadas corresponden principalmente a los géneros *Desulfotomaculum* y *Desulfovibrio*. Durante la operación del biorreactor reductor de sulfato con el sistema de pre-columna de piedra caliza, se observaron cambios en la composición microbiana y las comunidades procariotas fueron gradualmente menos diversas y más predecibles en términos metabólicos.

Este estudio contribuye al desarrollo de sistemas ecológicos autosustentables para el tratamiento del drenaje ácido de minas en Ecuador.

Palabras clave: biorremediación anaeróbica, drenajes ácidos de mina, bacterias sulfato reductoras, qPCR, metagenómica

ABSTRACT

Bioprecipitation of metals is a passive bioremediation process driven by sulfate-reducing bacteria, SRB, live in anaerobic environments and typically forming consortia with other microbial communities. Biological degradation or immobilization of environmental pollutants in wastewater treatment systems requires understanding the interactions between involved microorganisms; bioprecipitation is not an exception. This research addresses the study on changes of microbial composition during the removal of copper and zinc from a synthetic acid mine drainage in a sulfate reducing bioreactor with a limestone pre-column, using acetate as carbon source and electron donor.

Research activities included: Operation and monitoring of bioreactor, in three periods during a year. Validation of analytical method and construction of standard curves for real/time PCR, qPCR, for enumeration of SRB, based on *dsrA* and a*psA* genes amplification. And, molecular analyses of sludge samples at three bioreactor operation stages by qPCR and Metagenomics.

High removal efficiencies of Cu (II) and Zn (II), upper than 99%, were observed in the sulfate-reducing bioreactor and consume of acetate exceeded 70% of the initial chemical oxygen demand. qPCR method was successfully validated using DSR1F and RH3-dsr-R primer set for environmental and engineered sludge samples. During operation of bioreactor, sulfate-reducing bacteria displayed a lower concentration in the first days of operation, about 1E+05 cells mL^{-1}, and higher starting day 150, about 1E+06 cells mL^{-1}, stabilizing that value until end of operation.

According to the microbial diversity analysis of the sludge from the sulfate reducing bioreactor, the most abundant microorganisms are methanogen archaea affiliated with the genus *Methanosarcina*, however there was not methane production. Identified SRB correspond mainly to the genera *Desulfotomaculum* and *Desulfovibrio*. During operation of the sulfate reducing bioreactor with the limestone pre-column system, changes in microbial composition were observed and prokaryotic communities were gradually lesser diverse and more predictable in metabolic terms.

This study contributes to development of self-sustainable environmental friendly systems for the treatment of acid mine drainage in Ecuador.

Key words: anaerobic bioremediation, acid mine drainage, sulfate reducing bacteria, qPCR, Metagenomics

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CHAPTER I

SCIENTIFIC PAPER 1

Improved method for rapid and sensitive quantification of sulfate-reducing bacteria in environmental and engineered sludge samples using qPCR

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ABSTRACT

Sulfate-reducing bacteria (SRB) are ubiquitous and anaerobic microorganisms that form sulfide. SRBs use sulfate as a terminal electron acceptor in the degradation of organic compounds leading to the production of sulfide. Research of SRB is relevant from the ecological and industrial point of view, since they can remove heavy metals from wastewaters through bioprecipitation, which is a passive bioremediation process. In order to quantify SRB from environmental and engineered sources (e.g. sludge), direct counting or anaerobic cultivation methods are normally used. These conventional methods can take too long (range from 28 to 30 days) and give variable results. This study aimed to improve a fast-molecular method for quantification of SRB in environmental and engineered sludge samples using a method based on quantitative real-time polymerase chain reaction (qPCR). Four sets of primers that amplified genes (*dsrA* and *apsA*) encoding two key enzymes in the dissimilatory sulfate reduction pathway were tested. qPCR standard curves were based on plotting of Ct (cycle threshold) values using genomic DNA from SRB suspensions and dilutions of enriched sulfate-reducing sludge versus bacterial counts of viable SRB cultivated under anaerobic conditions. According to the sensitivity, accuracy, scope and reproducibility of the analytical method validated in this study, the DSR1F/RH3-dsr-R primer set ensure specific and accurate quantification based on *dsrA* gene amplification. After contrasting SRB counting methodologies using Neubauer counting (total), plate culture (viable) and this improved qPCR method, the conclusion was that qPCR estimates well the abundance of SRB in samples coming from a range of origins. Hence, this study provides evidence that this improved qPCR-based method can be a faster (less than one day) and a sensitive molecular tool for quantitative detection of heterogenic SRB populations in engineered sludge and samples from environmental origin.

Keywords

Sulfate-reducing bacteria, *dsr*, *aps*, qPCR.

1 INTRODUCTION

Sulfate-reducing bacteria (SRB) are anaerobic prokaryotic organisms, which are an integral part of the global sulfur and carbon cycles, and their importance is based on ecological and technical reasons. SRBs belong to a morphologically diverse and heterogeneous group of microorganisms, which are present both in nature and in engineered anaerobic environments such as marine sediments or swamps, and industrial wastewater treatment systems or in oil and gas production facilities (Muyzer & Stams, 2008; Agrawal & Lal, 2009). SRBs are responsible for the biogenic sulfide generation as part of their metabolism since they use sulfate as a terminal electron acceptor (Muyzer & Stams, 2008; St-Pierre & Wright, 2017).

The population of SRBs in environmental samples is of great scientific interest in remediation processes of metal contaminated effluents such as acid rock drainage (ARD). ARDs are the result of sulfide minerals exposed to air and water as well as a product of the lixiviation process in the mining industry, and they are characterized by elevated heavy metal content, high concentration of sulfate and low pH values (Hiibel *et al*, 2008; Simate & Ndlovu, 2014; Nieto *et al*, 2007). SRBs catalyze the bioremediation of ARDs that is mainly based on bioprecipitation of metals (Kaksonen & Puhakka, 2007).

Bioprecipitation process consists on the generation of biogenic sulfide by SRBs, which reacts with metal ions to produce metallic sulfides of low solubility (Sierra-Alvarez, Hollingsworth & Zhou, 2007; Le Pape *et al*, 2017). Other effects of bioprecipitation of metals driven by SRBs are reduction of acidity and sulfate concentration in treated effluents (Al-Abed *et al*, 2017; Luo *et al*, 2008).

Nowadays, research projects and technical applications require accurate assessments regarding enumeration, occurrence, distribution, diversity and community structure of SRBs in a wide range of environments (Hiibel *et al*, 2008; Shen & Voordouw, 2015). The detection and

enumeration of SRBs can be done by employing both conventional and molecular methods (Oude Elferink *et al*, 1998). Conventional methods include culture methods and enumeration techniques based on microscope images, which both have limitations due to time consuming, repeatability or selectivity (Spence, Whitehead & Cotta, 2008). First, microorganisms can be associated to soil particles or biofilms; these are important limitations for direct counts under a microscope (Kirk *et al*, 2004). In addition, it is known that at least 99% of all microorganisms can not be cultured and that among those culturable in plates not all develop into a colony due to inhibition or differential colony spreading, depending on microbial growth rates (Stewart, 2012).

Molecular methods, on the other hand, have the advantage of providing fast and precise information about the amount and distribution of bacteria in a specific environment (Wagner & Loy, 2002; Freeman *et al*, 2008). Within the molecular techniques, the use of antibodies raised against SRB and 16 rRNA probes are challenging since SRB include members of several phyla and domains, and some samples can have interferences such as high background with auto fluorescence of inorganic particles (Zhang & Fang, 2006; Muyzer & Ramsing, 1995). Another molecular method is quantitative or real-time PCR (qPCR) which has been reported to be applicable to the SRB analysis in several studies (Foti *et al*, 2007; Kondo *et al*, 2008).

For SRB identification and quantification, phylogenetic markers are functional and highly conserved genes: *dsrAB* and *apsBA*. They encode two key enzymes in the dissimilatory sulfate reduction pathway, which are dissimilatory sulfate reductase (DRS) and adenosine-5´-phospho-sulfate (APS), respectively (Muyzer & Stams, 2008). Several studies have validated the use of these two genes as amplification targets by real-time or quantitative polymerase chain reaction (qPCR) for quantification of SRB and phylogenetic analysis (Hiibel *et al*, 2008). Ben-Dov *et al* (2007) designed four sets of primers from conserved regions of multiple alignment

of *dsrA* and *apsA* genes, and developed a method based on qPCR for quantification of SRB in complex environmental and industrial water samples (Ben-Dov *et al*, 2007).

The goal of our study was to validate and improve a molecular assay to quantify SRBs in environmental and engineered sludge samples based on a qPCR method, using four sets of primers for *dsrA* and *aps*A genes, previously designed by Ben-Dov *et al* (2007). Standard curves were plotted using genomic DNA from bacterial suspensions and dilutions of enriched sulfate-reducing sludge, and bacterial counts of SRB cultivated under anaerobic conditions from the same sample. In addition, SRB counting methodologies using Neubauer chamber counting (total), plate culture (viable) and this improved qPCR method were compared. The conclusion of that comparison was that qPCR estimated well the populations of SRB in samples coming from a range of origins such as bacterial suspensions, engineered or enriched sludge, environmental sludge from a lagoon and sludge from a bioreactor.

The present study provides an innovative improvement for quantitative detection of heterogenic SRB populations. The qPCR method validated here is an alternative and efficient molecular tool that does not require culture of target microorganisms for its application in environmental and engineered samples.

2 MATERIALS AND METHODS

Basal mineral medium (BMM)

The basal mineral medium contained (in mg L^{-1}): NH_4Cl (280); KH_2PO_4 (195); $MgSO_4$ (49); $CaCl_2$ (10); $NaHCO_3$ (3000); yeast extract (10), Na_2SO_4 (2900), CH_3COONa (5300) and 1 mL L^{-1} of a solution of trace elements. The trace element solution contained (in

mg L^{-1}): H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2$ (32), $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3$ (50), $CoCl_2 \cdot 6H_2O$ (2,000), $NiCl_2 \cdot 6H_2O$ (50), $CuSO_4 \cdot 5H_2O$ (44), $NaSeO_3 \cdot 5H_2O$ (100), EDTA (1,000), resazurin (200) and 1 mL L^{-1} of hydrochloric acid (36%) (Ochoa-Herrera, Banihani *et al*, 2009).

Samples

Environmental sample: Anaerobic sludge was collected from sediments at bottom, approximately 1.2 m deep, of an artificial lagoon at Universidad San Francisco de Quito, Ecuador. The sludge was stored at 4 ºC. The content of total suspended solids (TSS) and volatile suspended solids (VSS) in the sludge were 51.7% and 5.9%, respectively. The maximum specific sulfidogenic activity was 4336.7 mg S^{2-} kg^{-1} VSS d^{-1}.

Engineered sample: Enriched sulfate reducing (SR) sludge was obtained from anaerobic sludge, that was enriched through growing in a selective culture media for SRBs. This procedure was conducted under anaerobic conditions in triplicates using sterile liquid culture medium and 10% w/v or v/v of anaerobic sludge. The cultures were cultivated in 160 mL sterile glass serum bottles hermetically sealed with butyl rubber stoppers and aluminum crimp seals. Bottle headspace was flushed with nitrogen gas and all bottles were incubated during 45 days in darkness, in a climate-controlled chamber at 30±2°C. The enriched SR sludge was immediately analyzed by conventional methods. Likewise, genomic DNA extracts from serial dilutions of enriched SR sludge were obtained and kept at -80 °C until the beginning of the molecular tests.

Mixture of synthetic SRB suspension*:* Immediately after the plate count, isolated colonies from SR sludge were picked off and placed into bottles containing sterile BMM supplemented with acetate 2.5 g COD L^{-1} as organic substrate and 2.0 g SO_4^{2-} L^{-1} as sodium sulfate (1.25:1 ratio). After the incubation period, SRB suspensions were cultured and enumerated by Neubauer chamber. Then, genomic DNA from mixture of synthetic SRB suspension was extracted and kept at -80 °C until the beginning of the molecular tests.

Neubauer chamber cell enumeration

In order to quantify total SRB in the enriched SR sludge and mixture of synthetic SRB suspension, they were enumerated by Neubauer chamber counting (Pérez *et al*, 2010). At the same time that the calibrators (mixture of synthetic SRB suspension and enriched SR sludge) were plated cultured, they were diluted in a sterile saline solution (0.9% NaCl w/v) for cells counting using a Neubauer chamber (BOECO, Germany) with 0.1 mm depth. Bacterial stain was not required due to the characteristic black color of SRB cells (Mohd Rasol *et al*, 2014). The number of total SRB per milliliter (mL) was calculated following supplier instructions.). Total SRB cells include those viable and non-viable. Each sample was analyzed in triplicates.

Plating culture and bacteria counting

In order to quantify viable SRB, 1 mL aliquot of sample was serially diluted in 9 mL of sterile BMM supplemented with acetate and sulfate in a 1.25:1 ratio. An aliquot of 100 µL of raw sample and each corresponding dilution (10E-01 to 10E-09) were pour-plated in three replicates. Culture medium was supplemented with 1.5% agar w/v (Bacto™ Agar, Difco

Laboratories, France) cooled to 45ºC, and a volume of 20 to 25 mL was dispensed per plate. The agar plates were incubated in an anaerobic chamber (atmosphere N_2 80%; CO_2 20%) at 30±0.5°C for 28-30 days and then the colonies were counted. The number of viable SRB per milliliter (mL) was calculated dividing number of colonies by sample volume and dilution factor.). Viable SRB cells were expressed as colony forming units (CFU). Each sample was analyzed in triplicates.

DNA extraction

DNA was extracted by standard protocols well documented in the literature (Ben-Dov *et al*, 2007; Méndez *et al*, 2015). A volume of 12 mL of sludge or bacterial suspension was centrifuged at 6000 rpm during 30 minutes. The supernatant was discharged and the settled fraction (pellet) was used for DNA extraction utilizing DNeasy® PowerSoil® Kit (QIAGEN GmbH., Germany) according the protocol provided by the supplier but washing twice with solution C5 (Ben-Dov *et al*, 2007). The purity, as concentration of the resulting DNA preparation, was determined spectrophotometrically by Qubit® system (Thermo Fisher Scientific Inc. USA). DNA integrity of molecular weights over 2000 pairs of bases (bp) were evaluated using 2.0% agarose gels (Bioline, London, UK) with Invitrogen SYBR® Safe DNA gel stain (Thermo Fisher, Carlsbad, CA, USA) and TAE (mixture of Tris base, acetic acid and EDTA) buffer. Electrophoresis was carried out at 80 mV for 30 minutes in Gel XL Enduro™ chamber (Labnet, Edison, USA) and using 2µL of DNA per well. Additionally, DNA of *E. coli* ATCC® 25922 was extracted using E.Z.N.A.® Bacterial DNA kit (Omega bio-tek, USA), following supplier directions.

qPCR analysis

qPCR assays were performed on PrimeQ thermal cycler system (Cole-Parmer, Staffordshire, UK), based on fluorescence resonance energy transfer, with a SYBR green fluorophore, in a 96-well optical plate at the Laboratory of Agricultural and Food Biotechnology, Universidad San Francisco de Quito USFQ. Four primer sets for qPCR amplification of *dsrA* and *apsA* genes were employed as previously evaluated by Ben-Dov *et al* (2007): DSR1F and RH3-dsr-R (size: 222 bp), APS7-F and RH2-aps-R (279 bp), RH1-dsr-F and RH3-dsr-R (164 bp), and RH1-aps-F and RH2-aps-R (191 bp) (Ben-Dov *et al*, 2007). Invitrogen™ (Thermo Fisher, Carlsbad, CA, USA) manufactured the four primer sets, their characteristics are presented in Appendix A. Reaction mixture consisted of 7.5 µL of SsoFast™ EvaGreen® Supermix (BioRad, Hercules, USA), 150 nM each forward and reverse primers, 1.5 µL of DNA template, and PCR water to a final volume of 15 µL. PCR cycles were 2 min at 50ºC, 15 min at 95ºC, followed by 40 rounds of 15 s at 95ºC (Ben-Dov *et al*, 2007) and 1 min at 62 ºC as annealing step. The stage of extension stage was 30 seconds at 72 ºC. Melting or dissociation curves (negative derivative of fluorescence versus temperature) were determine for the presence or absence of nonspecific amplification products. Size of PCR products were verified with SYBR® Safe-stained 2.0% agarose gels. Electrophoresis conditions were 80 mV for 60 minutes. All runs included a blank or no-template control (PCR water: Ultrapure™ Distilled Water, Invitrogen, Grand Island, NY, USA) and a negative control from non-SRB strain (DNA of *E. coli* ATCC® 25922) for each primer set.

Construction of standard curves

Standard curves were generated using different concentrations of template DNA obtained from mixture of synthetic SRB suspension and serial dilutions of enriched SR sludge. First, DNA template obtained from bacterial suspensions cultured with isolated SRB colonies was used in six serial dilutions points, in steps of ten-fold. Other calibrator consisted of extracted DNA from each serial dilution of enriched SR sludge. Calibrators were normalized according number of total SRB cells. Standard curves were obtained by plotting the qPCR threshold cycle (Ct) of each dilution point for the DNA templates obtained by the two different ways versus corresponding normalized units (viable SRB cells). Data analysis was performed in QuanSoft® software (Cole-Parmer, Staffordshire, UK). The qPCR reaction conditions were the same for each primer set. All calibrators were run independently in triplicates and standard deviation was calculated for each point.

Statistical analysis

The effect of the method for direct enumeration of SRBs in bacterial suspension and in enriched SR sludge was determined by standard deviations calculated in SPSS Statistics software (IBM Corp, New York). Similarity, standard curves from two different SRB calibrators were compared through Fisher´s test (homogeneity of variances) and Student-*t* test (slopes similarity of linear regression). The different quantification methodologies were compared by analysis of variance, ANOVA test, with 95% of confidence interval, using in SPSS Statistics software (IBM Corp, New York).

3 RESULTS AND DISCUSSION

Evaluation of SRB cells concentration by standard plate culture (viable cells) and cell count (total cells)

For the generation of standard curves for further improvement of the quantification method using qPCR, a mixture of synthetic SRB suspension and SRB enriched sludge were constructed as mentioned in the Material and methods section. Both SRB samples were counted during their exponential stage of growth by platting (viable SRB) and enumerated microscopically by Neubauer chamber (total SRB) simultaneously (Cotta *et al*, 2003).

Firstly, in the mixture of synthetic SRB suspension, concentration of SRB estimated by Neubauer chamber was 2.21E+06 total SRB cells per mL (Figure 1.1). SRB suspension was also plate cultured and enumerated to check their viability and the concentration of viable cells in SRB suspension was 1.79E+06 CFU per mL (Figure 1.1), corresponding to 81% of total cells. The measurements showed no statistical difference when counted with both methods, suggesting that, when it comes to the mixture of synthetic SRB suspension, the viable/culturable fraction and total count are similar since the suspension had been grown previously in culture media from isolated SRB colonies. Consequently, SRB suspension was homogenous in the number of bacteria, which reduced competition for nutrients facilitating the developing of SBR (Janssen *et al*, 2002). This similarity between enumeration of SRB in bacterial suspension by plating (viable) and by Neubauer chamber (total) indicates that both methods are valid for SRB enumeration in bacterial suspensions, during exponential growth.

Figure 1.1 shows that the concentration of viable SRB cells in enriched SR sludge, obtained by platting culture was 8.70E+05 CFU per mL and 7.63E+06 for total SRB cells per

mL, showing that only 12% of the total cell was grew. The total count of SRB in enriched SR sludge was significantly higher to the concentration of viable cells by one order of magnitude, being the concentration of total SRB significantly higher than concentration of viable SRB. Enriched SR sludge contained other microorganisms and mineral particles that can lead to overestimation of total SRB when counted with Neubauer chamber method (Kogure *et al,* 1978). In enriched cultures, the growth of certain non-SRB microorganisms can be favored, (Anderson *et al,* 2003). Moreover, the enumeration by Neubauer chamber was done under visible light illumination without contrast dyes, therefore total microorganisms counts were recorded (Anderson *et al*, 2003; Pérez *et al*, 2010).

Average relative reproducibility (standard deviation) of colony counts was ±24% and Neubauer counts was ±33%. These values were comparable with results reported in other studies (Battersby *et al*, 1985; Rath *et al,* 2001), showing that although culture and counting techniques for bacterial enumeration are time demanding, these methodologies continue to be reproducible for enumeration of microorganisms. Therefore, in this study bacterial counts for qPCR standard curves were based on values from colony counts.

Correlation of viable bacterial count versus qPCR data (Ct)

Standard curves were constructed by plotting the mean of Ct values obtained from qPCR versus Log10 of viable SRB cells from samples coming from bacterial suspensions and synthetic sludge. These curves were generated by assuming that SRB have only one *dsrA* gene copy and one aps gene copy (Kondo et al, 2004). When bacterial counts (x-axis) were correlated with Ct values (y-axis), linear regressions were obtained and the fitness of the qPCR method and its performance was validated by the qualitative real-time PCR method proposed by Broeders *et*

al (2014). Figures 1.2(a) and 1.2(b) show standard curves for *dsrA* gene with sets of primers DSR1-F/RH3-dsr-R and RH1-dsr-F /RH3-dsr-R, respectively; and Figures 1.2(c) and 1.2(d) show standard curves for *apsA* gene with sets of primers APS7-F /RH2-aps-R and RH1-aps-F/RH2-aps-R, respectively. In each plot, data points represent the average of three measurements and the standard deviations were represented with error bars.

Standard deviation of Ct values is inherent to inter-run variation and it is a good indicator of method reproducibility/precision (Bustin *et al*, 2009). Replicates of standard curves plotted for all four pairs of primers, RSDr criteria did not exceed 10%, validating precision of our analytical method (data now shown). Relative reproducibility standard deviation (RSDr) should be lower than 25% (Broeders *et al*, 2014).

There were no statistical differences in the slopes of standard curves obtained from two different calibrators of the same primer set (Appendix B). Additionally, this comparison contributed to evaluate the applicability of the qPCR method, since results were similar using two different set of primers (Broeders *et al*, 2014).

Linearity and proportional range of the fast-molecular method develop in this study were evaluated using a correlation coefficient (R^2) of the linear regression analysis. During validation of qPCR methods, R^2 values higher than 0.98 are desirable, but that criterion is referential (Broeders *et al*, 2014). Standard curves for *dsrA* gene (primers DSR1F/RH3-dsr-R and RH1-dsr-F/RH3-dsr-R) showed an R^2 over 0.98, in a concentration range between 1.79E+02 and 1.79E+06, and 7.63E+03 and 7.63E+07 viable SRB cells using a mixture of synthetic SRB suspension as calibrator and serial dilutions of enriched SR sludge (Figure 1.2(a) and Figure 1.2(b)), respectively. On the other hand, in the same ranges, standard curves for *apsA* gene (Figure 1.2(c) and Figure 1.2(d)) showed an R^2 between 0.94 and 0.97, which it was not an acceptable parameter according to R^2 threshold established by Broeders and co-workers

(2014). This can be explained since, primers for *dsrA* gene display high specificity to most SRB species, unlike primers for *apsA* gene showed poor specificity to some genera such as *Desulfacinum* and *Desulfotomaculum* (Ben-Dov *et al*, 2007).

Primers selection based on accuracy from theoretical values and bacterial counts

In order to validate if standard curves between qPCR values (Ct) and viable counts were accurate, independent samples from synthetic SRB suspension and enriched SR sludge were analyzed. The concentration of SRB in a control standard from a mixture of synthetic SRB suspension was 2.31E+06 CFU per mL while the concentration of SR sludge sample was 5.63E+05 CFU per mL (Table 1.1) and differences between concentration of total SRB and concentration of viable SRB were significant (Figure 1.1).

Criteria of accuracy is based on a relative error, Ɛt, which was calculated using SRB concentration determined by enumeration based on plate culture as theoretical value (true value) (Broeders *et al*, 2014; Ben-Dov *et al*, 2007). Accuracy can be assessed by comparing the value obtained from qPCR (estimated count) and the theoretical value (viable count). If we compare the estimated value obtained from qPCR using a set of primers validated in Figure 1.2, in some cases SRB concentrations may be overestimated by over 100-fold from the theoretical value depending on a primer set used (Tables 1.1 and 1.2). Amplification reactions using primer sets DSR1F/RH3-dsr-R and RH1-dsr-F/RH3-dsr-R of *dsrA* gene, are closer to the theoretical values than those concentrations determined using standard curves with primer sets APS7-F/RH2-aps-R and RH1-aps-F/RH2-aps-R of *apsA* gene (Tables 1.1 and 1.2).

The most accurate enumeration of SRB by qPCR was obtained with primers DSR1F/RH3-dsr-R primer set (Table 1.1 and 1.2), with values in the range of 6.3E-02 to 1.4E-

02 times higher than theoretical concentrations of both calibrators (error values ranging from 6.3 to 14.1%). In other study, using genomic DNA of *Desulfovibrio vulgaris* from pure culture and with plasmids containing *dsrA* and *apsA* genes, Ben-Dov *et al* (2007) developed qPCR standard curves that overestimated from 2.7 up to 10.5 fold for DSR1F/RH3-dsr-R pair, which was chosen in this study as the best primer set for accurate enumeration of SRB.

Even though environmental samples such a sludge are microbiologically diverse, the left DSR1F primer is highly specific to many species of SRB (Ben-Dov *et al*, 2007; Spence *et al*, 2008). However, the right RH3-dsr-R primer aligned consensus region of all SRB genera tested by Ben-Dov *et al* (2007) as suggested by Kondo *et al* (2004). This fact also indicates that qPCR analysis using the DSR1F/RH3-dsr-R primer set does actually target a larger and heterogenic SRB population in complex sludge from environmental origin.

Comparison of enumeration methods in synthetic, enriched and environmental samples

In this study, total SRB were quantified in six independent samples: two SRB suspensions, two enriched SR sludge samples, an environmental sample and a sludge from a SR bioreactor. SRB suspensions and serial dilutions of enriched SR sludge were prepared according to the experimental procedures described in Materials and Methods section. The environmental sludge sample was taken from the sediments of an artificial lagoon and SR sludge from a lab-scale bioreactor for bioprecipitation of copper (Mendez *et al*, 2015). All samples were analyzed by the fast-molecular qPCR method on genomic DNA. Figure 1.3 shows enumeration of each sample by Neubauer chamber (total count), platting culture (viable count), and the quantification by qPCR with primers DSR1F and RH3-dsr-R using both standard curves of synthetic SRB suspension and enriched SR sludge as standards (Tables 1.1 and 1.2).

According to ANOVA analysis, the two SRB suspensions samples (1 and 2) did not show significant statistical difference in SRB concentrations, independently of the quantification method (Figure 3). In both samples of enriched SR sludge, results of enumeration by Neubauer chamber (total SRB) were statistically different to those of other quantification methods, showing that enumeration by Neubauer chamber under visible light gives higher values for enumeration of SRB in sludge samples because it overestimates the bacterial concentration. Viable count and qPCR using both standard curves gave no significant differences, showing that viable count and qPCR values produce comparable results. In the environmental sludge (sample from lagoon) there was 1.27E+03 and 6.23E+03 cells per mL determined by qPCR using SRB suspension and serial dilutions of SR sludge as calibrators, respectively (Figure 1.3). No significant differences were found in both samples when qPCR as used. However, viable count was statistically different from total count, showing large differences between those two methods. qPCR analysis provided counting values that are in between viable (performed by plate) and total (counting performed by Neubauer) cells.

In the sludge from a SR bioreactor, the means of the counts of SRB quantified by qPCR using both calibrators were 7.48E+05 and 1.79E+06 SRB cells per mL, respectively. In both cases, there were no statistically significant differences between the samples as in the case when the environmental samples were tested. Also, total and viable SRB counts shown to be statistically different in each of these samples.

Regardless of the calibrator used in the fast-molecular qPCR method, the quantified SRB concentrations were similar to the data reported in other studies (Ben-Dov *et al*, 2007). Our results were consistent for low and high levels of SBR concentration of environmental and SR sludge samples, respectively. Therefore, the fast-molecular qPCR method developed in this

study is an improved, accurate and reproducible molecular alternative to quantify SRB in environmental and engineered sludge samples.

4 CONCLUSIONS

In the present study, a fast and robust qPCR method was improved for the quantification of SRB in sludge samples. Standard curves were plotted using genomic DNA from diverse SRB species. No interference from genomic DNA of other prokaryotic microorganisms was detected. After qPCR validation using standard curves and testing four sets of primers, primers DSR1F/RH3-dsr-R based on *drsA* gene showed more stability and accuracy. This method was validated based on acceptance criteria and it was shown to be specific, accurate and precise for the quantification of SRB in several samples from different origins.

5 ACKNOWLEDMENTS

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Table 1.1 Quantification of SRB in four dilutions of a control standard (SRB suspension) using standard curves for sets of primers DSR1F/RH3-dsr-R and RH1-dsr-F/RH3-dsr-R for *dsrA* gene, and APS7-F/RH2-aps-R and RH1-aps-F/RH2-aps-R for *apsA* gene, obtained with mixture of synthetic SRB suspension as calibrator.

Primer set	SRB concentration determined by qPCR standard curves[a] and percentage of relative error[b]							
	Equation of standard curve	Theoretical value[c] (SRB cells mL^{-1})							
		2.31E+06		2.31E+05		2.31E+04		2.31E+03	
		SRB cells mL^{-1}	$\%\mathcal{E}_t$	SRB cells mL^{-1}	$\%\mathcal{E}_t$	SRB cells mL^{-1}	$\%\mathcal{E}_t$	SRB cells mL^{-1}	$\%\mathcal{E}_t$
DSR1F/RH3-dsr-R	y = -2.23x + 30.42	2.10E+06	8.95	2.08E+05	9.89	2.46E+04	6.30	2.08E+03	9.89
RH1-dsr-F/RH3-dsr-R	y = -2.75x + 32.30	3.83E+04	98.3	1.26E+04	94.5	1.30E+03	94.4	1.07E+02	95.4
APS7-F/RH2-aps-R	y = -3.12x + 32.16	1.02E+06	55.8	3.09E+05	33.9	2.79E+04	20.9	1.28E+03	44.6
RH1-aps-F/RH2-aps-R	y = -3.03x + 31.11	8.88E+05	61.6	3.58E+05	54.8	2.65E+04	14.5	1.48E+03	35.9

a qPCR standard curves were obtained using mixture of synthetic SRB suspension as calibrator.

b Relative error was calculated as the absolute error (difference between the measured value and theoretical or true value) divide to true value.

c Theoretical or true SRB concentration was determined by was determined by plate culture.

Table 1.2 Quantification of SRB in four dilutions of a control standard (SR enriched sludge) using standard curves for sets of primers DSR1F/RH3-dsr-R and RH1-dsr-F/RH3-dsr-R for *dsrA* gene, and APS7-F/RH2-aps-R and RH1-aps-F/RH2-aps-R for *apsA* gene, obtained with serial dilutions of SR sludge coming from enriched subcultures as calibrator.

Primer set	SRB concentration determined by qPCR standard curves[a] and percentage of relative error[b]							
	Equation of standard curve	Theoretical value[c] (SRB cells mL^{-1})							
		5.63E+05		5.63E+04		5.63E+03		5.63E+02	
		SRB cells mL^{-1}	$\%\varepsilon_t$	SRB cells mL^{-1}	$\%\varepsilon_t$	SRB cells mL^{-1}	$\%\varepsilon_t$	SRB cells mL^{-1}	$\%\varepsilon_t$
DSR1F/RH3-dsr-R	y = -2.57x + 33.26	6.27E+05	11.4	5.19E+04	7.73	4.84E+03	14.1	6.05E+02	7.45
RH1-dsr-F/RH3-dsr-R	y = -2.57x + 34.51	4.55E+05	19.3	2.28E+04	59.5	1.27E+04	125	8.63E+02	53.2
APS7-F/RH2-aps-R	y = -2.97x + 32.61	3.94E+04	93.0	7.73E+03	86.3	8.72E+02	84.5	1.65E+02	70.7
RH1-aps-F/RH2-aps-R	y = -2.69x + 30.72	1.05E+04	98.1	3.58E+03	93.6	1.34E+02	97.6	1.40E+01	97.5

a qPCR standard curves were obtained using SR sludge coming from subcultures as calibrator.

b Relative error was calculated as the absolute error (difference between the measured value and theoretical or true value) divide to true value.

c Theoretical or true SRB concentration was determined by plate culture.

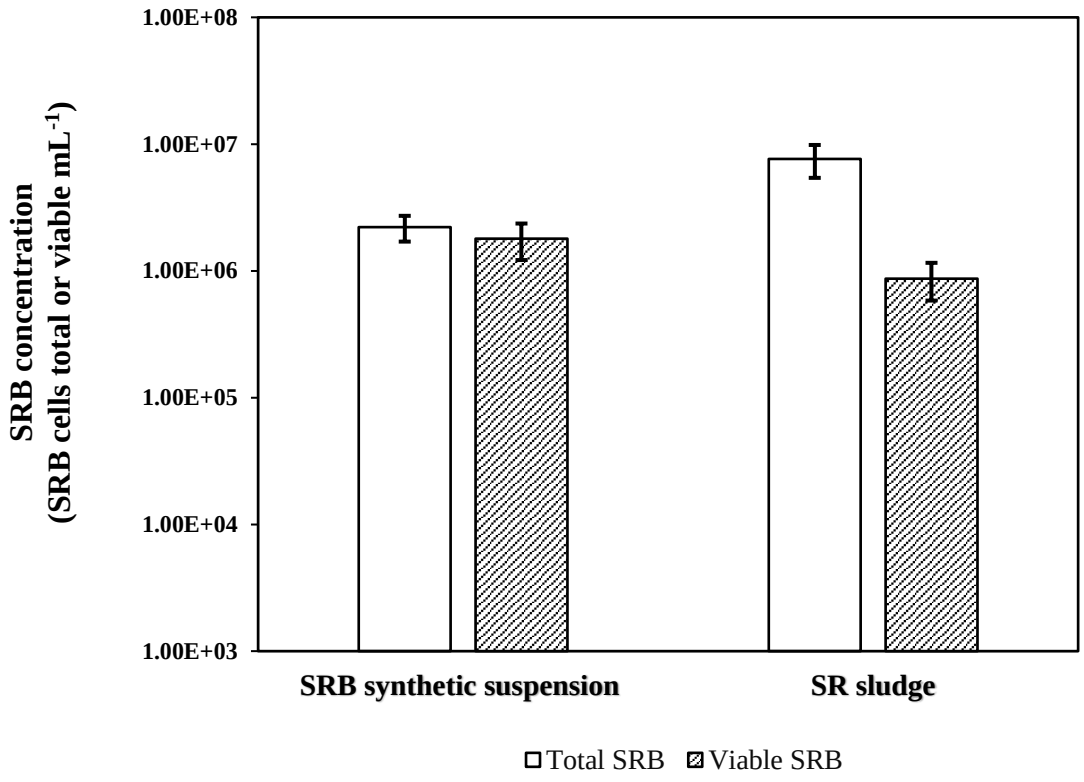

Figure 1.1 Bacterial counts using conventional methods. SRB concentration in a mixture of synthetic SRB suspension and SR sludge, using Neubauer chamber in open square (total) and by plating culture in closed square (viable). Bars represent the average of measurements, and errors bars are the standard deviations of each mean.

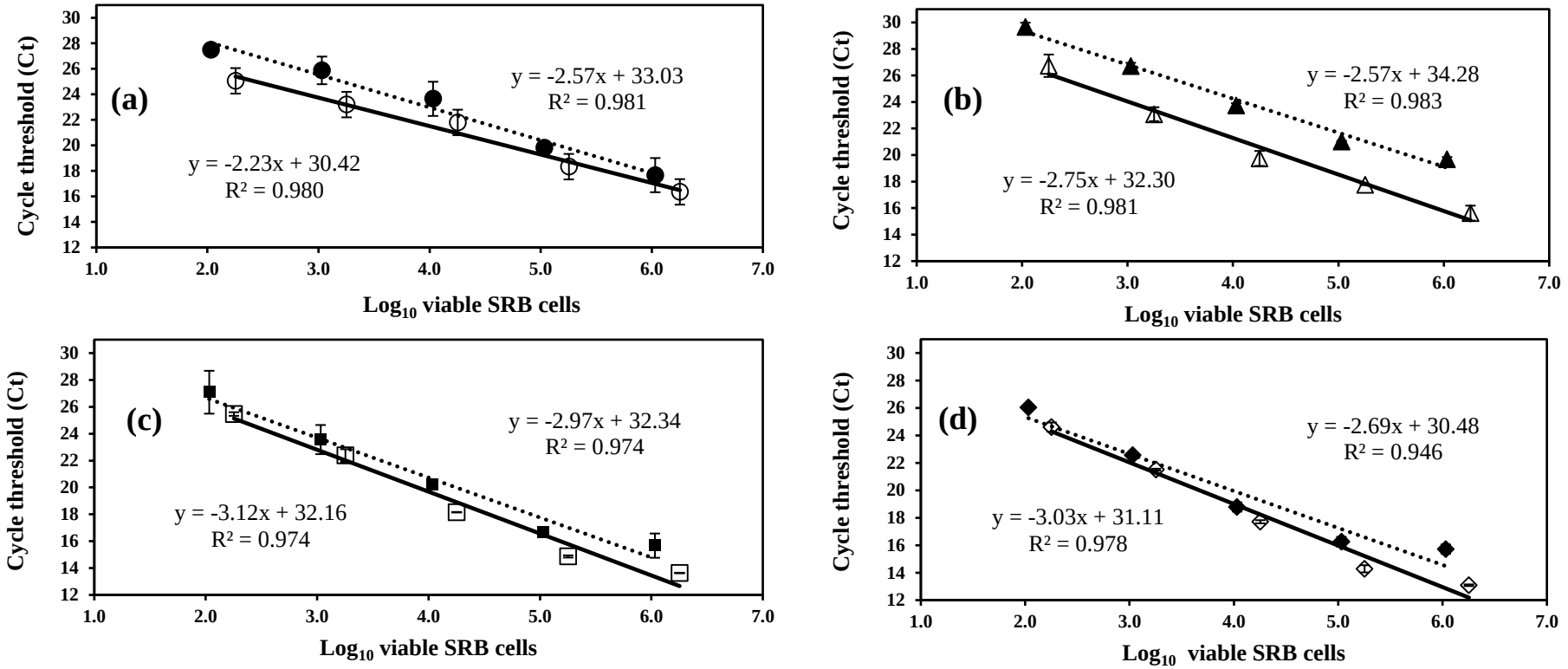

Figure 1.2 qPCR standard curves using mixture of synthetic SRB suspension (solid line, open markers) and serial dilutions of SR enriched sludge (dotted line, filled markers) as calibrators. *dsrA* gene: **a)** DSR1F/RH3-dsr-R (circles) and **b)** RH1-dsr-F/RH3-dsr-R (triangles). *apsA* gene: **c)** APS7-F/RH2-aps-R (squares) and **d)** RH1-aps-F/RH2-aps-R (diamonds). Data points represent the average of measurements and the errors bars are the standard deviations. There are not statistically significant differences between slopes of each primer set according ANOVA test (Supporting information).

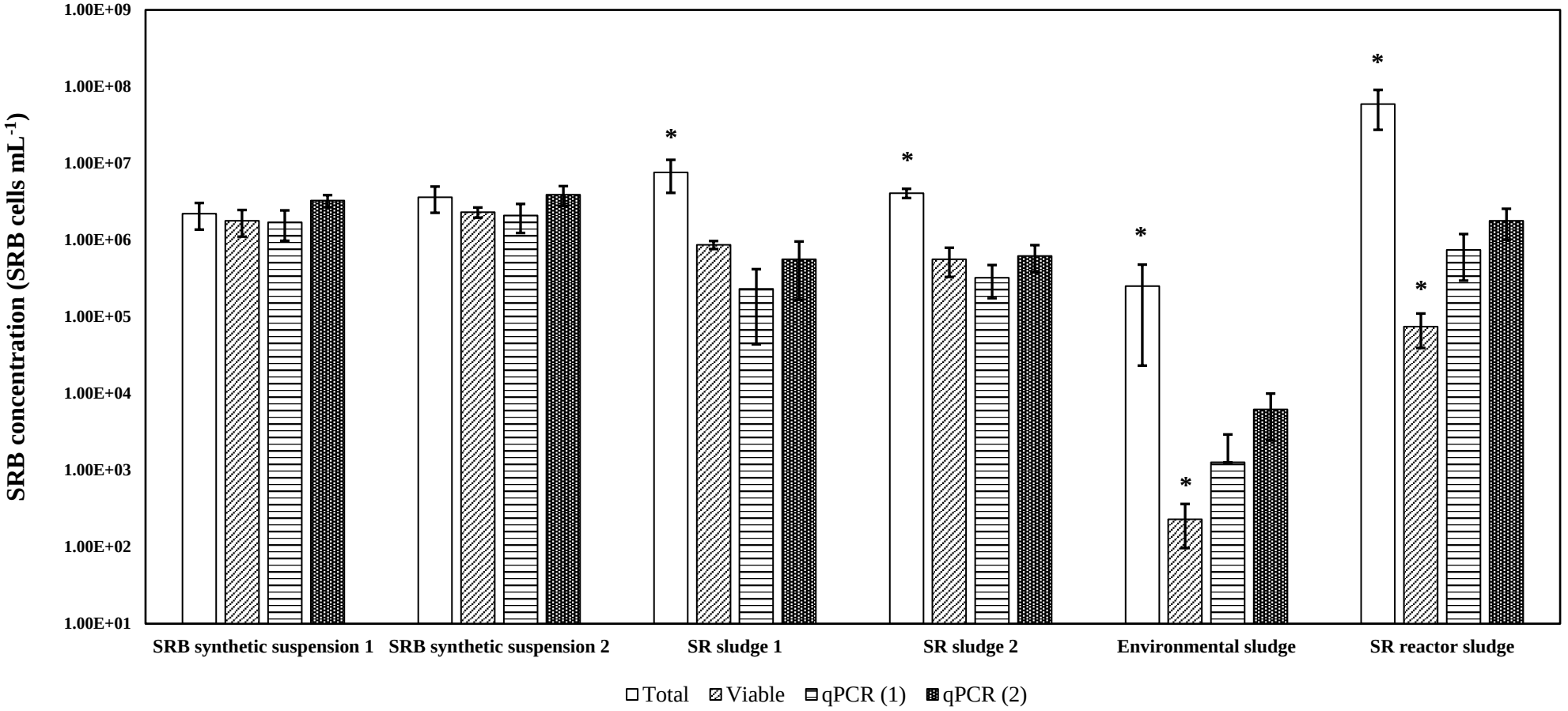

Figure 1.3 Quantification of SRB by qPCR using primers DSR1F and RH3-dsr-R and a mixture of synthetic SRB suspension (1) and serial dilutions of SR sludge (2) for standard curves plotting, Neubauer counting (total) and plate culture (viable) in four samples: a synthetic SRB suspension, two SR sludge samples, an environmental sludge and a sludge from a SR bioreactor. Bars represent the average of measurements and errors bars are the standard deviations. Asterisk (*) indicates a statistically significant differences according ANOVA test ($p<5\%$).

7 SUPLLEMENTARY INFORMATION

APPENDIX A. Characteristics of sets of primers used for qPCR analysis.

Set[a]	Primer pair[b]	Sequence (5´→ 3´)[c]	Target genes	qPCR product size (bp)	Melting point[d] (°C)
1	DSR1F	ACSCACTGGAAGCACG	*dsrA*	222	87
	RH3-dsr-R	gGTGGAGCCGTGCATGTT			
2	RH1-dsr-F	GCCGTTACTGTGACCAGCC	*dsrA*	164	87
	RH3-dsr-R	gGTGGAGCCGTGCATGTT			
3	APS7-F	GGG***Y***CT***K***TCCGC***Y***ATCAA***Y***ACATGA	*apsA*	279	89
	RH2-aps-R	ATCATGATCTGCCAgCGgCCGGA			
4	RH1-aps-F	CGCGAAGACCT***K***ATCTTCGAC	*apsA*	191	90
	RH2-aps-R	ATCATGATCTGCCAgCGgCCGGA			

a Sets of primers were designed and evaluated in study of Ben-Dov *et al* (2007).

b F: direct or forward primer. R: reverse primer.

c Boldface represents mixed bases, which are also known as degenerate or wobble bases. Lowercase represents bases that do not match appropriate sequences (NCBI, 2000).

d Values of this study.

APPENDIX B. Statistical comparison among slopes of qPCR standard curves using as calibrators: synthetic SRB suspension and serial dilutions of SR sludge coming from enriched subcultures, for four sets of primers for *dsrA* and *apsA* genes.

Set	Primer pair	Test for homogeneity of variances		t-test for equality of slopes			
		F calculated	F tabulated	Calculated t-value	Degrees of freedom, *df*	Tabulated t-value and significance 5% (2-tailed)	Comparison
1	DSR1F RH3-dsr-R	5.461	9.000	0.927	4	2.776	N.S.[a]
2	RH1-dsr-F RH3-dsr-R	2.367	9.000	1.239	4	2.776	N.S.
3	APS7-F RH2-aps-R	1.184	9.000	0.695	4	2.776	N.S.
4	RH1-aps-F RH2-aps-R	1.066	9.000	2.539	4	2.776	N.S.

[a] Differences are not statistically significant (95% confidence interval).

CHAPTER II

SCIENTIFIC PAPER 2

Changes in microbial composition during the removal of copper and zinc in a bioreactor with a limestone pre-column system

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ABSTRACT

Acid rock drainage (ARD), which is an environmental problem related to metal mining industry, causes deterioration of water sources worldwide. Bioremediation process to treat ARD is mediated by sulfate reducing bacteria (SRB) which reduces acidity, sulfate and metal concentrations. SRBs form consortia with other microorganisms to promote organic compounds degradation, nutrients assimilation and sulfate-reduction activity. Therefore, a key step for the successful implementation of SRB-based ARD bioremediation systems is to understand interactions and dynamics of these microbial populations. The objective of this research was to study the changes of microbial composition during the removal of copper and zinc from a synthetic acid mine drainage in a sulfate reducing bioreactor with a limestone pre-column system.

The treatment system was operated during 372 days and fed with synthetic AMD using acetate as carbon source and electron donor, and copper and zinc, each one in concentration of 15 mg L^{-1}. In sludge samples, SRB concentration was determined by qPCR using the DSR1F/RH3-dsr-R primer set for *dsrA* gene amplification. Additionally, by Metagenomics, the 16S rRNA genes from V3 and V4 regions were sequenced. The collected data were used to estimate alpha and beta diversity and abundance of microbial species in the sludge samples from the sulfate reducing bioreactor.

High removal efficiencies of copper (II) and zinc (II), 96.8-99.8% and 99.9%, respectively, were observed in the bioreactor, and the consumption of acetate exceeded 40% of the initial chemical oxygen demand. During the operation of the sulfate reducing bioreactor, SRB displayed a lower concentration in the first days of operation, about 1E+05 cells mL^{-1}, and they reached a value in the order of 1E+06 cells mL^{-1} at day 150, stabilizing that value until the end of operation.

According to the microbial diversity analysis of the sludge from the sulfate reducing bioreactor, the most abundant microorganisms are methanogen archaea affiliated with the genus *Methanosarcina*, however there was not methane production. Identified SRB correspond mainly to the genera *Desulfotomaculum* and *Desulfovibrio*. During operation of the sulfate reducing bioreactor with the limestone pre-column system, changes in microbial composition were observed and prokaryotic communities were gradually lesser diverse and more predictable in metabolic terms.

Keywords

Sulfate-reducing bacteria, acid mine drainage, copper, zinc, qPCR, Metagenomics, anaerobic microbial diversity

1 INTRODUCTION

Mining industry is one of the most productive activities worldwide. However, there are several environmental impacts as a consequence of mining production, small or large scale. Precisely, among these environmental impacts, the generation of acid rock drainages (ARDs) is one of the most relevant because of its heavy metal content, low pH values and high concentration of sulfate (Simate & Ndlovu, 2014) (Nieto *et al*, 2007) (Zipper & Skounsen 2014).

In nature, metals are required in small quantities for many biochemical processes; however, effluents rich in metals could pose a threat to human health because metals could generate toxicity and tend to bioaccumulate in organisms in the food chains, causing environmental deterioration and damage to human health (Bonnail *et al*, 2017). Low pH values of ARD cause increased acidity, behavioral and habitat modifications of living beings, death of sensitive species and higher solubility of metals (Gray, 1997). Sulfate is not toxic and, in general, it is considered an inert compound (Muyzer & Stams, 2008).

Bioremediation of ARD, mediated by sulfate reducing bacteria (SRB), has demonstrated to be an excellent alternative for removal of metals from effluents, through precipitation (Dar *et al*, 2006) (Muyzer & Stams, 2008). In that process, sulfate, in presence of SRB, with an electron donor compound and under anaerobic conditions, is transformed into sulfide which reacts with metals (cations) and forms metallic sulfides (Muyzer & Stams, 2008). Sulfate reduction produces metal precipitation because while sulfates of metals such as zinc, copper, cadmium or nickel are highly soluble, their corresponding sulfides have low solubility (Muyzer & Stams, 2008) (Giloteaux *et al*, 2012). In addition, as a consequence of the microbial oxidation of the electron donor, sulfate reduction generates alkalinity and increases pH (Kaksonen e*t al*. 2004) (McCauley, 2013).

Due sulfide production is driven by microorganisms, high concentrations of metals and acidity could inhibit essential SRB-assisted processes; for these cases, limestone is widely use as neutralization agent in the pre-treatment of ARDs (Sani *et al*, 2001) (Iakovleva *et al*, 2015).

Precipitation of metals driven by SRBs have proven to be efficient in the bioremediation of wastewater from mining industry, semiconductor manufacturing and groundwater treatment systems (Méndez *et al*, 2015) (Freeman *et al*, 2008) (Giloteaux *et al*, 2012) (Dar *et al*, 2006) (Pol *et al*, 2001).

Likewise, SRBs can use an organic compound or hydrogen as electron donors and sources of energy. SRB can be organotropic or chemolithotropic because they can use an organic compound or carbon dioxide as carbon source, respectively (Freeman *et al*, 2008). SRB constitute a heterogeneous microbial group affiliated into seven lineages, are ubiquitous and free-living microorganisms that form consortia with others, such as methanogen archaea and acetogenic bacteria (Muyzer & Stams, 2008). Due to the difficulty of cultivating and isolating environmental microorganisms, anaerobic bioreactors are typically inoculated with sludge from wastewater plants, lakes, swamp or marine sediments, manure, among others (Schmidtova, 2010). Typically, sludge is formed by consortia of microorganisms. Knowledge about the changes of microbial populations that conform sludge from bioreactors, the synergisms or antagonisms between members of consortia, and the interactions between contaminants, metabolites and inhibitory substances, leads current bioremediation approach; and, it is related to improvement of yield of treatment systems (Schmidtova, 2010) (Dar *et al*, 2006) (Satoh *et al*, 2013).

Different techniques have been used for enumeration and study of the diversity of microbial consortia. First, culture and microscopic techniques, also called conventional

methods have been employed; and, more recently, molecular techniques have been developed. Culture and microscopic techniques are direct and successful for microorganism enumeration; however, they are time-consuming and limited since only small fraction of environmental bacteria can be cultivated. Molecular techniques, on the other hand, are fast, provide approaches to overcome problems associated with cultured dependent methods, and allow analysis of diverse and complex communities of microorganisms from different environments (Muyzer & Stams, 2008) (Dar *et al*, 2006) (Wagner & Loy, 2002). Although promising, some of these molecular techniques involve high costs and specialized equipment, are difficult to use *in situ* or these techniques require be validated for analysis of specific type of samples (Ben-Dov *et al*, 2007) (Zamora & Malaver, 2012). Molecular techniques include fluorescence *in situ* hybridization (FISH), PCR-denaturing gradient gel electrophoresis (DGGE), DNA microarrays, stable isotope probing (SIP), real-time PCR (qPCR) and Metagenomics (Muyzer & Stams, 2008).

In the metabolic path of dissimilatory sulfate reduction, *dsrAB* genes encode the subunits alpha and beta of dissimilatory sulfate reductase that catalyzes the six-electron reduction of sulfite to sulfide (Dar *et al*, 2006). In addition, *apsBA* genes encode subunits that form a 1:1 beta alpha heterodimer of adenosine-5´-phosphosulfate (APS) reductase from bacteria. Both, *dsrAB* and *apsBA* genes, are excellent molecular markers for SRB detection and quantification because they are highly conserved. For that reason, these two genes are widely used in primers construction for SRB quantification by qPCR analysis (Ben-Dov *et al*, 2007).

In order to study of interactions, organization and, taxonomic and metabolism diversity, Metagenomics, based on amplification of variable regions of 16S ribosomal RNA (rRNA) genes, is a powerful tool for research of heterogenic microbial communities (Li *et al*, 2009)

(Plugge *et al*, 2011). 16S rRNA genes are general molecular markers for identification of prokaryotic microorganisms, which include bacteria and archaea (Muyzer & Stams, 2008).

The goal of this study was to characterize microbial diversity and changes of communities responsible of the biological treatment of ARD in a bioreactor with a limestone pre-column system fed with synthetic ARD composed of cooper (II) and zinc (II), sulfate and acetate as organic carbon source. The present research introduced a molecular approach, through qPCR and metagenomics analysis, to observe population dynamics in microbial consortia of sludge samples taken at three different periods of bioreactor operation. In addition, physical-chemical parameters were periodically analyzed to monitor performance of bioreactor with limestone pre-column system. This study is an innovative contribution to the knowledge of bioremediation applications based on sulfate reduction process mediated by microorganisms and ARD treatment in Ecuador.

2 MATERIALS AND METHODS

Basal mineral medium

The basal medium consisted of (in mg L^{-1}): NH_4Cl (280); KH_2PO_4 (195); $MgSO_4$ (49); $CaCl_2$ (10); $NaHCO_3$ (3000); yeast extract (10), Na_2SO_4 (2900), CH_3COONa (5300) and 1 mL L^{-1} of a solution of trace elements. The trace element solution contained (in mg L^{-1}): H_3BO_3 (50), $FeCl_2{\cdot}4H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2$ (32), $(NH_4)_6$ $Mo_7O_{24}{\cdot}4H_2O$ (50), $AlCl_3$ (50), $CoCl_2{\cdot}6H_2O$ (2,000), $NiCl_2{\cdot}6H_2O$ (50), $CuSO_4{\cdot}5H_2O$ (44), $NaSeO_3{\cdot}5H_2O$ (100), EDTA (1,000), resazurin (200) and 1 mL L^{-1} of HCl (36%) (Ochoa-Herrera, Banihani *et al*, 2009).

Bioreactor with a limestone pre-column operation and synthetic ARD composition

The laboratory-scale treatment system consisted of a 0.40 L limestone pre-column coupled to a 0.49 L biological reactor as described in our previous study (Méndez *et al*, 2015). The initial inoculum was obtained from an artificial lagoon at Universidad San Francisco de Quito, Ecuador. The content of total suspended solids (TSS) and volatile suspended solids (VSS) in the sludge were 52.8 and 6.2%, respectively. The biological reactor was packed with 116 g of sediments of the artificial lagoon (15 g VSS L^{-1}) and 371 g of sand with a density of 1.26 g mL^{-1} for support microbial growth (Méndez *et al*, 2015). In this study, the limestone pre-column was replaced with fresh limestone as recommended by Méndez and coworkers (2015). The column was filled with 1009 g of limestone ($CaCO_3 \geq 98\%$) pre-sieved in mesh # 8 and 16, which retained particles between 1 and 3 mm. The sieved limestone was washed to release any residual dust or impurities; and then it was dried at 90°C for 6 h in an oven (Precision Scientific, Winchester, VA, USA).

The treatment system was fed with a synthetic ARD composed of basal mineral medium supplemented with sulfate (2000 mg SO_4^{2-} L^{-1}), acetate as electron donor (e-donor) (2500 mg COD L^{-1}), copper (15 mg L^{-1}), zinc (15 mg L^{-1}) and the pH was regulated to pH 2.7, using concentrated hydrochloric acid (HCl). The temperature was maintained at 30±2°C during the operation of the treatment system. In period I, the bioreactor was operated as a stand-alone reactor, which means only bioreactor operation, without metal addition nor acidification, for 24 days with a volumetric loading rate of 2.8 g acetate-COD L^{-1} d^{-1} and a hydraulic retention time (HRT) of 2.0±0.37 days. In period II and III, the bioreactor was operated during 196 and 147 days with the limestone bed reactor as a pre-column, respectively. In period II, 15 mg L^{-1} of Cu (II) was added (as $CuCl_2.2H_2O$); whereas in period III, in addition to Cu (II), 15 mg L^{-1} of Zn

(II) was suministrated (as $ZnCl_2$). The volumetric loading rate in period II and III was of 2.8 g acetate-COD L^{-1} d^{-1} with a HRT of 2.0±0.37 days. Concentrations of copper and zinc where defined according to toxicity tests conducted in batch bioassays employing the same SRB sludge (Calderón & Ochoa-Herrera, 2016). Influent and effluent samples were analyzed for sulfate, sulfide (H_2S), total COD, conductivity, pH, soluble copper (II) and soluble zinc (II).

Physical-chemical analysis

Sulfate was determined using turbidimetric method 4500-SO_4^{2-}, by adding of $BaCl_2$. (APHA, 2012). Sulfide was analyzed colorimetrically using the methylene blue method (Truper & Schlege, 1964).

Chemical oxygen demand (COD) was determined by the colorimetric method 5220 D with potassium dichromate as described in standard methods (APHA, 2012). Samples for sulfate and COD were filtered previously through a 0.45 µm filter. COD removal and sulfate reduction were calculated as the difference between the influent and the effluent COD and sulfate concentrations, respectively.

Conductivity and pH were determined with electrodes and a portable multi-parameter Thermo Scientific Orion 5-Star (Thermo Scientific, Beverly, MA, USA) according to standard methods 2510A and 4500A, respectively (APHA, 2012).

Copper and zinc in liquid samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) Thermo Scientific ICAP 7400 (Thermo Scientific, Beverly, MA, USA), at Laboratory of Environmental Engineering, Universidad San Francisco de Quito. Calibration curves were conducted for each metal in 2% HNO_3. Samples were analyzed in triplicates, according method 3120B (APHA, 2012).

Methane (CH_4) generated in the bioreactor was measured using the liquid displacement method following biogas scrubbing through a sodium hydroxide (NaOH) solution (2%) to remove CO_2 and H_2S. The H_2S concentration in the biogas was calculated from the H_2S concentration in the liquid assuming equilibrium between phases and a dimensionless Henry's factor of 0.36 (Sierra-Alvarez, Hollingsworth *et al*, 2007). The percentage of electron equivalents of reducing power fed to the reactor (CODin, as g COD L^{-1} reactor d^{-1}) utilized for methane (% CH_4-COD) and sulfide (% H_2S-COD) generation were calculated as described in our previous publication (Sierra-Alvarez, Hollingsworth *et al*. 2007).

Chemicals

Sodium sulfate (100% purity) and sodium acetate were supplied by JT Baker Chemical Company (Phillipsburg, NJ, USA). Copper (II) chloride hydrate and ammonium iron (III) acetate (99%) was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). LOBA Chemie (Mumbai, India) supplied zinc chloride dry. Sulfuric acid (95.0-97.0%) was obtained from Merck KGaA (Darmstadt, Germany). DMP (oxalate N, N-dimethyl-p-phenylenediamine) (> 99%) was obtained from J.T. Baker (Zedelgem, Belgium). All reagents were used in the state in which they were received.

Sample collection

Six-bioreactor sludge samples were collected between June 17th of 2016 and July 6th of 2017; two biological replicates in each operation period. Samples were collected in sterile plastic recipients, opening a sampling port in the bottom of the bioreactor. Approximately 15-20 g of

material were collected for each sample. Genomic DNA form each sample was immediately extracted and maintained at −80°C prior to molecular tests.

DNA extraction

12 mL samples or standards were centrifuged at 4000 g during 30 minutes. The supernatant was discharged and settled fraction (pellet) was used for DNA extraction utilizing the utilizing DNeasy® PowerSoil® Kit (QIAGEN GmbH., Germany) according the protocol provided by the supplier but washing twice with solution C5 (Ben-Dov, Brenner & Kushmaro. 2009). The purity and concentration of the resulting DNA preparation were determined spectrophotometrically by Qubit® system (Thermo Fisher Scientific Inc. USA). DNA integrity of molecular weights up to 2000 pairs of bases (bp) was evaluated using 2.0% agarose (Bioline, London, UK) gels stained with Invitrogen SYBR® Safe DNA gel stain (ThermoFisher, Carlsbad, CA, USA) and TAE (mixture of Tris base, acetic acid and EDTA) buffer. Electrophoresis was carried out at 80 mV for 30 minutes in Gel XL Enduro^{TM} chamber (Labnet, Edison, USA) and using 2µL of DNA per well. DNA samples were maintained at −80°C prior to molecular analysis. For metagenomics analysis, DNA samples were lyophilized in Freeze Dryer (ilShinBioBase, Netherlands), at the Laboratory of Agricultural and Food Biotechnology, Universidad San Francisco de Quito.

qPCR analysis

qPCR assays were performed on PrimeQ thermal cycler system (Cole-Parmer, Staffordshire, UK), at the Laboratory of Agricultural and Food Biotechnology, Universidad San Francisco de

Quito. Primer set used for qPCR amplification of *dsrA* gene, DSR1F and RH3-dsr-R, was developed by Ben-Dov and coworkers (2007) and previously validated (Zambrano-Romero *et al*, 2018). This set of primers was manufactured by Invitrogen™ (ThermoFisher, Carlsbad, CA, USA).

Reaction mixture consisted of 7.5 µL of SsoFast™ EvaGreen® Supermix (BioRad, Hercules, USA), 150 nM each forward and reverse primers, 1.5 µL of DNA template, and PCR water to a final volume of 15 µL. Reaction conditions were 2 min at 50ºC, 15 min at 95ºC, followed by 40 rounds of 15 s at 95ºC and 1 min at 62 ºC (Ben-Dov *et al*, 2007). The stage of extension stage was 30 seconds at 72 ºC. Melting or dissociation curves analysis were performed to control specificity of qPCR method. PCR product sizes were verified with SYBR® Safe-stained 2.0% agarose gels. Electrophoresis conditions were 80 mV during 60 minutes. All runs included a blank or no-template control (PCR water: Ultrapure™ Distilled Water, Invitrogen, Grand Island, NY, USA).

Metagenomics

Analysis of the sequences of the V3 and V4 variable regions of the 16S rRNA genes, was obtained by high-throughput pyrosequencing of the six genomic DNA samples. Analysis was performed by Macrogen (Seoul, South Korea) using MiSeq Reagent Kit, an Illumina MiSeq platform (Albany, NY, USA) and MCS Sequencing Control Software; yielding a total of ∼120.3M bp of metagenomic reads.

After removing noise, a total of 94566 reads were assigned to their original samples using Quantitative Insights into Microbial Ecology (QIIME) software package version 1.9 (Caporaso *et al*, 2010), satisfying the quality criteria (length <300 bp and 800 bp). The

downstream primer region was manually removed. Reads in the reverse direction were reverse complemented and then combined with reads in the forward direction.

The combined reads were clustered into their operational taxonomic units (OTUs) with QIIME 1.9 at 97% sequence similarity.

Microbial diversity and statistical analysis

After removing singletons and those OTUs with lesser than 100 sequences, relative abundance was calculated dividing each number of sequences by total number of sequences. Alpha and beta diversity indices were calculated using QIIME 1.9. Alpha (α) diversity was calculated through Shannon index for diversity and Chao1 index for richness; while, beta (β) diversity was calculated through Bray-Curtis index.

Shannon or entropy index (D) (Wolda, 1981), Chao1 or richness (S_{Chao1}) (Jost *et al*, 2010) and Bray-Curtis o similarity index (S_{BC}) were calculated as follows (Wolda, 1981):

$$D = \sum_{s=1}^{S} \left(\frac{n_s\,(n_s - 1)}{N\,(N-1)} \right) \qquad \text{[Eq.1]}$$

$$S_{Chao1} = S_{obs} + \frac{F_1^2}{2F_2} \qquad \text{[Eq.2]}$$

$$S_{BC} = 1 - \frac{\sum_{s=1}^{S} |M_{i1} - M_{i2}|}{\sum_{s=1}^{S} (M_{i1} + M_{i2})} \qquad \text{[Eq.3]}$$

where, N = total number of sequences; n_s = number of sequences of species *s*; *S* = total number of species; S_{obs} = N of species in the sample; F_1 = number of singletons; F_2 = number of doubletons; M_{i1} = number of individuals of species *i* in sample 1; M_{i2} = number of individuals of species *i* in sample 2; *S* = total number of species

Diversity statistics was conducted using non-metric multidimensional scaling plots (NMDS) performed in Phyloset in R package (R Core Team, 2013), thorough Bray-Curtis and Canberra distance. Stress values were lesser than 0.2. Comparisons of prokaryotic communities in the course of operation periods I, II and III of the bioreactor with a pre-column system were done through parametric statistics in SPSS Statistics software by Fisher´s test and analysis of variance (one factor ANOVA).

3 RESULTS AND DISCUSSION

Performance of the bioreactor with a limestone pre-column system

Performance of the laboratory-scale bioreactor with a limestone pre-column treatment system was assessed through reduction of sulfate, increase of sulfide production, COD removal, methanogenesis, and copper and zinc removal. pH increase from 2.7 to 7.4 was monitored in the effluent of the system as a measure of the neutralization capacity of limestone pre-column and the increment of alkalinity in the SRB bioreactor due to the production of bicarbonate by oxidation of acetate during microbial sulfate reduction (McCauley, 2013) (Figure 2.1).

Méndez *et al* (2016) reported continuous increase of sulfate reduction reaching a maximum value of 41%, when synthetic ARD was supplemented with copper (II)

concentrations of 10, 20, 30 and 40 mg L^{-1} in the same SRB bioreactor. In addition, Kieu *et al* (2011) reported that sulfate-reducing activity is not affected in presence of copper (II), zinc (II), nickel (II) and chromium (VI) when concentrations are lower than 150 mg L^{-1} of each metal and reactor is operated with a hydraulic retention time of 20 days for 12 weeks. Other studies indicated that use of zero-valent iron (ZVI) promotes sulfate-reduction and bioprecipitation of other metal such as copper (II), cadmium (II) and lead (II) (Dinh *et al*, 2004) (Ayala-Parra *et al*, 2016).

Figure 2.2 shows the time course of sulfate reduction and sulfide production in the sulfate-reducing bioreactor fed with a pH-2.7 synthetic ARD during the three periods of operation of the treatment system. In the same manner, Table 2.1 presents average concentrations of sulfate in the influent and effluent, including the biogenic sulfide formation expressed as mg H_2S-S L^{-1}. In the time course of period I, adaptation phase, the SRB bioreactor operated stand-alone and it was fed with basal mineral medium. During this period, sulfate reduction achieved a maximum performance of 42.5% at day 15. In period II, when 15 mg L^{-1} of copper (II) were added, sulfate removal was 39.1%. In period III, when 15 mg L^{-1} of copper (II) and 15 mg L^{-1} of zinc (II) were added simultaneously, sulfate removal efficiency was 40.7%. No significant differences between the sulfate reduction efficiencies during the three operation periods were observed. Sulfate reduction is the best indicator of SRB activity and it is related to the capacity of treatment system to promote bioprecipitation of metals (Chen *et al*, 2008); therefore, our results suggest that growing and sulfidogenesis have not been affect by presence of copper and zinc.

Calderón and Ochoa-Herrera (2016) studied the inhibitory concentrations of copper (II), zinc (II) independently and together during the sulfate-reducing activity of the microorganisms

present in the sludge inoculated in the bioreactor with the limestone pre-column system. Those results indicated that 15 mg L^{-1} of copper (II) cause an inhibition of approx. 30% to the organisms responsible of the sulfate reducing activity. While 15 mg L^{-1} of copper (II) and 15 mg L^{-1} of zinc (II) causes lesser than 20% of inhibition. In addition, depending of metals mix, synergistic or antagonistic effects influence on anaerobic digestion (Chen *et al*, 2008). Despite that, no inhibitory effect of copper (II) and zinc (II) was observed during periods II and III of operation of the SRB bioreactor in the current study. This fact could be attributed, firstly, to the low concentrations of these metals in the bioreactor since in the limestone pre-column occurs about 50% metal precipitation as hydroxides and carbonates (Ayala-Parra *et al*, 2016). And, secondly, a possible chemisorption in the solid phase (sand) in the bioreactor, which provides support material and protection to anaerobic microorganisms of the inoculated sludge through biofilm forming (Jarrell & Saulnier, 1987).

Table 2.1 summarizes the fraction of organic substrate acetate, as %COD, used primarily by SRB for H_2S production (no methane was detected). The organic COD removal efficiency averaged approximately 70% in all operation periods with acetate concentrations ranging from 554 to 342 mg COD L^{-1} in the treated effluent. While, sulfate reduction removal was 48.7, 50.9 and 42.5% of organic COD during periods I, II and III, respectively.

The absence of methane in the SRB bioreactor is not surprising taken into consideration that SRB and methanogens compete for common organic and inorganic substrates (Chen *et al*, 2008). Typically, the organic substrate affinity of the SRB for acetate is ten-fold higher than that of methanogens; consequently, the result of this competition is the methanogenesis inhibition (Muyzer & Stams, 2008). On the other hand, a secondary inhibition effect is due to toxicity of sulfide produced by sulfate-reducing bacteria over other microbial groups (Chen *et*

al, 2008). For example, Alvarez-Sierra *et al* (2007) reported that an excess of sulfate, ratios upper than 0.6 g COD / g SO_4^{2-}, and high pH levels ranging from 7.4 to 8.0, promote the dominance of SRB over methanogens. In addition, the presence of copper and/or zinc, in periods II and III, could affect methanogens and other microorganisms, through different mechanisms, such as production of reactive species of oxygen, transmembrane interferences that affect nutrient and energy transport, among others, consequently, methanogenesis could be affected (Chen *et al*, 2008) (Gonzalez-Estrella *et al*, 2015). Figure 2.3 illustrates the concentrations of soluble Cu (II) and soluble Zn (II) in influent and effluent as a function of operation time of the sulfate-reducing bioreactor with the limestone pre-column system. In addition, Table 2.2 shows average performance during the three periods of operation. Limestone pre-column drives AMD neutralization and promotes metal precipitation as carbonates and hydroxides (Medírcio *et al*, 2006) (Ayala-Parra *et al*, 2016). Copper removal, achieved in the limestone pre-column, was 54.5 and 50.3% in period II and III, respectively; while zinc removal was 48.2% during period III.

In the sulfate reducing bioreactor, the average concentration of soluble copper (II) was reduced from 6.98 to 0.22 mg L^{-1} in period II and from 7.54 to 0.01 mg L^{-1} in period III. Therefore, copper (II) removal efficiencies of the complete system was 98.5 and 99.9%, in periods II and III, respectively. In the case of zinc removal, 51.8% occurred in the bioreactor; consequently, zinc (II) removal efficiency of the complete system was close to 100% during period III.

Other studies have reported high metals removal efficiencies during ARD treatment through bioprecipitation. Sierra-Alvarez *et al* (2007) reported copper (II) removal efficiencies higher than 99% in a system integrating a sulfate-reducing reactor with a fluidized bed

crystallization reactor for semiconductor manufacturing wastewater treatment. Ayala-Parra *et al* (2016) obtained high removal efficiencies of copper (II), cadmium (II) and lead (II) in bioremediation of acid rock drainage in flow-through columns testing zero-valent iron (ZVI). In bioreactors for AMD treatment, fed with very high initial concentrations of metals, excellent efficiencies in the order of 90% and higher have been obtained during the bioprecipitation of Cu (II), Zn (II), Cd (II), Pb (II), Ag (II), and Fe (II) (Neculitaa *et al*, 2006).

Our results show that the biogenic sulfide production was sufficient to assure copper (II) and zinc (II) removal by bioprecipitation (formation of insoluble sulfides). Nevertheless, pH neutralization and abiotic metal precipitation that occurs in limestone pre-column, significantly contributes to heavy metals immobilization (Ayala-Parra *et al*, 2016). For that reason and according to the recommendations of Méndez *et al* (2015), the limestone in the bed reactor should be replaced when is exhausted by dissolution or by encrustation with metals compounds and gypsum.

Quantification of SRB

In the current study, quantification of SRB present in the bioreactor with the limestone pre-column system was done using qPCR assays and SybrGreen detection, based on *dsrA* gene amplification. The standard curve, with DSR1F and RH3-dsr-R primer set, was constructed using serial dilutions of SR sludge, the same matrix of the samples. Quantification method was previously validated as described in our previous work (Zambrano-Romero *et al*, 2018). Samples were collected from the sulfate reducing bioreactor and their DNA was extracted as already described in Materials and Methods section.

Figure 2.3 shows the concentration of SRB in six analyzed samples during the three periods of operation of the laboratory-scale bioreactor with the limestone pre-column treatment system. The measured values were transformed to SRB cells mL^{-1} using the standard curve obtained in the validation of the qPCR method.

During period I or adaptation phase, there were in average 4.82E+04 SRB cells mL^{-1} at day 15 and 2.61E+05 SRB cells mL^{-1} at day 21. These values are comparable with previous reports of SRB in granular sludge from a full-scale anaerobic reactor treating paper mill wastewater and using acetate and sulfate as substrates, whose bacterial concentrations enumerated by the most probable number (MPN) ranged from 4.10E+04 to 4.60E+05 SRB cells mL^{-1} (Oude Elferink *et al*, 1998). Our results are also consistent with SRB concentrations reported in samples from high salinity industrial wastewater evaporation ponds in the Negev desert, Israel (about 10E+04 to 10E+08 SRB cells per mL depending on seasonal conditions); using qPCR with SybrGreen detection and standard curve with DSR1F and RH3-dsr-R, for SRB enumeration (Ben-Dov *et al*, 2007).

During period II, the SRB concentrations were 2.39E+06 SRB cells mL^{-1} at day 181 and 3.17E+06 SRB cells mL^{-1} at day 217. Finally, during period III, the enumerated SRB were 4.33E+06 SRB cells mL^{-1} at day 356 and 2.22E+06 SRB cells mL^{-1} at day 372. The concentration of SRB increased in period I and while it was stable during periods II and III. Figure 2.3 shows significant differences between both samples of period I, while all SRB concentrations of sludge samples during periods II and III are statistically similar. Unlike period I, periods II and III were characterized by the presence of 15 mg L^{-1} of Cu (II), and 15 mg L^{-1} of Cu (II) plus 15 mg L^{-1} of Zn (II), respectively. Although, toxic effects of heavy metals over anaerobic microorganisms cause upset by changes in function and structure of enzymes that

intervene in different metabolic pathways (Chen *et al*, 2008). Our findings suggest that the tested metal concentrations have not significantly affected to the sulfate-reducing process, nor SRB growing. In fact, SRB populations directly increased in relation to the operation time until reaching a maximum concentration of approximately three million of SRB per milliliter, independently of the concentrations of metals in the synthetic ARD.

Metal toxicity attenuation could be explained by the fact that microorganisms could develop defense mechanisms, such as exclusion by barrier permeability, sequestration, enzymatic transformation, metal reduction to less toxic forms and efflux mechanisms, some of them plasmid-mediated or by chromosomal determinants (Besaury *et al*, 2012) (Gillian, 2016). A deeper biological knowledge of microbial resistance strategies requires evolutionary genetics, transcriptomics and metabolomics approach.

Finally, no correlations between SRB populations and remediation performance of the sulfate reducing bioreactor were observed in this study. The reason for that can be explained in that passive sulfate-reducing systems for ARD treatment are successful operating under a wide range of conditions, configurations and scales (Neculitaa *et al*, 2006). Therefore, it is expected that stabilization of the treatment process play a preponderant role on microbial dynamics.

Microbial community analysis

A total of 43 992 OTUs were obtained but after exclusion of singletons and OTUs with lesser than 100 sequences, 248 OTUs remained for microbial community analysis.

Figure 2.5 shows the relative prokaryotic abundances, in phyla, order and/or family level, of six sludge samples obtained during the three different operation periods of the sulfate

reducing bioreactor with the limestone pre-column system. The most common OTU corresponds to the phylum Euryarchaeota, order Methanosarcinales, dominated by specie *Methanosarcina mazei*, which represented 100% of the total archaeal community and relative abundance was ranging from 9% to 36% in microbial communities present in the sludge samples. Followed by members of the phylum Bacteroidetes, family BA008, that increased from 0.01% to 12%. SRB population was constituted by members of Firmicutes, Chloroflexi, Synergistetes and Proteobacteria, which together represent 1.5% to 11% microbial communities. Phylum Thermotogae was present, containing 0.02% to 6.51% of sequences. Belonging to the family Thermovirgaceae and members of phylum Synergistetes, non-SRB, were not detected in sludge samples during period I or adaptation phase, but gradually increased their relative abundance until ∼5% with the operation time of the treatment system. Other grouped 239 OTUs, including Proteobacteria, Spirochaetes, Tenericutes, among other phyla, reduced from 90% to ∼40% in the time course of operation of bioreactor with the limestone pre-column system. Unassigned OTUs were also detected in all samples, but they were not upper than 0.1% of sequences.

In accordance with studies from sulfate-reducing sludge samples, methanogens archaea and few bacterial phyla were present, mainly Bacteroidetes, Firmicutes, Chloroflexi and Proteobacteria (Paulo *et al*, 2017) (Freeman *et al*, 2007). A loss of microbial diversity can be attributed to addition of sulfate together with metals, due some microorganisms in anaerobic bioreactors are affected and just a minor number of them are capable to resist those environmental conditions (Paulo *et al*, 2017).

Méndez *et al* (2015) reported the presence of genera *Methanosarcina* (15% abundance) and *Methanosaeta* (3% abundance) among others, in sludge samples of the sulfate bioreactor

with the limestone pre-column system. That means that during seven months of operation previous to current study, *Methanosarcina mazei* completely dominated the archaeal community, displacing all other methanogens. De Vrieze *et al* (2012) indicated that growth kinetics of *Methanosarcina* spp. is higher than those *Methanosaeta* species in mesophilic anaerobic digestion with acetate concentrations upper than 100 mg L^{-1}. Differences of growing rates would cause dominance of *Methanosarcina* populations over other methanogen species in archaeal community. In this study, the archaeal community is mainly constituted by *Methanosarcina mazei*, which is the most abundant OTU in the prokaryotic communities of all sludge samples evaluated. The archaeon *Methanosarcina mazei* belongs to the group of acetoclastic methanogens that are robust and tolerant against different stressors compared to other methanogens (De Vrieze *et al*, 2012).

The following three possible scenarios could hypothesize the absence of methane: **(1)** Metal concentrations could have affected the metabolic pathways related to methane production, but not necessarily methanogens growing (Paulo *et al*, 2017). Suggesting that methanogens improved their fitness under environmental conditions of biorreactor and successfully grew, although they did not drive the methanogenesis. **(2)** The second proposed scenario is the biological oxidation of methane, also called methanotrophy, which has been little studied and is produced by a combination of biotic and abiotic factors. Methanotrophy is driven by methanotrophs that live in a wide range of environments, including marine and freshwater sediments, soils, sludge, landfills, among others. In fact, more than 50% of annual production of methane in the oceans and soils is oxidized by anaerobic methanotrophs. Some bacterial genera affiliated with Gammaproteobacteria and Alphaproteobacteria have been identified as methanotrophs and more recently anaerobic methanotrophic archaea (ANME)

have been described as methanotrophic microorganisms (Serrano-Silva *et al*, 2014). **(3)** The third option is the reverse methanogenesis. This chemical mechanism occurs when SRB consume entirely hydrogen, then methane concentration increase and the reverse reaction is thermodynamically possible (Serrano-Silva *et al*, 2014; Tate, 2015).

In this study, SRB populations were constituted by species belonging to the genera *Desulfotomaculum*, SHD-231 from Anaerolinaceae family, HA73 from Dethiosulfovibrionaceae family, *Desulfovibrio* and members unidentified of the family Desulfobacteraceae. Which is, in general, consistent with predominant taxa identified in our previous study in the sludge of the bioreactor with the limestone pre-column system (Méndez *et al*, 2016). *Desultomaculum* and *Desulfovibrio* species are capable of using hydrogen and a wide range of organic compounds that includes ethanol, formate, lactate, pyruvate, succinate and malate. Typically, both are incomplete oxidizers of acetate, which means that they do not degrade organic compounds completely to carbon dioxide (Muyzer & Stams, 2008). Species of *Desulfotomaculum* genus are more resistant to thermic stress, drought, and exposure to air than species of other genera (Morasch *et al*, 2004).

Genus SHD-231 has been reported as part of ruminal microbiota of sheep in West Africa (Omoniyi *et al*, 2014) and cows in Brazil (Soares, 2016). Genus HA73 was found in bacterial community composition of a stable operating mesophilic bench-scale dairy manure digester where copper sulfate was added (Jordaan *et al*, 2015). In addition, HA73 has been reported in granular sludge of anaerobic treatment of pulp mill (Yang, 2015).

Members of the family Desulfobacteraceae are mainly found in freshwater, brackish water, marine, and haloalkaline habitats. Most of them are complete oxidizers of organic substrates to CO_2 and they can be mesophilic or psychrophilic SRB (Kuever, 2014).

Opposite to what was expected, acetate addition to ARD, as organic substrate, and the presence of sulfate and the metals did not decrease methanogens populations versus SRB, suggesting that the sulfide generated as product of sulfate-reducing process, diminished the toxic effects of metals (Paulo *et al*, 2017). However, methane production was not detectable.

Microbial community of sludge samples in the three different periods of operation showed to be consistently anaerobic and oxygen exposure was not significant since aerobic, facultative aerobic were not present. During study of microbial community of two field-scale sulfate-reducing bioreactors treating mine drainage, Hiibel *et al* (2008) demonstrated that the exposure to oxygen resulted in presence of *Thiobacillus* spp. and *Desulfovibrio aerotolerans* in a bioreactor known to have experienced repeated aerobic condition.

Alpha (α) diversity is a measure of the entropy of a biological system and species richness. Thus, a community in which every organism is different or not redundant, have maximum entropy (Bent & Forney, 2008). Instead, beta (β) diversity represents the degree of similarity between two biological systems and it refers to the heterogeneity of their composition (Tuomisto, 2010).

To estimate α diversity, we calculated Shannon or entropy index using Equation 1 and Chao1 index using Equation 2 (data not shown), respectively. These indices did not present significant difference in the overall OTUs between the sludge samples obtained in the three operation periods of bioreactor with the limestone pre-column system.

Bray-Curtis similarity index is one of the most frequently used to calculate the abundance-based compositional similarity measure of β diversity (Jost *et al*, 2010), which is obtained according to Equation 3. Then, a matrix of pairwise distances or dissimilarities is calculated to analyze multivariate data and differentiation between two sets of quantitative

variables (Warton *et al*, 2012). Bray-Curtis index is the complement of normalized Manhattan distance (Jost *et al*, 2010). Figure 2.6 shows microbial diversity statistics using non-metric multidimensional scaling plot (NMDS) that represents Bray-Curtis and Canberra distances. These graphics revealed significant differences in microbial composition of two sludge samples obtained during period I or adaptation phase. Otherwise, there were no significant differences in microbial communities between samples of periods II and III, showing cluster patterns in each case.

Bray-Curtis and Canberra are measures of dissimilarity considered suitable for microbial species abundance data. While an average of pair-wise Bray-Curtis values, based on Euclidian or Manhattan distance, represent an overall similarity measure that do not consider abundances, Canberra distance submits higher stress conditions, such as abundances, for differentiation of samples (Jost *et al*, 2010). So, in Canberra distance plot of Figure 6 is evidenced a better differentiation among assemblages. Consequently, during operation of the sulfate reducing bioreactor with the limestone pre-column system, prokaryotic community changes throughout stabilization of the sulfate-reducing process under anaerobic conditions.

4 CONCLUSIONS

Results of the present study confirm that the sulfate reducing bioreactor with the limestone pre-column system is a successful technology for the removal of copper (II) and zinc (II) from synthetic ARD using acetate as organic carbon source and sulfate. Metals were removed both in the limestone pre-column and in the sulfate reducing bioreactor, showing metal removal efficiencies higher than 99.9% for the complete system. Sulfate reduction driven by SRB

removed 49.4, 51.3 and 42.4% of organic COD, while methanogenesis was not observed. In addition, pH increased from 2.7 to approximately 7.5, mainly by neutralization in the limestone pre-column.

SRB concentration in sludge samples increased from the beginning of operation, reaching maximum values of 3.0E+06 cells mL^{-1}. These results indicate that at tested conditions, inhibition effects due to the presence of metals were not observed in the sulfate reducing bioreactor.

Archaeal and bacterial populations constituted prokaryotic community. The most abundant OTU (9-36%) corresponds to *Metanosarcina mazei* and represents 100% of the total archaeal community. Bacterial community includes members of phyla Bacteroidetes, Firmicutes, Chloroflexi and Proteobacteria. SRB populations were represented mostly by genera *Desultomaculum and Desulfovibrio*. Analysis of taxon diversity demonstrated changes throughout stabilization of the sulfate-reducing process under anaerobic conditions. Gradually, microbial community in sludge of biorreactor became less diverse and, phenotypically and metabolically more predictable.

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Table 2.1 Average performance of the sulfate-reducing bioreactor with the limestone pre-column during the various periods of operation.

Period	Affluent Sulfate (mg S L^{-1})	Effluent Sulfate (mg S L^{-1})	Time of operation[b] (d)	Effluent pH	H_2S (mg H_2S L^{-1})	%CODin[a]		
						Formed H_2S	CH_4	Organic COD removal
I[c]	672.0 ± 4.3	414.8 ± 19.1	24	8.04 ± 0.36	160.4 ± 21.4	49.4 ± 7.1	0.0 ± 0.0	68.1 ± 7.48
II	669.1 ± 7.5	414.9 ± 95.9	196	7.45 ± 0.15	191.0 ± 20.8	51.3 ± 12.6	0.0 ± 0.0	74.0 ± 5.09
III	667.2 ± 2.9	341.9 ± 85.1	152	7.43 ± 0.21	158.4 ± 19.1	42.4 ± 10.1	0.0 ± 0.0	73.0 ± 4.82

[a] Values are expressed as percentage of the initial wastewater COD (CODin).
[b] Days of each period. Time total of operation: 372 days.
[c] Adaptation, stand-alone reactor.

Table 2.2 Concentration of soluble copper (II) and zinc (II) in the influent and average removal of both metals attained by the sulfate-reducing bioreactor with the limestone pre-column during the various periods of operation.

Period	Time of operation[a] (d)	Influent metal concentration (mg L-1)		Removal of soluble metal (%)					
				Limestone reactor		Bioreactor		Complete system	
		Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
I	24	-	-	-	-	-	-	-	-
II	196	15.33 ± 0.37	-	54.5 ± 0.8	-	96.8 ± 0.8	-	98.5± 0.6	-
III	152	15.17 ± 0.35	15.54 ± 0.60	50.3 ± 1.9	47.1 ± 0.7	99.8 ± 0.9	99.9± 1.0	99.2 ± 0.4	>99.9± 1.0

[a] Days of each period. Time total of operation: 372 days.

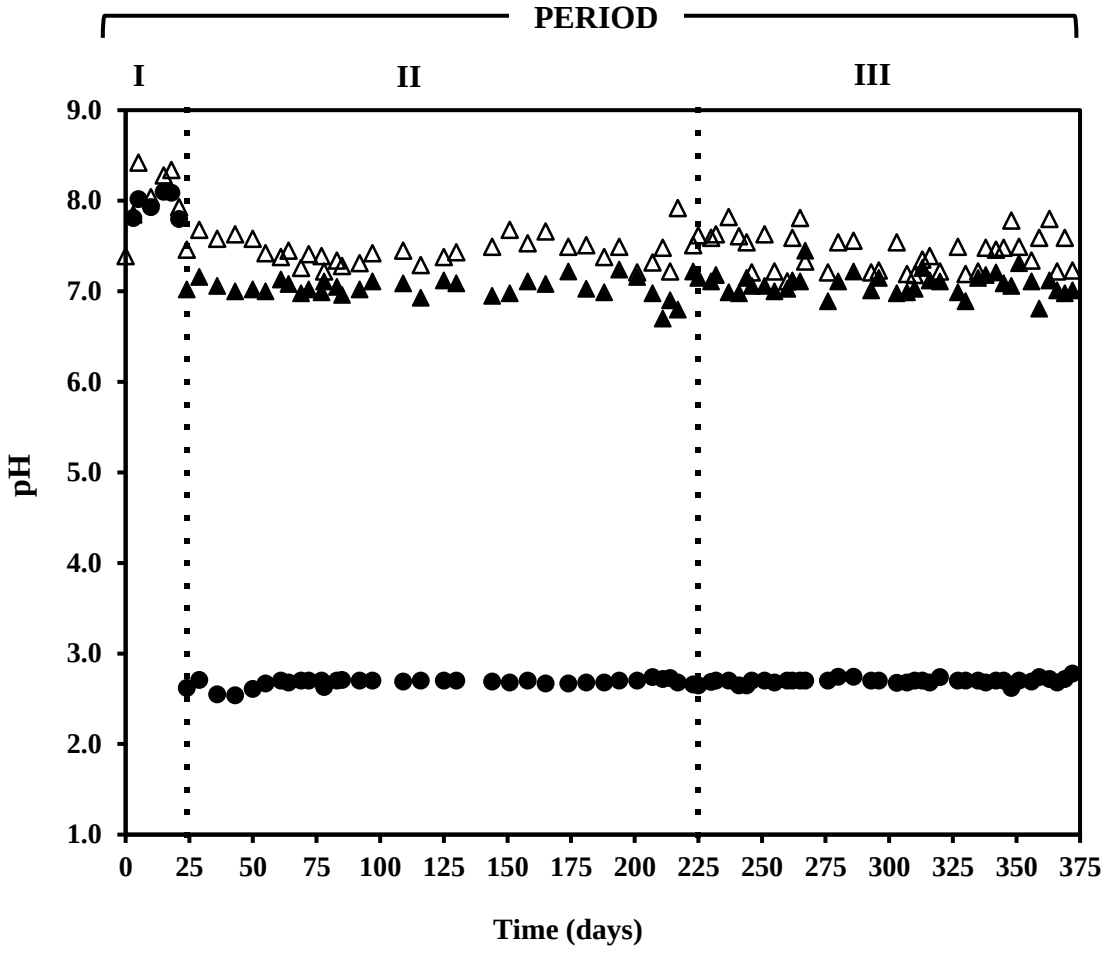

Figure 2.1 Time course of pH variation in the sulfate-reducing bioreactor with a limestone pre-column system fed with a pH-2.7 synthetic ARD containing sulfate (2000 mg L^{-1}), acetate as electron donor (2.5 g COD L^{-1}), copper II (15 mg L^{-1} during periods II and III) and zinc II (15 mg L^{-1} during period III): limestone pre-column influent (●), limestone pre-column effluent/bioreactor influent (▲), and bioreactor effluent (∆).

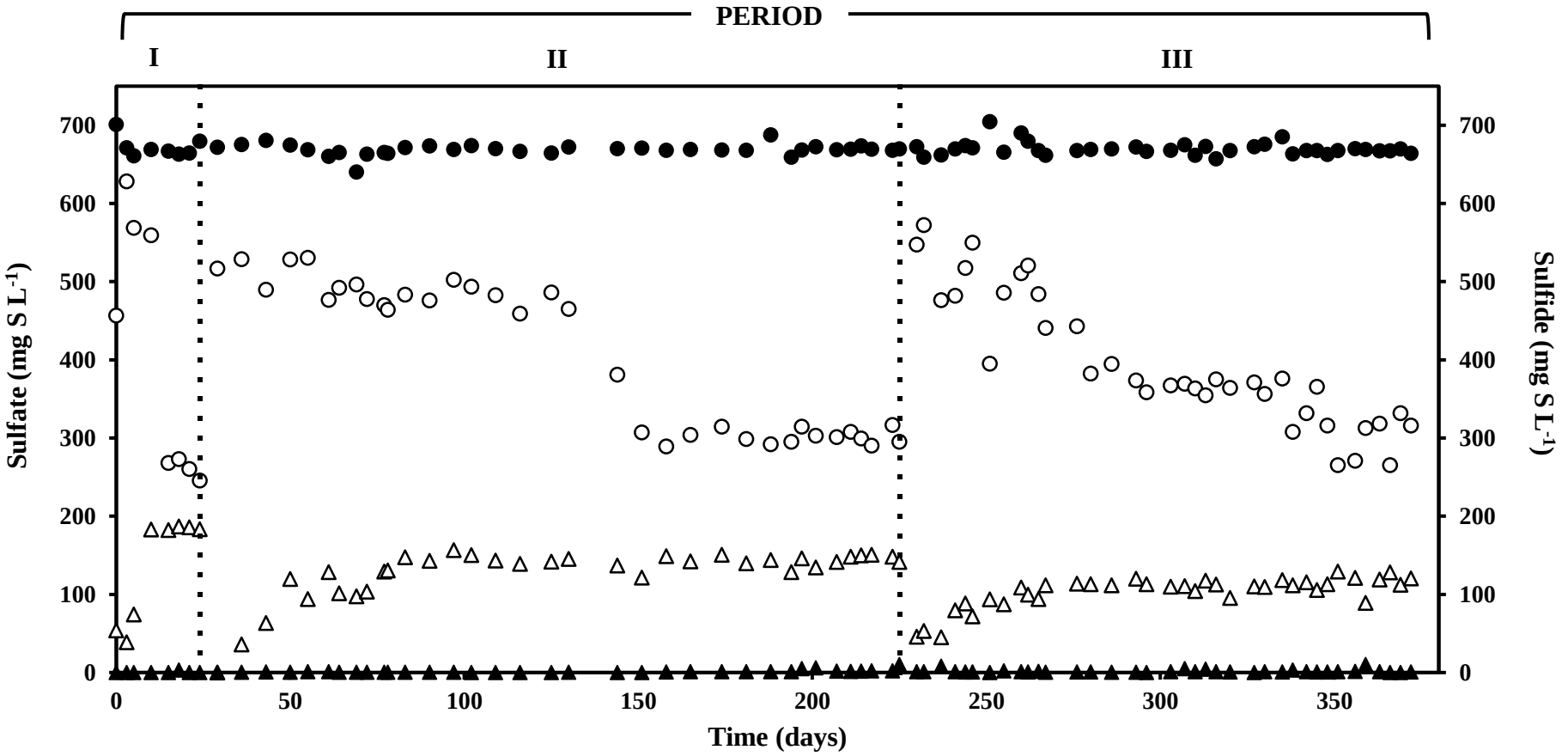

Figure 2.2 Time course of sulfate reduction (primary axis) and sulfide production (secondary axis) in the sulfate-reducing bioreactor fed with a pH-2.7 synthetic ARD containing sulfate (2000 mg L^{-1}), acetate as electron donor (2.5 g COD L^{-1}), copper II (15 mg L^{-1} during periods II and III) and zinc II (15 mg L^{-1} during period III): sulfate (●) and sulfide (▲) in the influent and sulfate (○) and sulfide (Δ) in the effluent.

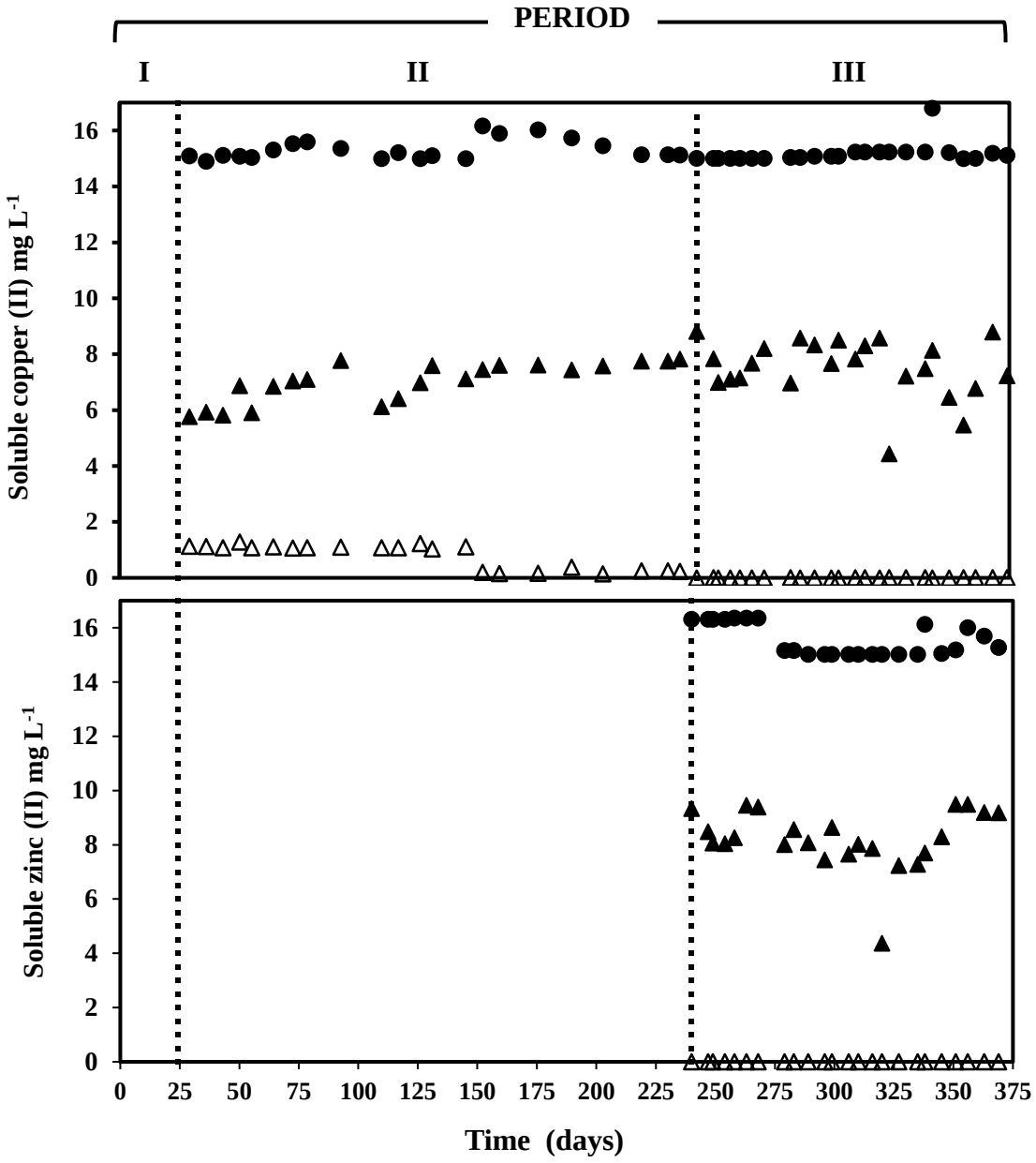

Figure 2.3 Time course of concentration of soluble Cu (II) and soluble Zn (II) in the sulfate-reducing bioreactor with a limestone pre-column system fed with a pH-2.7 synthetic ARD containing sulfate (2000 mg L^{-1}), acetate as electron donor (2.5 g COD L^{-1}), copper (II) (15 mg L^{-1} during periods II and III) and zinc (II) (15 mg L^{-1} during period III): limestone pre-column influent (●), limestone pre-column effluent/bioreactor influent (▲), and bioreactor effluent (Δ).

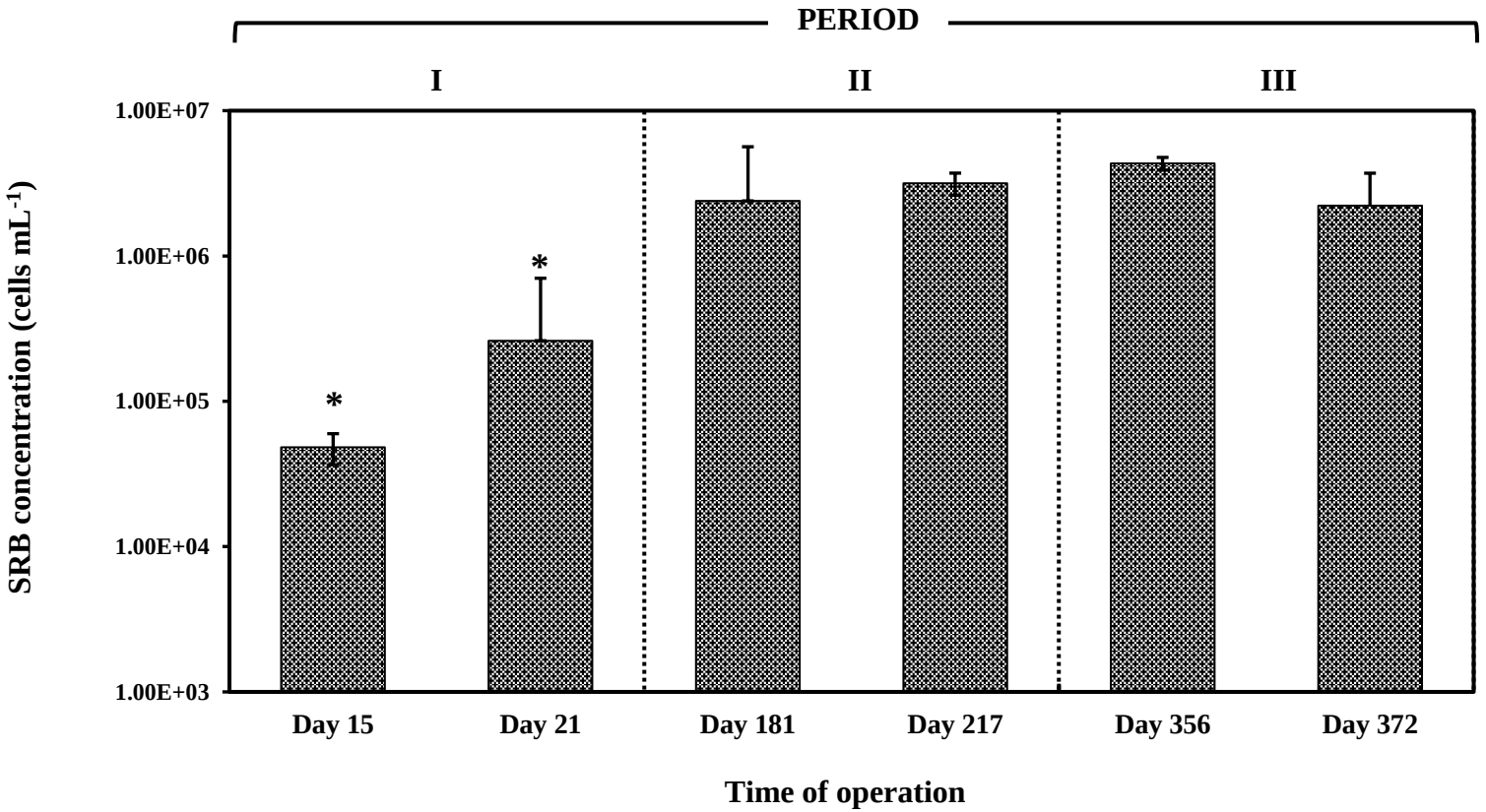

Figure 2.4 Enumeration of SRBs in sludge samples by qPCR during three different operation periods of the sulfate reducing bioreactor with the limestone pre-column system. Bars represent the average of measurements and errors bars are the standard deviations. Asterisk (*) indicates a statistically significant difference from the resting values according ANOVA test ($p<5\%$).

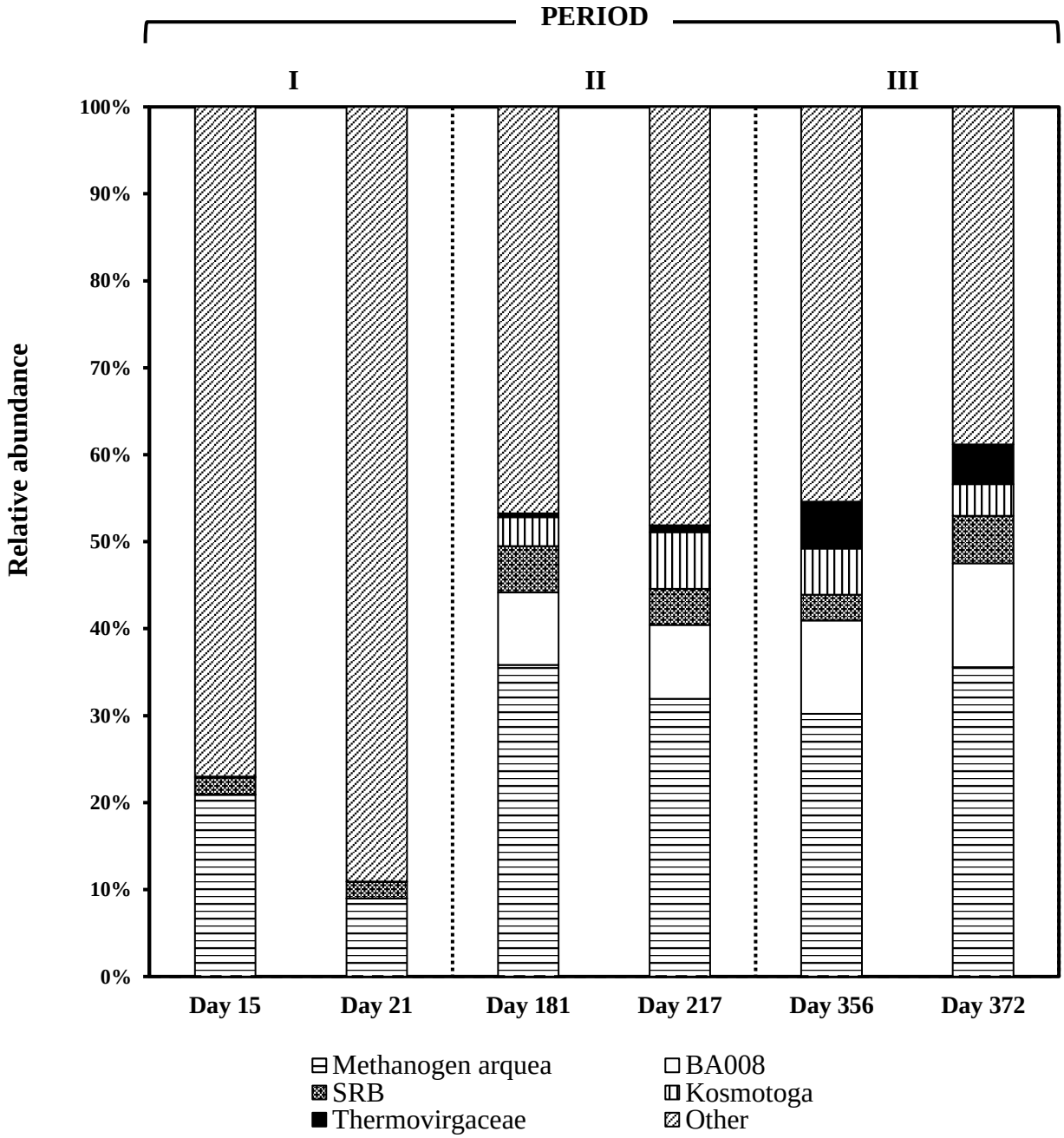

Figure 2.5 Relative abundances of most abundant OTUs (phyla, order and/or family level) in six sludge samples during the three different operation periods of the sulfate reducing bioreactor with the limestone pre-column system. Two samples or biological replicates were collected in each period.

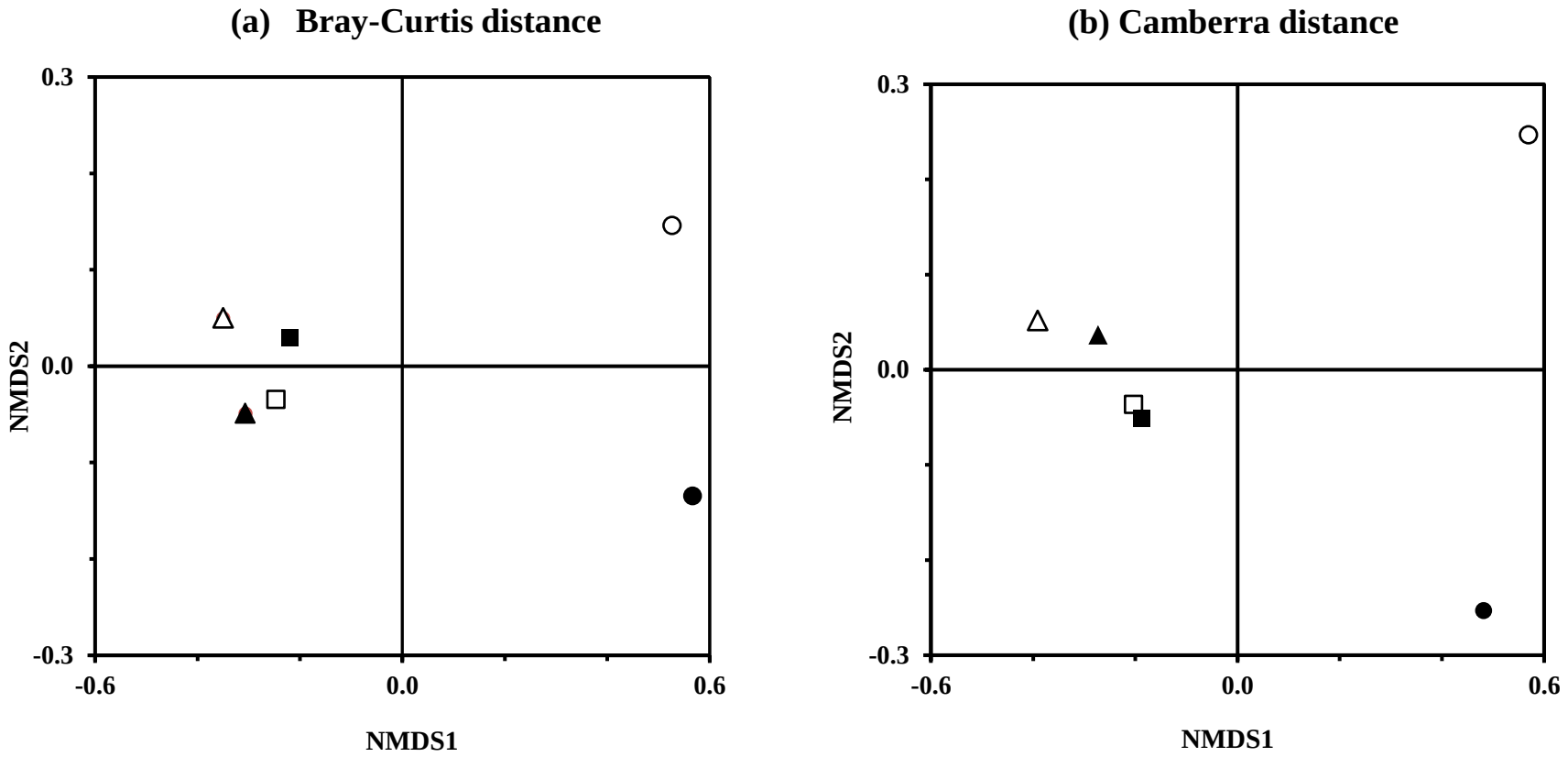

Figure 2.6 Analysis performed on Bray-Curtis (a) and Canberra (b) distances or dissimilarities (stress<0.2) for six sludge samples during the three different operation periods of the sulfate reducing bioreactor with the limestone pre-column system. Two samples or biological replicates were collected in each period. Period I (adaptation phase): day 15 (●) and day 21 (○). Period II (15 mg Cu L^{-1}): day 181 (■) and day 217 (□). Period II (15 mg Cu L^{-1} and 15 mg Zn L^{-1}: day 356 (▲) and day 372 (Δ).