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Removal of copper in a sulfate reducing bioreactor with a limestone pre-column system

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Sulfate reducing activity of anaerobic sediments for the treatment of acid mine drainage (AMD)

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ABSTRACT

The potential environmental hazard of acid mines drainages (AMD) is a problem that need to be addressed in mining regions worldwide; biological treatments catalyzed by sulfate reducing bacteria (SRB) present an interesting alternative because they are much cheaper and more efficient than conventional chemical and physical treatments. The objective of this research was to evaluate the metabolic activity of sulfate-reducing bacteria present in anaerobic samples collected near mining areas and different inoculum in batch assays amended with sulfate (2000 mg L^{-1}) as electron acceptor and acetate (2.5g COD acetate L^{-1}) as carbon source under environmental control conditions of pH and temperature. A physical, chemical and microbiological characterization of samples collected near mining regions was also carried out with samples collected from different sectors of Portovelo-Zaruma the most important mining region in Ecuador.

Then, the presence of sulfate-reducing bacteria (SRB) in the same samples was evaluated through the measurement of sulfide production and sulfate reduction. Bioassays were conducted to determine the highest sulfate-reducing activity between the granular sludge of: the treatment plant of the National Brewery, the artificial lagoon of the University San Francisco de Quito (USFQ); and the sample collected near Oroporto mine (homogenized of the sediments and supernatant). The best inoculum evaluated was the sediment of the artificial lagoon at University San Francisco de Quito, showing a sulfate reducing activity of 7821.67 mg SO_4^{2-} kg^{-1} VSS d^{-1} and a sulfide production of 4855.69 mg S^{2-} kg^{-1} VSS d^{-1}. The production of methane in the sediments of the artificial lagoon at USFQ was also monitored to study microbial competition under anaerobic conditions; obtaining a methanogenic activity of 0.0115 of g COD-CH_4 g VSS^{-1} d^{-1} .

According to acetate consumption the SRB showed a value of 111.35 mg COD-acetate L^{-1} d^{-1}, while methanogens consume 53.27 mg COD-acetate. $L^{-1.}$ d^{-1}; concluding that acetate was a highly effective substrate during the microbial sulfate reduction and could be used as substrate for this process.

These results suggest that the sediments from the artificial lagoon can be potentially used as the microbial inoculum during the production of biogenic sulfide in a bioreactor for the treatment of acid mine drainage through the precipitation of heavy metals.

Keywords

Sulfate reducing bacteria, sulfate, sulfide, acid mine drainages, methanogenesis.

1 Introduction

Acid mine drainage (AMD) are naturally generated in the environment from abandoned mines and mine tailings by chemical reaction between water and rocks containing sulphur-bearing minerals [1]. AMD have contaminated water bodies and created large acidified lakes all over the world [2], being a major environmental problem in many mining areas [3]. AMD typically pose an additional risk to the environment because they often contain elevated concentrations of metals (iron, aluminum, copper and manganese, and possibly other heavy metals) and metalloids (of which arsenic is generally of greatest concern) [4] Being the presence of copper in AMD an important problem in mining industry, that despite being an abundant trace element found in the earth's crust and a micronutrient for both plants and animals at low concentrations, it may become toxic to some forms of aquatic life at elevated concentrations. Copper concentrations from 1 to 8000 µg L^{-1} have been shown to cause environmental problems as inhibition of growth of various freshwater plant species[5]. So that, acid mine drainage causes environmental pollution that affects many countries having historic or current mining industries [4].

In Ecuador, the mining activity make up a large portion of the country´s economy with the four main gold districts concentrated in the south region of Portovelo-Zaruma, Nambija, Ponce Enriquez, and Santa Rosa. Portovelo-Zaruma is the most important because of the exploitation of gold with several hundred years old of mining tradition [6], [7]. The Portovelo-Zaruma district is located in the province of El Oro, southwest of Ecuador, in the foothills of the Western ¨Cordillera de Los Andes" The mining activity carried out in the district is formal and informal. The first main mining company established in Zaruma-Portovelo was the American company Sadco (South American Development Co.), that later was followed by the invasion of local miners, which are located to north and south of the confluence of the Calera and Amarillo rivers. These two rivers are tributaries of Puyango river which flow south-west, eventually through Peruvian territory and enters the ocean at the city of Tumbes [6]. In 1999, the Portovelo–Zaruma mining district consisted of about 400 mines, 66 plants for crushing, grinding and amalgamation, and 80 cyanidation plants [8]. Currently mining and processing are occurring at numerous locations in a 16 km by 9 km area situated north and south of the confluence of the Calera and Amarillo rivers [8]. The most exploited mines in Zaruma city are Sexmo, Miranda, Pillacela, Palacios, Vizcaya, among others, while in the canton Portovelo can be found Portovelo, Abundance, Oroporto, Cantabria mines and holdings of gold in the sands of the Amarillo river [9].

The environmental impacts associated with the mining activities of the Portovelo-Zaruma district were first acknowledged in a number of studies in the early 1990s and being currently the areas more affected by discharges of process tailings, in the canton Zaruma: the Pache region; and the banks of Calera, Amarillo and Pindo rivers, tributaries of the Puyango river [6, 10, 11]. Most mineral processing plants are located along the Calera river and the mining wastes are discharged into the river [6].

The same applies to the Amarillo River due to the presence of numerous small plants that unload their tails into the river without any type of treatment [12]. These discharges cause that regularly the metals and cyanide levels in rivers water exceed environmental quality criteria downstream of the mining district. However, metals are mainly associated with sediments and suspended solids due to the prevalently neutral to slightly alkaline river waters. The Calera river located downstream of the mining area is considered a dead river with no fish nor invertebrates, whereas the Amarillo river has had its fauna severely diminished [6]. Moreover, fish and aquatic invertebrates are nonexistent more than 20 km after the confluence of the two rivers. Further downstream, some life reappears, although the invertebrate fauna is still severely reduced up to 160 km downstream of the mining area, within Peruvian territory [6].

Several treatment methods have been proposed in the literature to minimize environmental problems caused by AMD, keeping water away from acid generating materials and preventing AMD formation [1]. Preventing the formation or the migration of AMD from its source is generally considered the preferable option, although this is not feasible in many locations, and in such cases, it is necessary to collect, treat, and discharge mine water. There are various options available for remediating AMD, which may be divided into those that use either chemical or biological mechanisms to neutralize AMD and remove metals from solution. The abiotic and biological systems include those that are classified as active or passive [4]. Active treatment involves installing a water treatment plant, where the AMD is first dosed with lime to neutralize the acid and then passed through the settling tanks to remove the sediment and particulate metals. While passive treatment aims to develop a self-operating system that can treat the effluent without constant human intervention [1]. The majority of bioremediation options for AMD are passive systems, and of these,

only constructed wetlands and compost bioreactors have so far been used in full-scale treatment systems [1].

The basis of biological remediation of AMD derives from the abilities of some microorganisms to generate alkalinity and immobilize metals, thereby essentially reversing the reactions responsible of for the formation of AMD [13]. Microbiological processes that generate net alkalinity are mostly reductive processes and include denitrification, methanogenesis, sulfate reduction, and iron and manganese reduction [1]. Bacteria that catalyze the dissimilatory reduction of sulfate to sulfide generate alkalinity by transforming a strong acid (sulfuric) into a relatively weak acid (hydrogen sulfide) by the action of sulfate reducing bacteria (SRB) [1]. SRB are anaerobic prokaryotic microorganisms that can utilize carbon sources such as organic acids (acetate) or alcohols as electron donors for the reduction of sulfate to hydrogen sulfide through dissimilatory way of sulfate [14, 15]. The ability of sulfate reducers to couple acetate oxidation to sulfate reduction is of particular interest during the treatment of AMD given also the importance of acetate as an intermediate in methanogenesis [16]. The higher energy yields enables the sulfate reducers to grow at lower hydrogen concentrations; in addition, they can utilize a much boarder range of substrates and therefore outcompete the methanogens [17].

Besides the ameliorative effect on AMD brought by the resulting increase in pH, the reduction of sulfate is an important mechanism for removing toxic metals from AMD, since as many ions such as e.g., zinc, copper and cadmium form highly insoluble metal sulfides [1]. Due to the combined removal of acidity, metals and sulfate; sulfate-reduction appears to be the most promising bioprocess for AMD treatment and metal recovery [13].

Much research work has been focused on characterizing and evaluating the application of sulfate reducing processes to remediate contaminated AMD sites. For example, Moosa *et al*, studied the activity of SRB with acetate as carbon source in a continuous stirred tank reactor obtaining sulfate reduction rates of 43 and 19 mol $SO_4^{2-}m^{-3}d^{-1}$, in 2002 and 2006, respectively [18], [19]. Also in a study conducted by Koschorreck and co-workers, in 2010 about the accumulation and inhibitory effects of acetate in a sulfate reducing in situ reactor for the treatment of an acidic Pit Lake, sulfate reduction rates $\leq 65g\ SO_4^{2-}L^{-1}d^{-1}$ were obtained [20]. Also, in 2005, Vallero *et al* evaluated the rate of sulfate reduction in a submerged anaerobic membrane bioreactor (SAMBaR) at high salinity, where acetate was used as carbon source, obtaining sulfate reducing rates of $69g\ SO_4^{2-}L^{-1}d^{-1}$ [21].

As acetate can be a substrate for methanogens and SRB, studies of competition between these two groups has been made. For example, Santegoeds, C.M., *et al*, in the study of microsensor measurements in methanogenic-sulfidogenic aggregates revealed that the activity of sulfate-reducing bacteria was of 2 to 6 mmol of S^{2-} m^{-3} s^{-1} or 2 × 10−9 mmol s−1 per aggregate. While methanogenic activity in the methanogenic-sulfidogenic aggregates was of 1 to 2 mmol of CH_4 $m^{-3}s^{-1}$ [22]. Also, in the study of influence of acetate and propionate on sulphate-reducing bacteria activity by van den Brand, T.P.H., and coworkers, in an acetate fed reactor, a complete COD substrates removal was achieved, but no sulfate reduction occurred, while with pure acetate feeding methanogens outcompeted the SRB. However on the mixed substrate of acetate and propionate a culture of SRB dominated.[23]. Also is known that in the presence of adequate sulfate concentrations, SRB typically out-compete methanogens due to kinetic and thermodynamic advantages. However, the coexistence of SRB and methanogens has been observed in anaerobic sewer biofilms in the presence of sulfate. In the study of

Sun, *et al* in an annular biofilm reactor, was found that sulfide and methane were produced simultaneously in the reactor, with concentrations of sulfide (13.0 to 18.6 mg S $liter^{-1}$) and methane (9.3 to 14.9 mg $liter^{-1}$) and a COD utilization per gram of sulfide and methane formed of 2 g COD/g H_2S-S and 4 g COD/g CH_4, respectively. Therefore, sulfidogenesis accounted for 36.0% of the COD loss in the wastewater whereas methanogensis accounted for 60.0% .[24]

As biological sulfate reduction is increasingly replacing chemical unit processes in mining biotechnology, the objective of this study was to evaluate the metabolic activity of sulfate-reducing bacteria present in anaerobic samples collected near mining areas and different inoculum in batch assays and its possible applications in the bio treatment of acid mine drainage. Samples collected from mining areas were also characterized based on physical, chemical and microbiological parameters. In addition, microbial competition between sulfate reducing bacteria and methanogens was evaluated in the presence of acetate as electron donor.

2 Materials and methods

Chemicals

Sodium sulfate (100 % purity) was obtained from JT Baker (Phillipsburg, NJ, USA). Sulfate of ammonium and iron (III) and sodium acetate were obtained from Chemical Laboratories H.V.O. (Quito, Ecuador). Sulfuric acid (95.0 - 97%) were obtained from Merck KGaA (Darmstadt, Germany). The DMP (oxalate N, N -dimethyl- p-phenylenediamine) (> 99%) was obtained from Acros Organics (Geel, Belgium). Zinc chloride (97.1 %) was obtained from JT Baker (Zedelgem , Belgium). The N2 gas was delivered from AGA Ecuador (Guayaquil, Ecuador). All reagents were used in the state in which they were received.

Sediment samples

Four samples were collected near mining areas in the south of Ecuador, in the mining district Portovelo-Zaruma. The samples correspond to sector "Agua Dulce", near Oroporto mine, surrounding areas of Amarillo river and sector "El Pache", in Canton Portovelo (Figure *1*). The samples were collected in 1 gallon plastic bottles (80% sediments and 20% supernatant). Sediments and supernatants were characterized based on physical, chemical and microbial parameters. All samples were kept in refrigeration.

The sulfate reducing metabolic activity bioassays were conducted with supernatants and sediments from the mining areas, anaerobic sediments from the artificial lagoon at Universidad San Francisco de Quito and granular sludge from a wastewater treatment plant in Quito (National Brewery). The content of total suspended solids (TSS) and of volatile suspended solids (VSS) of the sediments evaluated were: in sector "Agua Dulce" (59.5, 2.5%), near Oroporto mine (68.9, 3.8%), surrounding areas of Amarillo river (77.6, 2.4%), sector "El Pache" (77.7, 1.9%), granular sludge of National Brewery (9.7, 8.4%) and artificial lagoon of the USFQ (52.8, 6.2%), respectively.

Basal medium

The basal mineral medium used in the bioassays of sulfate-reducing activity and methanogenic activity, contained (in mg L^{-1}): NH_4Cl (280); KH_2PO_4 (195); $MgSO_4$ (49); $CaCl_2$ (10); $NaHCO_3$ (3000); yeast extract (10) and 1 mL L^{-1} of a solution of trace elements. The solution elements trace was composed of (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%), similar to other batch assays

carried out by Ochoa-Herrera and co-workers [25]. The pH of the basal mineral medium was adjusted to 7.1-7.3 with HCl and NaOH, as required.

Analytical Methods

Ammonium, nitrate, conductivity and pH were measured using a portable multi-parameter Thermo Scientific Orion 5-Star (Thermo Scientific, Beverly, MA, USA) and according to protocols established in Standard Methods for Examination of Water and Wastewater [26]. Biological Oxygen Demand (BOD) was determined using the OxiTop system and OxiTop Box incubator (WTW, Weilheim, Germany) for five days at a temperature of 20°C [26]. Chemical Oxygen Demand (COD) was determined using a colorimetric method [26]. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined by the method of crucibles and filters according to Standard Methods for Examination of Water and Wastewater [26]. Sulfate was determined by the method of barium sulfate precipitation [26]. Sulfide in cultures of sulfidogenic bacteria was determined by the methylene blue reaction [26]. All measurements were conducted in triplicates. Prior to the analysis, the sediments and supernatant of miming samples were homogenized; in addition for the measurement of sulfate and dissolved sulfide, the samples were centrifuged at 6000 rpm for 15min [27]. The presence of sulfate-reducing bacteria in sediments of artificial lagoon at USFQ was determined quantitatively by the most probable number method (Most Probable Number, NMP, for its acronym in English)[28]. Detection of coliforms and *E. coli* (MPN/100 mL) were determined by two techniques: membrane filtration, with 0.45 μm filter, which were placed in Petri dishes with chromocult agar [29]; and the most probable number technique per 100 mL [30], [31]. Five dilutions (10^{-1} to 10^{-5}) were done for quantification of total numbers of bacteria (CFU mL^{-1}).

Batch Microbial Bioassays

Bach microbial bioassays were conducted in triplicates using 160 mL glass serum bottles sealed with butyl rubber stoppers and aluminum crimp seals. The headspace was flushed with N_2 gas to assure anaerobic conditions. Flasks lacking microorganisms were also incubated and served as abiotic controls. All bioassays were incubated in a home-made climate-controlled chamber at 30±2°C. In sulfate reducing activity and methanogenic activity bioassays, each flask was supplemented with 100 ml basal mineral medium, 2000 mg SO_4^{2-} as sodium sulfate, 10% w/v or v/v of microbial inoculum and acetate 2.5 g COD L^{-1} as organic substrate. For the case of the assays with the supernatant of samples collected near mining areas, each flask was supplemented with 90 mL basal mineral medium concentrated. In methanogenic activity, the methane production was measured by a liquid displacement method [32] by an inverted flask of 125 mL with an alkaline solution of 2% NaOH [33],[34]. The reduction of sulfate to sulfide was periodically monitored by measuring the S^{2-} concentration in aqueous phase.

The sulfate reducing activity and specific methanogenic activity were calculated from the slope obtained by plotting sulfide or methane concentration against time, expressed as COD and the amount of VSS utilized in the bioassays. Maximum specific activity of sulfate reduction, sulfide generation and methanogenic production were expressed in mg SO_4^{2-} kg^{-1} VSS d^{-1}, mg S^{2-} kg^{-1} VSS d^{-1} and g COD-CH_4 g^{-1} VSS^{-1} d^{-1}, respectively.

3 Results and Discussion

In the first stage of this study, different samples collected near mining regions were analyzed based on physical, chemical and microbiological parameters. Table 1 presents

the results of the characterization of samples (sediments and supernatant) collected in the mining district Portovelo-Zaruma.

The concentrations of nutrients in samples collected near mining regions ranged from 112.2 to 6414.2 mg L^{-1} of ammonium and 415.9 to 3515.3 mg L^{-1} for nitrate. The phosphate concentration was very similar for all samples ranging from 105.2 to 172.3 mg L^{-1} as well as the pH values that varied from 5.6 to 6.9; while conductivity values ranged from 420 to 918 µS cm^{-1}.

Sulfide concentration varied widely, the sample collected near Amarillo river showed the highest value of 47.2 mg L^{-1}. The values of chemical oxygen demand were very high between 1418.9 to 14012.1 mg L^{-1}. In contrast, biological oxygen demand values ranged from 25 to 250 mg L^{-1}.

Regarding sulfate, one of the characteristic parameters of AMD, the values varied from 1083.86 to 2085.41 mg L^{-1}. These values are similar to other mining areas, as example in Finland, in Vehkankuilu mine the sulfate concentration was 1100 mg L^{-1} [35].

The ratio of volatile suspended solids (VSS) and total suspended solids (TSS) of the samples collected were: in sector “Agua Dulce” (0.04), near Oroporto mine (0.05), surrounding areas of Amarillo river (0.03), sector “El Pache” (0.02), granular sludge of National Brewery (0.8) and artificial lagoon of the USFQ (0.1). The determination of these parameters is necessary as a common measure of effectiveness of anaerobic digestion process [36] The relationship between SSV/SST of the samples collected near mining areas is below the values reported in the literature (0.5-0.6) for healthy inoculum[37]. This indicates that the sample contains a large amount of inert material, represented as ash, and the concentration of microorganisms is small [38] compared to the others microbial inoculants evaluated.

The microbiological results showed the presence of coliforms in all samples evaluated with values >1600 MPN/100 mL. On the other hand, samples collected near Oroporto mine and Amarillo river were the only ones that presented fecal contamination. These results suggest a possible contamination with other kind of residues that are not necessarily discharge of mining process as it is the case of sewage contamination because *E. coli* are commonly found in human and animal feces [39].

In terms of environmental legislation, it should be noted that the samples collected and analyzed in this study do not meet the local permissible limits for discharges to freshwater bodies as established in the Ecuadorian Legislation, in the Unified Text Secondary Environmental Legislation (TULMAS) [40]. In general terms, the majority of parameters evaluated show concentration values higher that the permissible maximum limits, as biological oxygen demand, chemical oxygen demand, sulfate, sulfide, total suspended solids, coliforms and fecal coliforms, although many of the parameters evaluated in this study are not regulated by the Ecuadorian legislation. However, based on these results, it can be concluded that samples collected near mining areas show contamination and presumably due to domestic and industrial effluent discharges.

The conditions of operation of the bioassays evaluated were determined according previous investigations. Andrade and Ochoa-Herrera evaluated in batch bioassays the efficiency of acetate, lactate, ethanol and peptone as electron donors (2.5 g substrate L^{-1}) and sulfate (4000 mg L^{-1}) as electron acceptor. The sulfate reduction rates (mg SO_4^{2-} g^{-1-1}sustrato $día^{-1}$) obtained, were: acetate (1.5 to 52.4), lactate (0.8-24.9), ethanol (0.8-5.3) and peptone (1.2-2.1) [41]. Being acetate the best electron donor under the conditions established. These results are supported by other studies in the literature,

Manous and co-workers determined that the addition of sodium acetate increased significantly the rate of sulfate removal from 24 mM to 16 mM[42].

Thereafter, Flor and Ochoa-Herrera evaluated the optimal sulfate concentration for biogenic sulfide production in a chemical-physical-biological system (CFB) for treating acid mine drainage at laboratory scale,. The results obtained determined that with a sulfate concentration of 4000mg L^{-1} there was a sulfide production of 167 mg S^{2-} L^{-1}; with a reduction to 3000 mg L^{-1} the sulfide stabilized in 170 mg L^{-1}; and with 2000 mg L^{-1} during the first 50 days the reactor do not show a significant change in sulfide production, maintaining in 170 mg L^{-1}, although the last days the sulfide production decrease to 74 mg L^{-1}, enough for an efficient copper removal of 98% and a chemical oxygen demand increased of 50 to 75% [43]. In base to these studies it was suggested to perform bioassays with different microbial inoculums to 2000 mg L^{-1} of sulfate as electron acceptor and 2.5 g L^{-1} of acetate as electron donor, with the objective of determine if these are the optimal concentrations for AMD treatment. Also was suggested a study of molecular identification of different groups of microorganisms present in the microbial consortium.

Table 2 showed the results of specific sulfide production activity and specific sulfate reduction activity of several microbial inoculum evaluated. Of the samples collect near mining areas, the sample that show betters results correspond to the sample collected near Oroporto mine, with the highest sulfate reducing activity (8222.2 mg SO_4^{2--} kg^{-1} VSS d^{-1}) and sulfide production activity (3534.1 mg S^{2-} kg^{-1} VSS d^{-1}). However, when this sample was compared with the sediments of the artificial lagoon and the granular sludge; the highest sulfide production activity (4855.7 mg S^{2-} kg^{-1} VSS d^{-1}) was obtained with the sediments of the artificial lagoon, along with the second best sulfate reduction activity (7821.7 mg SO_4^{2--} kg^{-1} VSS d^{-1}). While the granular sludge

showed the lowest values of specific activities among the three sediment evaluated. The specific sulfide production activity of the SRB present in the sediments of the artificial lagoon was 1.9 and 1.4 times greater than the granular sludge and the sample collected near Oroporto mine, respectively. While the specific sulfate reduction activity was 2.6 times greater than the granular sludge and had a similar value compared with the sample collected near Oroporto mine.

These results are comparable with literature studies. Mendoza in the study of remediation of acid rock drainage with permeable reactive barriers packed with compost-zero valent iron-limestone (C-Z-L) mixtures, reported a specific sulfate reduction activity of 486 mg $SO_4^{2-}\ kg^{-1}$ VSS d^{-1} [44]. In the case of the sediments of the artificial lagoon, the best acetate oxidizer microbial inoculum between the ones evaluated in this study, the maximum specific sulfate reduction activity was 16 times higher than the one reported by Mendoza under similar conditions. In the same study, the sulfate reduction activity of other carbon sources such as compost, the metabolic activity ranged from 2178 to 8647 mg $SO_4^{2-}kg^{-1}$ VSS d^{-1} for Erthfood and ARBICO composts, respectively [44]. In the case of using propionate as carbon source, Ghigliazza and co workers obtained values of 3200 mg $SO_4^{2-}kg^{-1}$VSS d^{-1} [45]. The specific biological activity of SRB is an important parameter, which provides information about the performance and microbial biomass properties. These results indicate that SRB present in the sediments of the artificial lagoon were highly efficient for the microbial sulfate reduction and are excellent candidates for the bio treatment of acid mine drainage in continuous systems.

Figure *2* illustrates the time course of sulfate reduction (primary axis) and sulfide production (secondary axis) in batch bioassays after 61 days of treatment in the presence of acetate (2.5 g COD L^{-1}) as substrate and 2000 mg $SO_4^{2-}\ L^{-1}$, in an abiotic

control (absence of microorganisms) and with the different sediments evaluated in this study. There was practically no sulfide production in the abiotic control, while in the treatments bioassay the production of sulfide gradually increased with incubation time, while sulfate concentration decreased. The sediments of the artificial lagoon showed a sulfate reduction of 55.8% and a maximum sulfide production of 37.3 mg L^{-1}, similar to results obtained by Andrade and Ochoa-Herrera where the maximum sulfide production was 35.9 mg L^{-1} after 45 days of treatment with similar characteristics (2.5 g COD acetate L^{-1} and 4000 mg L^{-1} of sulfate) [41]. In the same manner, Dev and Bhattacharya in the study about sulfate reduction and growth kinetics of SRB with marine waste extract, percentages of sulfate reduction varied between 31.9 to 48.1% with concentrations of 1500 to 5500 mg $SO_4^{2-}\ L^{-1}$, respectively [47]. These results show that SRB present in the samples evaluated used acetate as an electron donor in a greater or lesser extent to support microbial sulfate reduction. For the artificial lagoon, it was estimated an acetate oxidation of 679.3 mg COD-acetate for the sulfate reduction obtained.

Among the samples evaluated, the anaerobic sediments of the artificial lagoon were chosen as the best microbial inoculum because presented the highest sulfate reduction with 55.8%; while the other samples had values of sulfate reduction ranging between 0-48.1%. Considering that mining and other industries that use sulphur compounds, like metallurgical, pulp and paper, and petrochemical industries, are responsible for an increase in sulphate concentrations in wastewaters[46], a highest sulfate reduction in AMD treatment system is important.

The relation between the amount of sulfate reduction and the amount of sulfide generation in the form of H_2S, was established according to the following equation.

$$\frac{1}{8}CH_3COO + \frac{1}{8}SO_4^{2-} + \frac{3}{16}H^+ \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3 + \frac{1}{16}H_2S + \frac{1}{16}HS$$ Equation 1

In Table 3**,** the sulfur balance show the concentration of dissolved sulfide in the effluent, the sulfate concentration in the influent and the sulfate concentration in the effluent, resulting 37.3, 728, 321 mg L^{-1} sulfur., showing a loss of sulfur that could be due to the high volatility of sulfur that caused that the measurement of dissolved sulfide was lower than the theoretical values.

The determination of the presence of sulfate reducing bacteria in the sediments of the artificial lagoon was performed with the most probable number method (MPN). During the microbial count of sulfate-reducing bacteria it could clearly see the formation of black precipitates in the iron nails evaluated. This demonstrates the presence and activity of sulfate-reducing bacteria, as generated sulfide necessary for the formation of iron sulfide precipitate black. The results indicate that there were $1.1x10^5$ SRB.

The metabolic activity of methanogens present in the sediments of the artificial lagoon was also evaluated, to study possible substrate competition between methanogens and sulfate reducing bacteria. The methane production started at 0.0001 mol CH_4 L^{-1} in the initial hours and reached 0.0042 mol CH_4 L^{-1} after 50 days of incubation. with a methanogenic activity of 0.011 g COD-CH_4 g^{-1} VSS^{-1} d^{-1} (Figure 3), a low rate in relation with typical values of specific methanogenic activity of granular sludge of industrial wastewater, that range from 0.5 to 1.0 g COD-CH_4 g^{-1} VSS^{-1} d^{-1} [48]. Also, it was estimated an acetate oxidation of 266 mg COD-acetate for CH_4 production.

With respect to acetate consumption was obtained for SRB a value of 111.3 mg COD-acetate L^{-1} d^{-1}, while methanogens 53.3 mg COD-acetate L^{-1} d^{-1} .These data show that SRB in the sediments of the artificial lagoon used acetate as a highly effective substrate for sulfate reduction, under the conditions set in the bioassay.

With the results obtain, different bacterial consortiums are expected to be present in the microbial inoculum evaluated in this study; even more, a potential competition between them by the substrate (acetate), the sulfate-reducing bacteria, and the methanogens mainly. As sulfate reduction and methanogenesis are both involved in the final step of the degradation of organic matter in anaerobic environments, the presence of a methanogenic activity is an indicator that exist the presence of several bacterial consortia in the anaerobic microbial inoculants evaluated.

Many sulfate reducing bacteria are metabolically much more versatile than methanogenic bacteria and can use all classical fermentation products and oxidize them to carbon dioxide, simultaneously reducing sulfate to sulfide. Although a few sulfate reducing bacteria that have been isolated recently can also use sugars or amino acids, these bacteria do not compete successfully with classical fermentative bacteria on the same substrates [17]. The sulfate-reducing bacteria predominate in the sediments of the artificial lagoon and that are oxidants of acetate could belong to the genus *Desulfobacter, Desulfotomaculum, Desulfococcus, Desulfosarcina and Desulfonema* [41] which subsequently can be confirmed using molecular techniques.

4 Conclusions

Sulfate reduction and sulfide production was catalyzed by SRB present in the sediments of the artificial lagoon, the granular sludge and the sample collected near Oroporto mine. However, the other samples collected near mining areas showed a low sulfate reduction and sulfide production under the conditions imposed.

SRB present in the sediments of the artificial lagoon showed predominance in acetate consumption for biogenic sulfide production as carbon source over the methanogens, observing a competition between these two groups of microorganisms.

It was determined that the sulfate-reducing bacteria present in the sediments of the artificial lagoon, constitutes an excellent option for biological treatment of acid mine drainage characterized by elevated concentrations of heavy metals, sulfates and acidity, because of the high percentage of sulfate reduction obtained.

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Table 1 Physical, chemical and microbial characterization of the sediments and supernatant of samples collected near mining regions in the mining district Portovelo-Zaruma, Ecuador.

Parameters	**Units**	**"Agua dulce"**		**Oroporto Mine**		**Amarillo river**		**Pache Sector**		**Permissible Limits[a]**
Ammonium	mg L^{-1}	710.0	± 60.2	6414.2	± 43.1	112.2	± 37.7	601.6	± 3.7	-
Biochemical Oxygen Demand	mg L^{-1}	50.0	± 1.5	60.0	± 2.1	250.0	± 5.4	25.0	± 0.6	100
Chemical Oxygen Demand	mg L^{-1}	14012.1	± 2759.1	4079.5	± 752.5	1418.9	± 501.7	7804.2	± 1064.2	250
Conductivity	µS cm^{-1}	859	± 0.5[b]	918	± 0.5[b]	796	± 0.5[b]	420	± 0.5[b]	-
Nitrate	mg L^{-1}	808.8	± 28.1	3521.2	± 15.4	1431.2	± 78.5	415.9	± 27.5	10
pH		5.6	± 0.01[b]	6.2	± 0.01[b]	6.8	± 0.01[b]	6.8	± 0.01[b]	5-9
Phosphate	mg L^{-1}	128.6	± 33.2	105.2	± 28.5	172.3	± 18.9	138.7	± 18.9	-
Sulfate	mg L^{-1}	2085.4	± 38.8	1207.3	± 38.8	1083.8	± 58.2	1660.1	± 252.2	1000
Sulfide	mg L^{-1}	0.2	± 0.0003	0.7	± 0	47.2	± 0.03	4.9	± 0.002	0.5
Total solids	g L^{-1}	82.8	± 3.3	9.7	± 0.06	6.4	± 0.4	547.1	± 15.6	1.6

Total suspended solids	g L^{-1}	55.5	± 0.5	8.9	± 0.7	4.8	± 0.2	462.1	± 2.3	0.1
Total suspended solids in sediments	%	59.5	± 0,2	68.9	± 0.1	77.6	± 0.2	77.6	± 0.3	-
Volatile solids	g L^{-1}	5.9	± 1.2	1.8	± 0.1	0.3	± 0.03	9.4	± 0.6	-
Volatile suspended solids	g L^{-1}	1.6	± 0.1	0.6	± 0.1	0.1	± 0.02	3.7	± 0.7	-
Volatile suspended solids in sediments	%	2.5	± 0.05	3.8	± 0.1	2.4	± 0.03	1.9	± 0.01	-
Total Bacteria	CFU mL^{-1}	5.90E+03		7.00E+04		1.48E+06		1,20E+05		-
Coliforms	MPN/100 mL	n/d		>1600		>1600		n/d		Remotion >99.9%
Fecal coliforms (*E.coli*)	MPN/100 mL	n/d		130		280		n/d		Remotion >99.9%

[a] Unified Secondary Environmental Legislation Text (TULSMA), Book VI, Annex 1, Table 12
[b] Standard deviation of the equipment
n/d = not detected

Table 2 Specific activities obtained with samples collected near mining areas and sediments, evaluated in batch assays, during 48 and 61 days in the presence of acetate (2.5 g COD L^{-1}) and sulfate (2000 mg L^{-1}).

Sample	Time of treatment (days)	Type of sample	Sulfate reduction (%)	Sulfide (mg L^{-1})	*Specific activity of sulfate reduction* (mg SO_4^{2-} kg^{-1} VSS d^{-1})	*Specific activity of sulfide production* (mg S^{2-} kg^{-1} VSS d^{-1})
Agua dulce Sector	48	S	0	1.1±0.01	0	146.1
		St	0	4.3±0.01	0	577.0
Amarillo river		S	0	0.1±0.01	0	19.4
		St	0	0	0	0
Pache Sector		S	0	4.0±0.09	0	511.9
		St	0	3.5±0.01	0	409.8
Oroporto Mine		St	26.8	15.1±3.5	3354.2	1063.7
Granular Sludge	61	S	15.5	65.8±4.1	2986.4	2474.0
Oroporto Mine		S	48.1	12.7 ±1.9	8222.2	3534.1
Artificial lagoon		S	55.8	37.3±1.1	7821.6	4855.7

S: sediments
St: supernatant

Table 3 Sulfur balance in the bioassay performed with the sediments of the artificial lagoon at the USFQ, with a fed of 2000 mg L-1 of sulfate.

	Influent sulfate ($mg\ L^{-1}$)	Effluent Sulfate ($mg\ L^{-1}$)	Dissolved Sulfide effluent ($mg\ L^{-1}$)
	2181	963	37.3
Sulfur ($mg\ L^{-1}$)	727	337	37.3

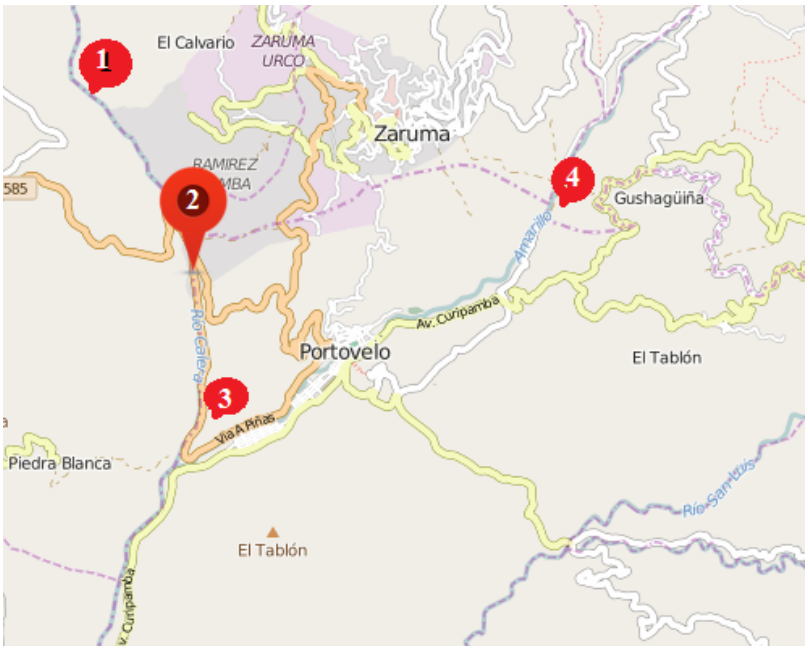

Figure 1 Mining district Portovelo-Zaruma at south of Ecuador. Red points correspond to sampling areas. (1) Sector Agua Dulce (Calera river), (2) Sector Pache (Calera river), (3) Oroporto mine (Amarillo river) and (4) Banks of Amarillo river.

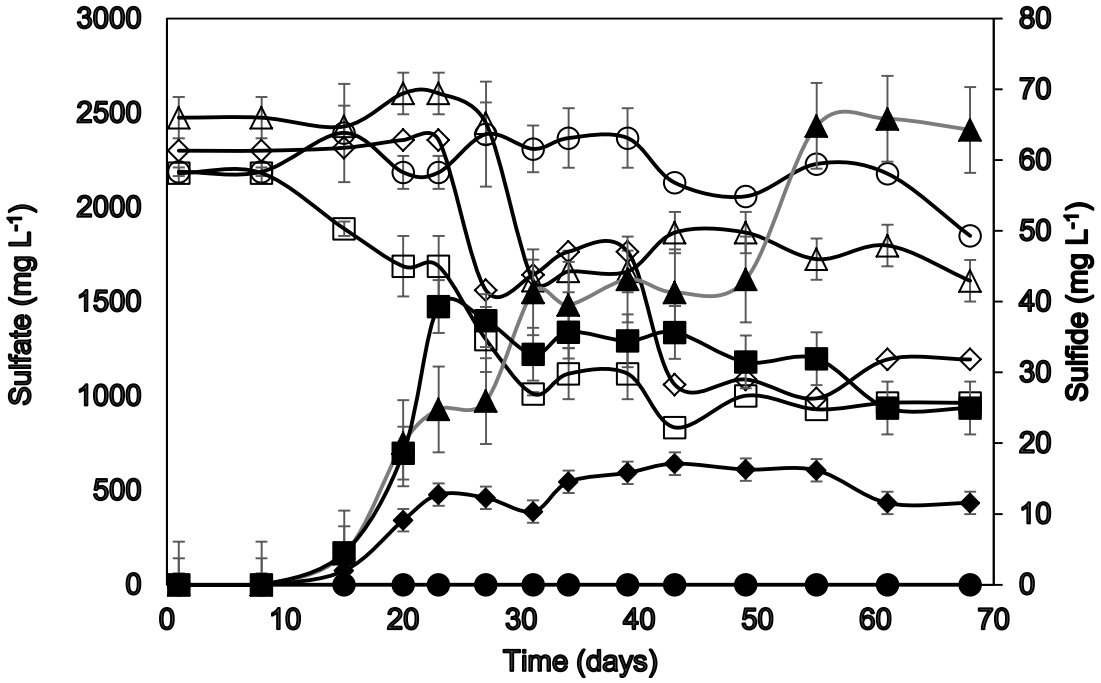

Figure 2 Sulfate reducing activity of sediments during batch assays, by a period of treatment of 61 days. Concentration of sulfide in secondary axis (filed figures), sulfate in primary axis (open figures): (● and o) control, (▲ and Δ) Granular sludge, (◆ and ◊) Sample collected near Oroporto mine, (▮ and □) Sediments of the artificial lagoon (USFQ).

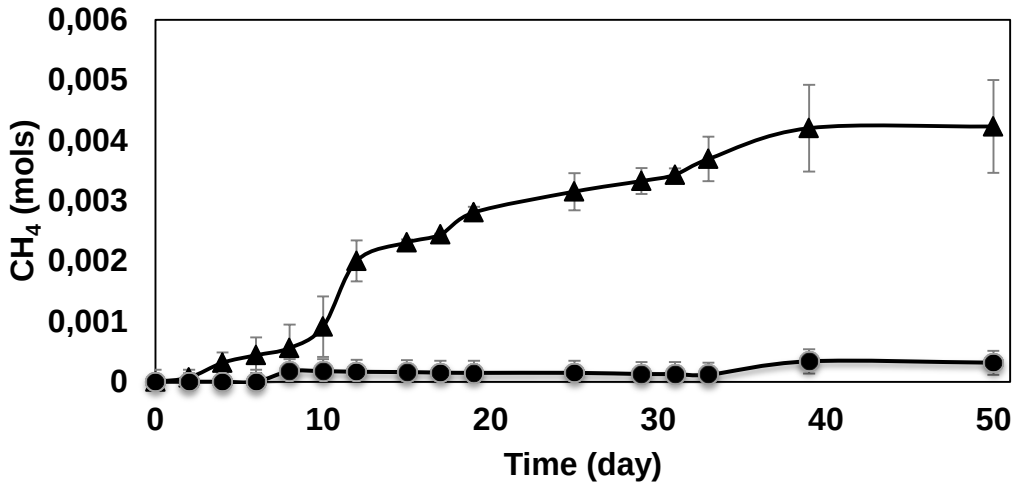

Figure 3 Methanogenic activity of sediments of the artificial lagoon of the University San Francisco de Quito (USFQ), after 50 days of treatment. (●) Control. (▲) artificial lagoon.

Removal of copper in a sulfate reducing bioreactor with a limestone pre-column system

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ABSTRACT

Acid mine drainage (AMD) is a major environmental problem threaten water resources worldwide. Passive treatments relying on the activity of sulfate reducing bacteria are efficient way to reduce acidity, metal and sulfate concentration of AMD water and improve the overall water quality. The objective of this study was to conduct a phylotyping of the microorganisms present in a sulfate reducing bioreactor with a limestone pre-column for the removal of copper in Ecuador. The system was fed with a synthetic acid mine drainage (AMD) with a pH of 2.7 containing high concentrations of Cu^{2+} (10-40 mg L^{-1}), sulfate (2000 mg L^{-1}), and acetate as electron donor (2.5 g COD acetate). Copper removal efficiencies ranged from 95 to 99%, with a final concentration of 0.53 mg L^{-1} of copper (II) in the two-stage system, with almost complete removal occurring in the limestone pre-column. The final sulfate reduction was 37% and chemical oxygen demand removal was 59%, with the predominance of COD consumption by methanogens during the first 153 days; an almost equal COD consumption by methanogens and SRB was obtained after 154 days and a dominance of SRB with a COD removal of 41.3% of the total (59%) was obtained at 228 days. Finally, a determination of bacterial taxonomic composition was conducted by analysis of 16s rRNA and *dsr* funcional gen at day 113. *Methanosaeta* and *Methanosarcina* were the most prevalent methanogens in the biological reactor while, *Desulfotomaculum, Syntrophobacter, Desulfosalsimonas, Desulfobulbus, Desulfacinum, Desulfosarcina and Desulfovibrio* were the most prevalent sulfate reducing bacteria (SRB). Among the sulfate-reducing bacteria which were identified, *Desulfotomaculum intricatum* (99% identity) and *Desulfotomaculum acetoxidans* (90%) were the most abundant SRB that used acetate, as only carbon source..

Key Words

Sulfate reduction, acetate, sulfate, sulfide, acid mine drainage, copper, heavy metals, sulfate reducing bioreactor with the limestone pre-column, methanogens 16s rRNA, *dsr* gene.

5 Introduction

Anthropogenic release of heavy metals in the environment is mainly related to wastewaters discharges from industrial and mining activities. In particular acid-mine drainage (AMD), which is recognized as the current largest environmental problem facing the mining industry, negatively impacts thousands of kilometers of waterways worldwide, affecting the aquatic and neighboring terrestrial environment [1]. In Ecuador, mining activities are primarily conducted in the district Portovelo-Zaruma (El Oro province); however, the lack of treatment systems for AMD and control by state authorities have caused several impacts in this mining district [2]. For instance, the discharges of process tailings to the Amarillo and Calera rivers affect severely the environment with metals and cyanide levels in river water that exceed environmental quality criteria [3].

AMD is generated through a combination of chemical and biological processes by which metal sulfates are converted to sulfide and metal hydroxides when exposed to fresh water and oxygen. AMD is characterized by low pH values with high concentrations of sulfate and heavy metals ions [4], [5]. Numerous passive AMD treatment systems have evolved over the past three decades, however the most common designs involve sulfate-reducing bioreactors, particularly in low-flow situations where water contains high concentrations of metals [6]. This bioprocess is based on biological hydrogen sulfide production by sulfate reducing bacteria (SRB), followed by metal sulfide precipitation and neutralization of the water by the alkalinity produced by the microbial oxidation of the electron donor which is typically an organic compound [7]. Limestone has also been studied extensively as AMD pH neutralizer and it is used mainly in passive wastewater treatment [8], primarily because of its effective dissolution rates, and its relative abundance near mine sites [1].

A vast majority of studies have been reported in the literature regarding the precipitation of metals such as copper by biogenic sulfide. Muhammad and co-workers reported copper removal as high as 99% in a passive treatment of metal and sulfate rich acid mine drainage (AMD) using mixed limestone, spent mushroom compost and activated sludge [9]; a similar value also obtained by Ňancucheo and co-workers who used a consortia of acidophilic sulfidogenic bacteria for metals removal [10]. Lower percentages of removal were obtained by Kiran and co-workers, reaching values of 70% of copper removal with 50 mg L^{-1} Cu (II), in batch assays with lactate and using sulfate-reducing biomass obtained from a lab-scale upflow anaerobic packed bed reactor [11].

Sulfate-reducing bacteria (SRB) involved in these remediation processes use a wide range of organic compounds as electron donors; these compounds could be ethanol, formate, lactate, pyruvate, malate, succinate [12-14] and short-chain fatty acids such as acetate [15]. SRB use two different pathways to oxidized acetate, a modified citric acid cycle used by *Desulfobacter postgatei*, and the acetyl-CoA pathway used by *Desulfobacterium, Desulfotomaculum, Desulfococcus and Desulfobacca acetoxidans* [12].

Sulfate reducing bacteria and their activity can be detected molecularly by amplifying 16s rRNA gene [12] or sulfate reducing genes such as *dsrAB* ,which encodes the dissimilatory sulfite reductase, or *aprBA*, which encodes the dissimilatory adenosine-5′-phosphosulphate reductase [12].

The objective of the study was determine the bacterial taxonomic composition present in a sulfate reducing bioreactor with a limestone pre-column for the removal of copper at concentrations typically found in mine sites in Ecuador (10-40 mg L^{-1}). The limestone pre-column allowed an increase of pH and copper removal while the reduction of sulfate and additional precipitation of copper were attained in the anaerobic sulfate reducing bioreactor fed with acetate as electron donor. AMD samples from mining district

of Ecuador were also characterized based on physic-chemical parameters. This study provides information regarding the composition of the microbial consortium responsible for the bioremediation of AMD in the presence of acetate which could be used in the future to develop treatment technologies adapted to local conditions with low operating costs.

6 Materials and methods

Acid Mine Drainage (AMD)

Four samples of AMD were collected in the south of Ecuador, in the canton Portovelo, in the mining district Portovelo-Zaruma. The AMDs were collected in amber 1L bottles filled to the top and kept in refrigeration at 4^{o}C. The samples corresponded to AMD of Oroporto Mine, Tramut Mine and mining areas near to Amarillo-Calera River and Amarillo river in the black bridge sector. AMDs were characterized as described in Analytical 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 was composed of (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) y 1 mL L^{-1} of HCl (36%) [16].

Reactor Operation

The treatment system consisted of a 0.397 L limestone pre-column coupled to a 0.487 L biological reactor (Figure 4). The sludge inoculum was obtained from the 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 limestone pre-column was supplied with 1009.3 g of limestone ($CaCO_3 \geq 94.7\%$) pre-sieved in mesh # 8 and 16, which retained particles between 1 and 3 mm. Sieved limestone

was washed to release any residual dust or impurities; and it was dried at 90°C for 6 hours in an oven (Precision Scientific, Winchester, VA, USA). The biological reactor was packed with 115.8 g of sediments of the artificial lagoon (15 g L^{-1} of volatile suspended solids), and 371.2 g of sand with a density of 1.3 g mL^{-1} as support for sulfate reducing bacteria. The bioreactor was operated according to the information described in Table 4 during the various periods of operation. The bioreactor operated as a stand-alone system until day 70 (Period I). After day 70, the bioreactor operated in series with the limestone bed reactor as a pre-column and Cu^{2+} addition (as $CuCl_2$ $2H_2O$) was initiated (Period II). The bioreactor was fed with acetate as electron donor during all operation of the treatment system. The reactor influent consisted of basal mineral medium, sulfate, acetate and increasing concentrations of Cu (II) (Table 4). Reactor feed and effluent samples were analyzed for sulfate, sulfide (H_2S), total COD, conductivity and pH.

Analytical Methods

Sulfide was analyzed by the colorimetric method of the methylene blue according to Truper and Schlege [17]. Sulfate was determined by the gravimetric method by adding a saturated $BaCl_2$ solution to form $BaSO_4$ precipitate according to standard methods [18]. Chemical oxygen demand (COD) was determined by the colorimetric method with potassium dichromate as described in standard methods [18]. samples for sulfate and COD were filtered previously; COD removal and sulfate reduction were calculated as the difference between the influent and the effluent COD and sulfate concentrations, respectively. Biological Oxygen Demand (BOD) was determined using the OxiTop system and incubator (WTW, Weilheim, Germany) for five days at a temperature of 20° C [18]. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according standard methods. [18].

Nitrate, ammonium, conductivity, fluoride, chloride and pH were determined with a portable multi-parameter Thermo Scientific Orion 5-Star (Thermo Scientific, Beverly, MA, USA) according to standard methods [18].

Determination of metals such as copper, iron, manganese, magnesium, potassium and zinc, in AMD samples collected and influent and effluent samples of the reactors were analyzed by an absorption spectrophotometer (AA) Buck Scientific Model 210 VGP (Norwalk, USA) with hollow cathode lamps. Calibration curves were conducted for each metal using the respective standards with 2% HNO_3. Samples were analyzed in triplicates.

Methane generated in the bioreactor was measured using the liquid displacement method following biogas scrubbing through a NaOH solution 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 [19]. The percentages of electron equivalents of reducing power fed to the reactor (CODin, in g COD/L reactor. d) utilized for methane (% CH_4-COD) and sulfide (% H_2S-COD) generation were calculated as described in our previous publication [19].

$$\% CH_4\text{-}COD = 100(M*F_m)/COD_{in} \quad \text{Equation 2}$$

$$\% H_2S\text{-}COD = 100(S*F_s)/COD_{in} \quad \text{Equation 3}$$

where M=methanogenesis (expressed as g CH_4/L reactor.d); S=sulfide generation (sulfide in the liquid phase + sulfide in the gas phase + sulfide precipitated as CuS, expressed as g S^{2-} L^{-1} reactor.d); Fm= 4 g CH_4-COD/g CH_4; and Fs= 2 gS^{2-} COD/g S^{2-} [19].

Chemicals

Sodium sulfate (100% purity) was obtained from JT Baker (Phillipsburg, NJ, USA). Ammonium iron (III) acetate (purity) and sodium sulfate (purity) were obtained from H.V.O. (Quito, Ecuador). Sulfuric acid (95.0-97.0%) was obtained from Merck KGaA

(Darmstadt, Germany). The DMP (oxalate N, N-dimethyl-p-phenylenediamine) (> 99%) was obtained from J.T. Baker (Zedelgem, Belgium). N_2 gas was delivered by AGA Ecuador (Quito, Ecuador). All reagents were used in the state in which they were received.

Identification of microorganism

DNA extraction

Samples of sediments (2 g) were taken from the bioreactor at day 113 corresponding to the end of period IIa. The DNA was extracted with the commercial kit for genomic DNA isolation PowerSoil®DNA Isolation Kit of MoBIO, according the protocol provided by the supplier. The purity and concentration of the resulting DNA preparation was determined spectrophotometrically at 260 nm using NanoDrop (2000).

16S Amplicon Library Preparation and Sequencing

PCR targeting 16S rRNA gene V4 variable region was performed using the primers for bacterial/archaea 16S rRNA, 515F and 806R. Sulfate reducing bacteria (SRB) were also detected by targeted the functional marker genes *dsrAB* (alpha- and beta-subunits of dissimilatory (bi) sulfite reductase), with taxa-specific DSR1F–DSR4R primers [20, 21]. The PCR primers were used in a 30 cycle PCR (5 cycle used on PCR products) employing the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads.

The pooled and purified PCR product was used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed

at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Operational taxonomic units (OTUs) were defined by 97% similarity. Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov,[22], http://rdp.cme.msu.edu).

Phylogenetic analysis of 16S RNA

A phylogenetic tree was assembled using Mega 6.0 for alignment of 30 most abundant OTUs, with the Maximum Likelihood using 100 pseudo-replicate bootstrap.

3 Results and discussion

Acid mine drainage (AMD) characterization

Four AMD samples were collected in the mining district Portovelo (southwest Ecuador). Table 5 presents the average composition of acid mine drainages characterized and the allowable limits established by the US EPA 40 CFR Part 434 for the coal mining sector [23] and the Ecuadorian Legislation for discharges to freshwater bodies [24]

The concentrations of nutrients measured in acid mine drainages analyzed ranged from 176.5 to 7480.3 mg L^{-1} for ammonium, 718.9 to 1299.2 mg L^{-1} for nitrate and 26.5 to 291.7 mg L^{-1} for phosphate. The pH values were very similar for all samples analyzed varying from 1.9 to 3.5, similar to the values reported by Jimenez Rodriguez in Rio Tinto [25] as well as in the mining area of Eveline located near Silverton, Colorado in San Juan County with pH values of 3.3 [26]. Conductivity ranged widely from 8.5 to 2160 µS cm^{-1}, comparable with values obtained in the zone of Rio Tinto that varied from 1.18 to 57 µS cm^{-1} [27]. The values for COD were between 13.7 to 521.4 mg L^{-1}, and for the BOD_5, the values were very similar around 45 to 50 mg L^{-1}. Regarding sulfate, values varied from

465.1 to 1831.6 mg L^{-1} which are typical values for AMDs, and the sulfide concentrations were very low up to 3.5 mg $L^{-1.}$

Copper concentration ranged between 20.9 to 117.6 showing similar values reported previously in an abandoned copper mines in S. Domingos, Mértola, Southeast Portugal, with a concentration of 44 mg L^{-} Cu^{2+} [28]. The concentrations of iron were between 82.5 and 168.9, values very high in comparison with others acid mine drainages. For instance samples collected along Dunkard Creek downstream of Taylortown, Pennsylvania with presented numbers of 3.6-19 mg L^{-1} [29] and AMD in the mining district of Tharsis (Spain) that reached values of 35.2 mg L^{-1} [27] or samples in in the Western Region of Ghana in the Central African gold sector at Bibiani where iron concentrations were 22.6-29.2 mg L^{-1} [30]. Magnesium in the samples collected was detected in concentrations ranging from 4.0 to 11.4; similar to the value obtained at an AMD at Eveline mine in Colorado of 10.8 mg L^{-1} [26].

Zinc concentrations reached values between 1.2 to 3.4 mg L^{-1}, a little higher comparing with those reported for large mine sites in British Columbia near Elk River Valley and Goddard Marsh where the concentrations of zinc, copper and iron were <1 mg L^{-1} [31].

The typical composition of water effluents resulting from sulfide and coal mine operations in USA had pH values of 2.6 to 6.3 and concentrations of iron 1-473 mg L^{-1}, aluminium 1-58 mg L^{-1} and manganese 1-130 mg L^{-1} [32].

All samples of acid mine drainages collected in Ecuador had pH values and copper concentrations higher than the limits set by the Ecuadorian regulation taking into account that the maximum allowable limits in Ecuador for copper (1 mg L^{-1}) are high in comparison to the ones established by the US EPA regulations (0.05); the China Water

Risk regulation (0.5) [33] and the national environmental agency of Singapore government (0.1) [34], among others.

Regarding the limits set for sulfates in mining effluents, it can be observed that the samples collected in the mining are near Amarillo-Calero river and in the Amarillo river-Puente Negro sector, exceeded the limits by a factor of 1.8 for the Ecuadorian legislation. The samples collected of Oroporto, Tramut mine and Amarillo-Calero river exceeded the maximum allowable limits for sulfide established by the Ecuadorian normative [24] by a factor of 6.9, 1.2 and 1.9, respectively. The AMDs collected in the areas of Tramut Mine and Amarillo-Calero River, also exceeded the maximum allowable limits for manganese by a factor of 1.2 and 2.6, respectively. The concentrations of magnesium is not regulated in effluents discharges in Ecuador, however these metals are regulated by the US EPA [32] and all samples exceeded the maximum allowable limits established in the American legislation.

Reactor performance

The performance of sulfate reducing reactor with the limestone pre-column was tested using a synthetic acid mine drainage prepared based on the characterization of raw AMD samples from the mining district in Ecuador. This synthetic AMD was formulated to simulate the effluents generated during mining operations. The efficiency of the treatment system was assessed through determination of sulfate reduction, sulfide generation, copper precipitation, COD removal and increase of pH in the effluent of the reactor.

Figure 2 shows the time course of sulfate reduction and sulfide production in the sulfate reducing bioreactor with the limestone pre-column. Sulfide production was attained in the anaerobic bioreactor. Period I corresponded to the stabilization of the biological reactor stand-alone for 70 days with an average final pH of 7.7 to 8.6 (Table 3). As expected, the decrease in the concentration of sulfate was accompanied by an increase in

the production of sulfide reaching a value of 41 mg S^{2-} L^{-1}, and a sulfate reduction of 34% at day 61. In period II, at day 74, a sample of the packed sediments was taken and the reactor was open which caused a decrease in sulfide production and sulfate reduction due to the presence of oxygen. In the followings days, a little increase in sulfide production was observed until day 77 when 10 mg L^{-1} copper (II) were added (Period IIa) which caused a decrease of sulfide production due to the precipitation of copper sulfide. In period IIb, a second sampling of sediments for molecular analysis was conducted at day 116 causing a decrease in the sulfide production attaining a final value of 1.4 mg S^{2-} L^{-1}.s. In the period (IIc), a continuous increase in sulfide production was observed, reaching a final concentration of 118.3 mg L^{-1} at day 195 with a sulfate reduction of 41%, the maximum percentage obtained along the operation of the treatment system. In the last period (IId) the sulfide production was stabilized at 88 mg L^{-1} at day 228 with a sulfate reduction of 37%. Table 3 presents the sulfur balance for the treatment system, where the concentrations of dissolved sulfide in the effluent, sulfate as sulfur in the influent and effluent of the reactor were 83.2, 685 and 404 mg S^{2-} L^{-1}, respectively. The loss of 23% of sulfur could be due to the high volatility of the compound during the addition of the reagents of methylene blue method to the sulfide solution [35], as was observed by Cline in a study of spectrophotometric determination of hydrogen sulfide in natural waters [36].The average substrate utilization in the sulfate reducing bioreactor with the limestone pre-column during the various periods of operation presented in Table 3 indicated that during Period I, IIa and IIb acetate was degraded mainly by methanogens, with a final organic COD removal efficiency of 65, 25 and 35%, respectively, which it was supported by molecular diversity analysis performed. In Period IIc, a very similar acetate consumption by SRB and methanogens was observed with just 8% of difference. However, in the last period (IId), the electron flow in the bioreactor was almost exclusively directed toward sulfate

reduction, corresponding to 41% of the total final COD removal (59%), concluding that sulfate reduction dominated over methane production in this period. It is important to note that during operation of the system there was not complete consumption of acetate, observing the presence of acetate in the effluent of the bioreactor.

The acidity of the system was reduced efficiently from pH of 2.7 to 7.3 due to the presence of limestone in the bed reactor and it was raised even more in the bioreactor by the production of bicarbonate from the oxidation acetate, that can be used to reduce neutralization costs in the treatment of AMD because it is produced simultaneously during sulfate reduction during bacterial metabolism [37]. However, an additional alkalinity source such as limestone is often augmented with the organic carbon source to improve bicarbonate generation[38].

The final sulfate reduction obtained of 37% was similar to the values reported by Celis (36%) in the start-up of a down-flow fluidized bed with acetate [39]. The 59% of COD removal obtained in the treatment system was similar to other studies; Li reported a 52% of removal in an up-flow anaerobic sludge blanket (UASB) reactor treating saline sulfate wastewater with acetate, sucrose and propionate as carbon sources [40]; Bharaty obtained a COD removal efficiency of 55% in a study with acetate as carbon source for sulfate reduction [13] and Alves and co-workers in a study of copper removal performed in an UASB using acetate as carbon source obtained a value of removal of 60% [41].

These results of this study indicate that when the system was operated at a ratio of 1.25 g COD g SO_4^{2-} with acetate as electron donor, a competition between SRB and methanogens was promoted. It is known that in anaerobic treatment processes, SRB and methanogens always compete for carbon source in greater or lesser degree. In a study with pure co-cultures of these two groups of microorganisms it was observed that acetate was converted into CH_4 and CO_2 during the incubation period, suggesting the coexistence of

acetoclastic methanogens and acetoclastic sulfate reducers [42]. Jing, Z. in a study performed for wastewater treatment in a UASB reactor that run for more than 180 days with presence of 3000 mg L^{-1} of sulfate, 1000 mg L^{-1} of ethanol and 1000 mg L^{-1} of acetate (about 3000 mg L^{-1} of COD in total) determined that the proportion of COD used for methane production was around 50% with a HRT in the range of 3–12 h and SRB accounted for 28.4–31.0% of electrons utilization showing a strong competition between these microorganisms[43].

Flaherty and co-workers observed sulfate-reducing, methanogenic, syntrophic and homoacetogenic bacteria in a full up, flow fully packed anaerobic digester sludge which was treating sulfate containing wastewater; they also found that methanogenic bacteria outcompeted sulfate-reducers for acetate [44]. In contrast, in our study it was observed that the growth of SRB was stimulated overtime, suggesting that SRB required some time to overcome the competition from other anaerobic species in order to reach a completely sulfatorreductor system. According to our results, literature studies have demonstrated that the predominance of SRB over methanogenic bacteria in a sulfate medium was only achieved after long term operation of bioreactors [45] [46]. For instance, Harada et al observed that in a UASB reactor with mesophilic digested sewage sludge the portion of electron flow used by SRB ranged from 38.9% at a loading rate of 1.0 kg COD m^{-3} d^{-l} to 74.9% at 3.0 kg COD m^{-3} d^{-1} in a period of 180 days; these result indicated that SRB had been gradually outcompeting methanogens during the long-term operation [45]. Omil and co-workers in a study about the competition between acetate utilizing methane-producing bacteria and sulfate reducing bacteria in a mesophilic upflow anaerobic sludge bed (UASB) reactors treating volatile fatty acids and sulfate; observed that SRB became predominant over methanogens after prolonged reactor operation (250-400 days), increasing the amount of acetate used by SRB from 50 to 90% [46].

Copper removal

Figure 6 shows the concentration of Cu^{2+} in the influent and effluent of the system as a function of time. The reactor system proved to be highly efficient for the removal of Cu^{2+} to the low ppm range. The average concentration of soluble Cu^{2+} in the wastewater was reduced from 10 to 0.5 mg L^{-1} in Period IIa, from 20 to 1.6 mg L^{-1} in Period IIb, from 30 to 0.5 mg L^{-1} in Period IIc and from 40 to 0.4 in Period IId (Figure 6). Therefore, the removal efficiency of copper in the reactor ranged from 95.7 to 99.8 %. Only at the end of period IIb and the beginning of period IIc, 95 and 91% removal efficiencies were observed; however, in the following days values between 97 to 99% were reached. This somewhat lowered performance was likely due to several interruptions in the reactor due to sampling of the sediments of the reactor for molecular analysis that caused reduction of sulfide production. Precipitation of the heavy metals by biogenic sulfides resulted in very low metal concentrations in the effluent. These results are similar to those reported by Chambe where 99% copper removal was obtained with an initial concentration of 106.6 mg L^{-1} Cu^{2+} in the acid drainage of the Production Unit of Cerro de Pasco (Peru) belonging to the mining company Volcan; using as carbon source for the growth of the SRB, the manures of corral birds, sheep and cattle [47]. In the same manner, Ňancucheo and co-workers in their study of selective removal of transition metals from acidic mine waters using a consortia of acidophilic sulfidogenic bacteria 99% of copper removal was attained. [10]

Finally, taking into consideration that 90% of the total copper removal occurred in the limestone bed reactor due to the formation and precipitation of the corresponding metal-carbonate ligands [48] ; it is recommended to added more limestone to the bed reactor if the limestone is exhausted by dissolution [49] or by encrustation with metals compounds and gypsum ($CaSO_4$ $2H_2O$) [50]. It is also done with other treatment systems

like wethlands that are used for treating AMD for a finite period, after which the system must be replenished or replaced [49].

Microbiological taxonomic composition

The microbial diversity present in the sulfate reducing bioreactor for the removal of copper at day 113 corresponding to period IIa was also evaluated by means of 16S rRNA and *dsrAB* gene analysis.

16S rRNA library analysis revealed a mixed community of archaeal and bacterial species; 61.278 sequences were assigned to taxonomic affiliations with homologies ranging from 73 to 100%. Members of the Archaea domain corresponded to 19% of the total sequences, Bacteria domain to 31% while Eukarya corresponded to the 0.094%.

The most abundant phyla were: Euryarchaeota, Protobacteria, Bacteroidetes, Firmicutes, Spirochaetes (Figure 7). The most abundant Archaea genera were: *Methanosarcina* (15% abundance) and *Methanosaeta spp* (3% abundance). While, for Bacteria, the predominant genera were: *Petrimonas, Spirochaeta, Desulfotomaculum, Desulfovibrio, Desulfococcus, Aminivibrio and Bacteroides*.

Methanogens clearly were most abundant that SRB at time of sampling as shown in Table 8. *Methanosaeta* and *Methanosarcina* were the predominant genera which are considered among the most important acetoclastic methanogens with higher affinity for acetate [51]. SRB were less prevalent; the main SRB were: *Desulfovibrio vulgaris, Desulfotomaculum sp (Desulfotomaculum intricatum* and *Desulfotomaculum acetoxidans).*

Desulfovibrio vulgaris can use acetate, pyruvate, formate, and certain primary alcohols as carbon source [52]. *Desulfotomaculum intricatum* is known to use acetate, n-butyrate, ethanol and H_2 as electron donors for the sulfate reduction [53]. Formate, fumarate and pyruvate are also utilized weakly by this species for sulfate reduction; moreover its growth is enhanced by the addition of yeast extract [53]. *Desulfotomaculum*

acetoxidans was one of the first sulfate-reducing bacteria known to use acetate for energy and carbon source; it oxidizes acetate via the acetyl-CoA/carbon monoxide dehydrogenase (CODH) pathway [54, 55]. This bacterium utilizes ethanol, butanol and butyrate as electron donors but not hydrogen, lactate, propanol or pyruvate [53, 56].

Sulfate reducers belonging to Archaea Domain were not identified, probably due to the fact that sulfate reducing archaea exhibits optimal growth temperatures above 80°C [54], while our reactor was maintained at 30°C.

In accordance with Elferink and co-workers, the most abundant SRB in their reactor were: *Desulfobacter,* followed by *Desulfotomaculum, Desulfovibrio, Syntrophobacter pfennigii and Desulfobulbus*. Although *Desulfobacter sp*. has been reported as an acetate-utilizing sulfate reducer, in our bioreactor this specie did not play an important role [57]. The main reason may be that *Desulfobacter spp*. requires high levels of sodium and magnesium chloride (like sea water)[58], which did not correspond to the conditions in our reactor.

Analysis of the *dsrAB* revealed (63.725 sequences, homologies ranging 71-98%) confirmed the results obtained by the16S sequence (Table 8). The main genera identified were: *Desulfotomaculum*, *Desulfovibrio, Syntrophobacter, Desulfosalsimonas, Desulfobulbus, Desulfacinum* and *Desulfosarcina*.

Among the microorganisms identified, *Syntrophobacter* is known to grow mainly in the presence of propionate and sulfate [57], *Desulfobulbus sp*. has been identified in reactors with carbohydrates and/or volatile fatty acids containing wastewater [57]. While, *Desulfotomaculum alkaliphilum sp* can grow in the presence of sulfate plus acetate, formate, ethanol, lactate or pyruvate [59].

Our study also concurs with the distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates reported by Santegoeds, C.M and co-workers [60] and so

was the composition of metal-removing consortium which was similar to that reported by Baldwin in sulfidogenic biochemical reactors (BCRs)[61]

Finally, it was expected that under the operational conditions applied in this study a complex community of methanogens and sulfate reducing bacteria would develop on the bioreactor, which was confirmed by molecular analysis. However, we found low SRB density in the sediment which was probably due to distinct adaptation times of different SRB; for instance *Desulfotomaculum* (most abundant SRB) when grows solely on acetate exhibits slower growth rate than other SRB probably because it possess a non-cyclic pathway that does not have substrate level phosphorylation [62]. Santegoeds and coworkers also found low SRB population density in UASB reactors fed with acetate, butyrate and propionate after 90 days of operation [60].

Although sulfate-reducers were restricted to only a few lineages at the time of sampling for molecular analysis in the study (Period IIa), there was evidence of sulfate reduction by the detection of sulfide during the treatment system operation. However it is important considered that at the end of the study the sulfide concentration increased notably and the methane production reduced significantly. This could be an indication that the system was shifting from methanogenesis to sulfidogenesis. For this reason, it is recommended to determine the microbial community present in the bioreactor at different operation times.

7 Conclusions

This work demonstrated that the application of a sulfate reducing bioreactor with a limestone pre-column can be used for the removal of copper (Cu^{2+}) from AMDs, with an efficacy between 95 to 99%, as only treatment objective or in combination with reduction of sulfate and COD concentrations. The final sulfate reduction obtained was 37% and

chemical oxygen demand removal was 59%, with the predominance of COD consumption by methanogens during the first 153 days; and the dominance of SRB with a COD removal of 41.3% at the end of the study (228 days). Also, the acidity of the system was reduced effectively from pH values of 2.7 in the influent to neutral pH values of 7.7. The alkalinity generated from the sulfate reduction process as well as bicarbonate alkalinity from acetate degradation can be used to reduce neutralization costs in the treatment of AMD

Finally, according to molecular analysis at day 116 of operation of the biological reactor, was observed the predominance of methanogens (*Methanosaeta* and *Methanosarcina),* while sulfate reducing bacteria (SRB) were restricted to the genera: *Desulfotomaculum, Syntrophobacter, Desulfosalsimonas, Desulfobulbus, Desulfacinum, Desulfosarcina and Desulfovibrio*; The species of SRB responsible for sulfate reduction in presence of acetate were probably *Desulfotomaculum intricatum* and *Desulfotomaculum acetoxidans*.

This microbial diversity is similar to others studies where acetate was used as carbon source, and was determined that the time of operation is important in the establishment of a SRB population that outcompete methanogens. Future studies must performed new molecular analysis to determine the microbial diversity present at different times of operation of the treatment system.

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Table 4 Average concentration of components in the influent and conditions maintained during the operation of the sulfate-reducing bioreactor.

Parameter	Units	Average value
Sulfate	g L^{-1}	2.0 (±0.1)
COD-acetate	g L^{-1}	2.5 (±0.3)
pH	-	7.8 [a] - 2.7 [b]
Cu 2^{+} (only periods IIa to IId)	mg L^{-1}	10-40[b] (±0.4)
Volumetric loading rate	g COD L^{-1} d^{-1}	3.8 [a] – 2.1 [b]
Temperature	ºC	30 (±2)
Hydraulic retention time (HRT)	d	1.4[a]-2.5[b]

[a] Period I: biological reactor stand-alone (70 d).
[b] Period II: Period IIa: 10 mg L^{-1} Cu^{2+} (36 d), Period IIb: 20 mg L^{-1} Cu^{2+} (40 d), Period IIc: 30 mg L^{-1} Cu^{2+} (40d), and Period IId; 40 mg L^{-1} Cu^{2+} (35d).

Table 5 Physical and chemical characterization of real acid mine drainages collected in the mining district Portovelo-Zaruma.

Analysis	Units	Oroporto Mine	Tramut Mine	Mining area near Amarillo –Calero river	Mining area near Amarillo river in puente negro sector	Permissible Limits
Ammonium	mg L^{-1}	7480.3 ± 21.0	176.4 ± 56.9	358.3 ± 38.3	232.2 ± 70.1	-
Biological oxygen demand	mg L^{-1}	50.0 ± 2.7	35.0 ± 1.1	45.0 ± 2.2	50.0 ± 3.1	100 [a]
Chloride	g L^{-1}	52.7 ± 2.3	75.7 ± 1.4	27.7 ± 1.6	15.3 ± 1.6	-
Chemical oxygen demand	mg L^{-1}	521.4 ± 65.1	16.0 ± 1.9	18.8 ± 2.4	13.7 ± 0.3	250
Conductivity	µS/cm	12.8 ± 0.5[c]	8.5 ± 0.5 [c]	1521 ± 0.5 [c]	2160 ± 0.5 [c]	-
Fluoride	mg L^{-1}	<0.5	<0.5	<0.5	<0.5	-
Nitrate	mg L^{-1}	721.8 ± 82.3	718.9 ± 37.8	1299.2 ± 58.1	917.2 ± 35.7	10
pH	-	1.9 ± 0.01 [c]	1.9 ± 0.01 [c]	3.5 ± 0.01 [c]	2.6 ± 0.01 [c]	5-9
Phosphate	mg L^{-1}	291.7 ± 27.7	59.2 ± 4.6	206.5 ± 9.2	26.4 ± 3.9	-
Sulfate	mg L^{-1}	465.1 ± 5.8	477.4 ± 10.4	1790.4 ± 29.1	1831.6 ± 2.9	1000
Sulfide	mg L^{-1}	3.4 ± 0..01	0.6 ± 0.001	0.9 ± 0.003	<0.05 ± 0	0.5
Total solids	mg L^{-1}	425.0 ± 14.1	197.5 ± 10.6	14632.5 ± 1219.7	13205.0 ± 2291.0	1600
Total Suspended Solids	mg L^{-1}	252.5 ± 3.5	70.0 ± 21.2	47.5 ± 10.6	455.0 ± 56.6	100[a]-70[b]

Volatile solids	mg L^{-1}	247.5	±	24.7	65.0	±	2.8	20.0	±	7.1	145.0	±	49.5	-
Volatile suspended solids	mg L^{-1}	250.0	±	14.1	62.5	±	17.6	10.0	±	1.0	115.0	±	21.2	-
Copper	mg L^{-1}	20.9	±	1.0	23.1	±	1.1	34.4	±	1,4	117.6	±	2.2	1.0 [a], 0.05 [b]
Iron	mg L^{-1}	148.8	±	1.4	125.0	±	2.3	82.4	±	1.7	168.8	±	0.2	10[a], 6[b]
Magnesium	mg L^{-1}	3.9	±	0.3	8.9	±	0.6	11.3	±	1.2	4.5	±	0..04	3.5[b]
Manganese	mg L^{-1}	1.3	±	0.1	2.4	±	0.4	5.3	±	1.3	1.1	±	0.3	2.0 [a,b]
Potassium	mg L^{-1}	9.8	±	0.5	20.2	±	1.9	23.4	±	1.3	35.3	±	1.4	-
Zinc	mg L^{-1}	1.2	±	0.1	1.9	±	0.01	4.2	±	0.1	3.4	±	0.2	5.0[a], 1.5

[a] Ecuadorian legislation: Text of Unified Secondary Environmental Legislation (TULSMA), Book VI, Annex 1, Table 12
[b] USEPA United States Environmental Protection Agency. Development document for final effluent limitations guidelines. 40 CFR Part 434
[c] Standard deviation of the instrument.

Table 6 Average performance of the sulfate reducing bioreactor with the limestone pre-column during the various periods of the system operation.

Period	Effluent sulfate, (mg S L^{-1})	Effluent pH	H_2S (mg H_2S L^{-1})	%COD in[a]		
				H_2S formed	CH_4	Organic COD removal
I	685[b]-459	7.7-8.6	41.2 (±0.4)	22.6 (±3.2)	45.4 (±6.2)	68 (±2.4)
IIa	653	7.0-7.7	4.2 (±0.1)	8.5 (±0.6)	16.5 (±4.1)	25 (±3.4)
IIb	526	7.0-7.8	11.3 (±0.1)	13.0 (±0.8)	21.9 (±3.7)	35 (±2.3)
IIc	438	7.0-7.5	70.9 (±0.7)	29.8 (±2,5)	21.2 (±3.5)	51 (±5.4)
IId	404	7.1-7.7	88.4 (±1.5) (83.2 mg S L^{-1})	41.3 (±3.1)	17.7 (±0.3)	59 (±1.8)

[a] Values are expressed as percentage of the initial wastewater COD (CODin).
[b] Sulfate of the influent

Table 7 Influent Cu^{2+} concentration and average removal of copper attained by the sulfate reducing bioreactor with the limestone pre-column during the various periods of operation.

Period	Time of operation (d)	Cu^{2+} conc. (mg L^{-1})	Removal soluble copper (%)		
			Limestone reactor	Bioreactor	Complete system
IIa	36	10 (±0.3)	93.5 (±5.7)	6.2 (±1.9)	99.7 (±1.2)
IIb	40	20 (±1.1)	87.8 (±6.3))	7.8 (±0.7)	95.7 (±3.4)
IIc	40	30 (±0.8)	90.5 (±2.5)	9.0 (±0.3)	99.5 (±3.2)
IId	35	40(±0.5)	94.5 (±1.9)	4.7(±0.2)	99.2 (±0.4)

Table 8 Predominant taxa identified by 16s RNA and dsr gene in the sulfate reducing reactor in Period IIa (day 116) of operation of the treatment system.

Taxonomic affiliation				16S rRNA		*dsr* gene	
Domain	Phylum	Genera	Specie	Homology (%)	Abundance (%)	Homology (%)	Abundance (%)
Archaea	Euryarchaeota	*Methanosarcina*	*Methanosarcina siciliae*	99	9	n/d	n/d
Archaea	Euryarchaeota	*Methanosarcina*	*Methanosarcina mazei*	100	6	n/d	n/d
Archaea	Euryarchaeota	*Methanosaeta*	*Methanosaeta sp.*	98	3	n/d	n/d
Bacteria	Bacteroidia	*Petrimonas*	*Petrimonas spp*	99	4	n/d	n/d
Bacteria	Synergistia	*Aminivibrio*	*Amnivibrio pyruvatiphilus*	99	2	n/d	n/d
Bacteria	Bacteroidia	*Bacteroides*	*Bacteroides sp*	99	2	n/d	n/d
Bacteria	Proteobacteria	*Desulfacinum*	*Desulfacinum infernum*	n/d	n/d	88	2.5
Bacteria	Proteobacteria	*Desulfobulbus*	*Desulfobulbus spp*	n/d	n/d	84	3.2
Bacteria	Proteobacteria	*Desulfococcus*	*Desulfococcus spp*	99	2	88	0.2

Bacteria	Proteobacteria	*Desulfosalcimona*	*Desulfosalsimonas propionica*	n/d	n/d	85	5
Bacteria	Proteobacteria	*Desulfosarcina*	*Desulfosarcina variabilis*	n/d	n/d	86	1.7
Bacteria	Firmicutes	*Desulfotomaculum*	*Desulfotomaculum sp*	99	2.5	91	53
Bacteria	Firmicutes	*Desulfotomaculum*	*Desulfotomaculum intricatum*	99	2	91	30
Bacteria	Firmicutes	*Desulfotomaculum*	*Desulfotomaculum acetoxidans*	96	0.1	84	21
Bacteria	Firmicutes	*Desulfotomaculum*	*Desulfotomaculum alkaliphilum*	97	0.1	73	1
Bacteria	Deltaproteobacteria	*Desulfovibrio*	*Desulfovibrio vulgaris*	99	2	98	11
Bacteria	Betaproteobacteria	*Delftia*	*Delftia acidovorans*	99	1	n/d	n/d
Bacteria	Deltaproteobacteria	*Syntrophobacter*	*Syntrophobacter*	99	7	87	11
Bacteria	Sphingobacteria	*Solitalea*	*Solitalea canadensis*	100	1	n/d	n/d

n/d not detected

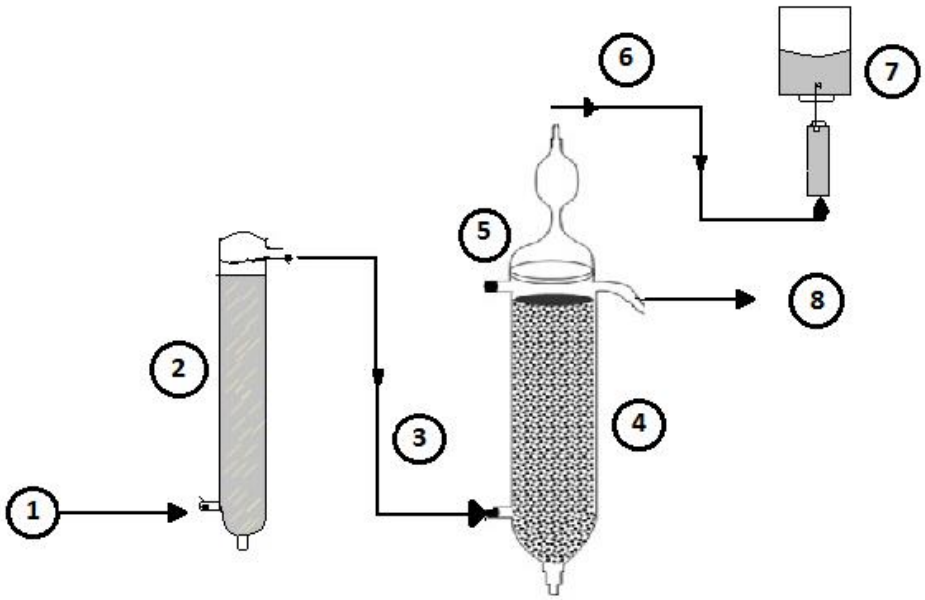

Figure 4. Schematic representation of the sulfate reducing bioreactor with the limestone pre-column. (1) Influent, (2) limestone pre-column (height, 25 cm; internal diameter (i.d.), 5.5 cm), (3) limestone pre-column effluent/bioreactor influent, (4) biological reactor (height, 43.2 cm; i.d., 5.5 cm), (5) gas-liquid-solid separator, (6) biogas, (7) biogas scrubber containing 1M NaOH; and (8) treated effluent..

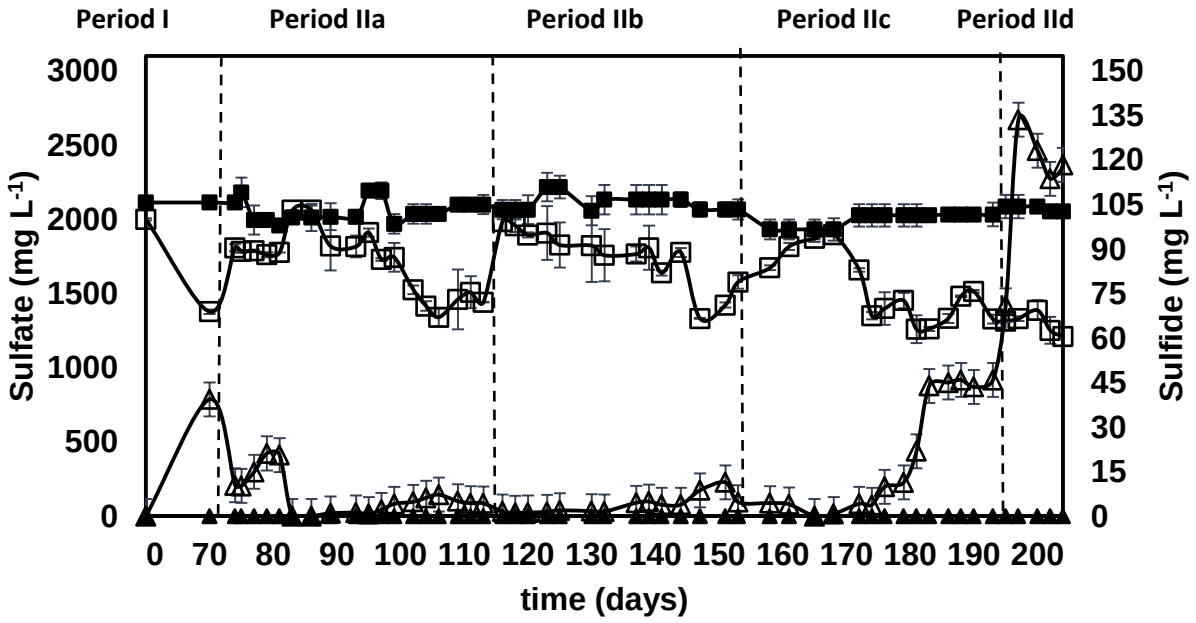

Figure 5 Time course of sulfate reduction and sulfide production in the sulfate reducing bioreactor during (204) days of operation: sulfate influent (filled square), sulfate effluent (open square), sulfide influent (filled triangle) and sulfide effluent (open triangles).

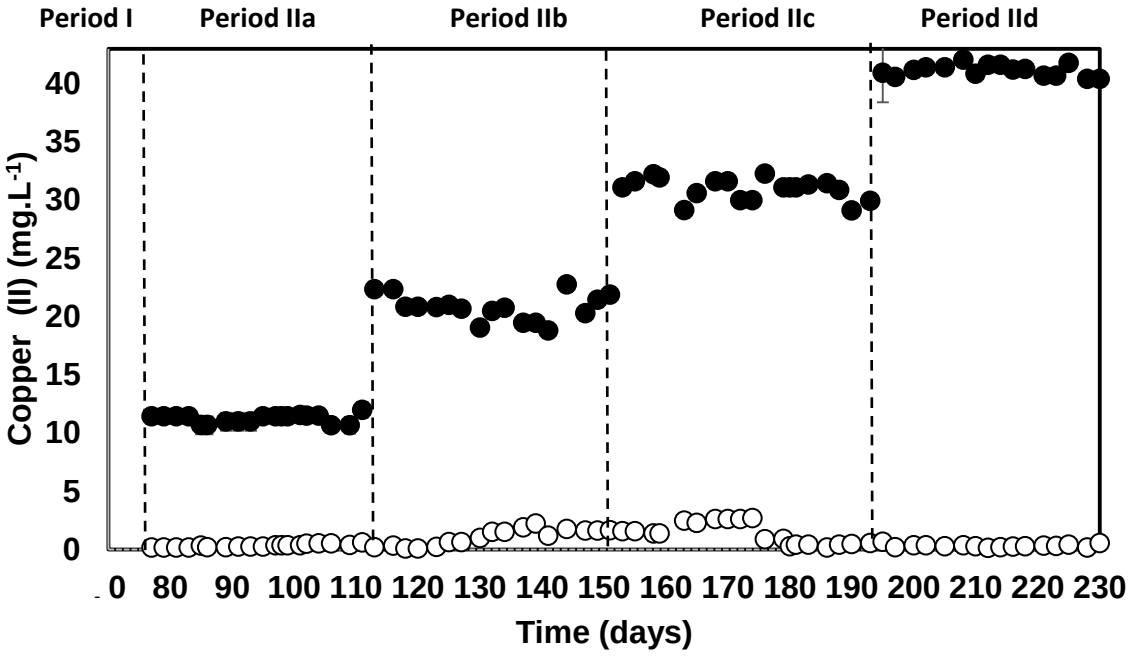

Figure 6 Concentration of soluble copper in the influent (filled circles) and effluent (open circles) of the sulfate reducing bioreactor with a limestone pre-column system as a function of time.

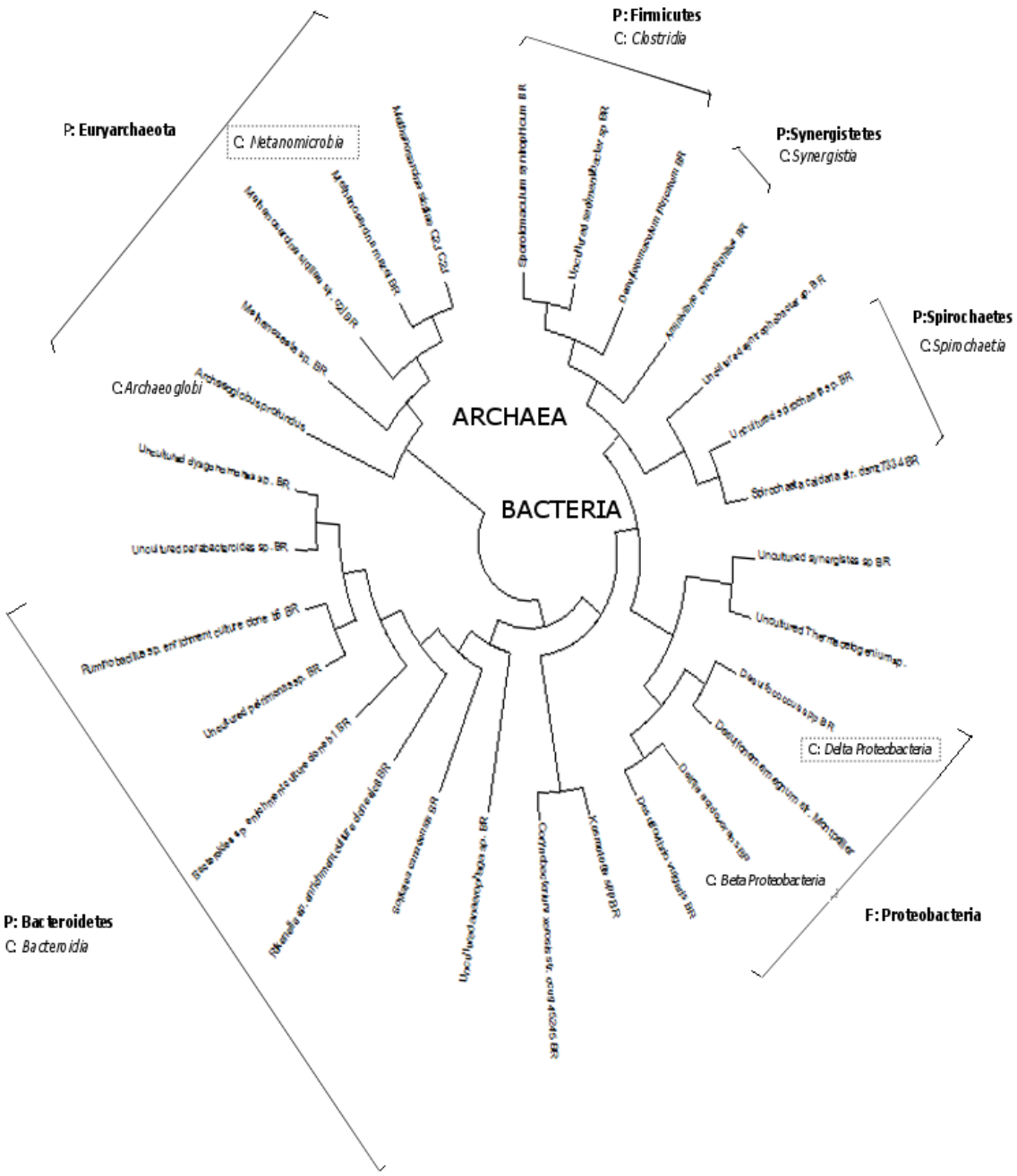

Figure 7 Molecular Phylogenetic analysis by Maximum Likelihood method: The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 28 nucleotide sequences. All positions

containing gaps and missing data were eliminated. There were a total of 269 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.