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## Effect of the substrate as electron donor during the microbial sulfate reduction and its possible applications in the biological treatment of acid mine drainage

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### Abstract

The uncontrolled release of acid mine drainage (AMD) characterized by elevated concentrations of dissolved metals, high levels of sulfate ions and low pH values threatens the quality of water resources nearby mining areas worldwide. The development of novel technologies based on the precipitation of metals as metal sulfides catalyzed by sulfate reducing bacteria constitute an important method for the bioremediation of AMD. The objective of this study was to evaluate in batch experiments the efficiency of different organic substrates such as acetate, lactate, ethanol and peptone as electron donors during the generation of biogenic sulfide by different microbial inocula and its possible applications in the bioremediation of AMD. The highest sulfide production activity was obtained with 2.5 g acetate-COD L<sup>-1</sup> as substrate, 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> as electron acceptor and the sediments of an artificial lagoon as bacterial inoculum. The final cumulative sulfide production was 463 mg S<sup>2-</sup> L<sup>-1</sup>, the maximum specific sulfide production activity was 9 mg S<sup>2-</sup> g acetate<sup>-1</sup> d<sup>-1</sup>, and the maximum specific sulfate reduction activity was 52 mg SO<sub>4</sub><sup>2-</sup> g acetate<sup>-1</sup> d<sup>-1</sup>. In terms of the substrate concentration, a 10-fold increase in the concentration of the electron donor resulted in substrate inhibition significantly decreasing the biogenic sulfide activities. The results of this study indicate that acetate was a highly effective substrate during the microbial sulfate reduction with a potential application in the remediation of acid mine drainage. Moreover, the use of acetate as electron donor favored the sulfate reducing activity through the inhibition of the methanogenic activity of the microorganisms present in the evaluated microbial inocula.

**Keywords.** sulfate reducing bacteria (SRB), acid mine drainage (AMD), organic substrates, electron donor, biogenic sulfide activity.

### Resumen

La descarga no controlada de los drenajes ácidos de mina (DAM) caracterizados por presentar elevadas concentraciones de metales disueltos, iones sulfato y pH ácido amenaza la calidad de los cuerpos de agua cercanos a las zonas mineras alrededor del mundo. Tecnologías basadas en la precipitación de metales en forma de sulfuros metálicos, catalizada por las bacterias sulfato reductoras encargadas de la producción desasimilatoria de sulfuros constituyen un importante método de remediación de los DAM. En este trabajo de investigación se evaluó la eficiencia de diferentes sustratos tales como acetato, lactato, etanol y peptona como donadores de electrones durante la sulfato reducción microbiana. Diferentes inóculos microbianos y sus posibles aplicaciones en el tratamiento biológico de los DAM fueron estudiados en experimentos batch. La mayor eficiencia de producción de sulfuro catalizada por las bacterias sulfato reductoras fue alcanzada empleando 2.5 g DQO-acetato L<sup>-1</sup> como donador de electrones, 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> como aceptor de electrones y los sedimentos de una laguna artificial como inóculo bacteriano. La producción final acumulada de sulfuro fue 463 mg S<sup>2-</sup> L<sup>-1</sup>, mientras que la actividad máxima específica de producción de sulfuro fue 9 mg S<sup>2-</sup> g acetato<sup>-1</sup> d<sup>-1</sup> y la actividad máxima específica de reducción de sulfato fue 52 mg SO<sub>4</sub><sup>2-</sup> g acetato<sup>-1</sup> d<sup>-1</sup>. El efecto de la concentración de sustrato también fue evaluado, un incremento de 10X en la concentración del donador de electrones resultó en una inhibición por sustrato disminuyendo significativamente la actividad biogénica de generación de sulfuro. Los resultados de este estudio indican que acetato fue un sustrato muy eficiente durante la sulfato reducción microbiana con un gran potencial de aplicación en la remediación de los DAM. Más aún, el empleo de acetato como donador de electrones favoreció la actividad sulfato reductora a través de la inhibición de la actividad metanogénica de los microorganismos presentes en los inóculos microbianos evaluados.

**Palabras Clave.** bacterias sulfato reductoras, drenajes ácidos de mina (DAM), sustratos orgánicos, donadores de electrones, producción biogénica de sulfuro.

## Introduction

The mining industry is one of the most important industries worldwide. However, the liquid discharges generated by this activity known as acid mine drainage (AMD) are considered, nowadays, one of the most serious environmental problems around the globe. AMD is characterized by high concentrations of heavy metals, sulfate ions and low pH values. AMD is generated through a combination of chemical and biological processes by which the metal sulfides from mining activities, such as pyrite ( $\text{FeS}_2$ ), arsenopyrite ( $\text{FeAsS}$ ), chalcopyrite ( $\text{CuFeS}_2$ ), and sphalerite ( $\text{ZnS}$ ) are oxidized and generate lixiviates which are very toxic to the ecosystem [1, 2].

The methods for the remediation of AMD based on the use of sulfate reducing bacteria (SRB) represent a promising technology for the biotreatment of mining effluents. SRB in the presence of sulfate and an organic substrate catalyzed the biogenic generation of sulfide that promotes the precipitation of heavy metals as metal sulfides, the increment of pH of the effluent due to the formation of bicarbonate during the oxidation of the organic substrate and the consumption of sulfate [1–3]. This type of treatment significantly reduces the negative impact of AMD in the ecosystems and human health.

Much research work has been focused on characterizing and evaluating the application of sulfate reducing processes to remediate contaminated AMD sites. The distribution and activity of SRB that utilizes acetate as substrate were studied by Karnachuk *et al.* using the sediments of the Norilsk mining area (Northern Siberia) [3]. Acetate was provided in concentrations of  $0.5 \text{ g COD L}^{-1}$  obtaining sulfate reduction rates averaged between  $0.0048$  and  $2.88 \text{ mg SO}_4^{2-} \text{ L}^{-1} \text{ d}^{-1}$ . In addition, a SRB number of  $2.5 \times 10^6 \text{ cells mL}^{-1}$  was determined by the most probable number (MPN) method [3]. In the study conducted by Manoues and coworkers with the sediments of Devils Lake in the northeast region of North Dakota, the importance of an adequate electron donor in the SRB activity was also illustrated. Bioassays conducted with  $1.92 \text{ g acetate-COD L}^{-1}$  with sulfate concentrations in the lake ranging between  $300$  and  $3000 \text{ mg L}^{-1}$  reported maximum specific sulfate reduction activities between  $1.1$  and  $8.5 \text{ mg SO}_4^{2-} \text{ L}^{-1} \text{ d}^{-1}$  [4]. These results demonstrated the efficiency of acetate as substrate with sulfate reduction maximum specific activities six times higher than when acetate was not used [4].

The presence of SRB has also been investigated. Vladár *et al.* conducted a study in the sediments of Velencei Lake located in the southeast of Budapest (Hungary) to determine the presence of SRB [5]. In that study, lactate was provided as the electron donor and the most probable number method was utilized for determining the amount of SRB present in the sediments which was found to be  $5.4 \times 10^5 \text{ cells g sediment}^{-1}$ . Among 47

SRB cultures were characterized through molecular methods. Different electron donors were also tested in order to obtain information of the substrate utilization capacity of the species isolated from the sediments [5].

The objective of this research work was to evaluate the capacity of SRB to utilize different substrates as electron donors during the microbial sulfate reduction and its possible applications in the biotreatment of acid mine drainage. Acetate, lactate, ethanol and peptone were evaluated in this study in the presence of sulfate in batch bioassays. The anaerobic microbial inocula tested included sludge and sediments from wastewater treatment plants, artificial and natural lagoons, and stabilization lagoons in Ecuador. Microbial competition between sulfate reducing bacteria and methanogens was also evaluated in the presence of acetate as electron donor.

## Materials and Methods

### Sludge and sediments

Anaerobic sludges from five sources were evaluated in the study. Three sludges came from wastewater treatment plants (WWTP) and two were lagoon sediments. An anaerobic sludge was obtained from the facultative lagoon of the WWTP of Ucubamba - ETAPA in Cuenca ( $S_1$ ). Sludge from a WWTP in Quito was also used as microbial inoculum: hydrocyclone sludge ( $S_2$ ) and sedimentation tank sludge or discharge sludge ( $S_3$ ). The sediments of an artificial lagoon at the Universidad San Francisco de Quito - USFQ ( $S_4$ ) and of a natural lagoon located in the Orellana Province ( $S_5$ ) in the Amazonian region were also collected. The content of total suspended solids (TSS) and of volatile suspended solids (VSS) in the sludge and sediments was (TSS, VSS):  $S_1$  (13.94%, 3.56%);  $S_2$  ( $8.94 \text{ g L}^{-1}$ ,  $5.92 \text{ g L}^{-1}$ );  $S_3$  ( $2.88 \text{ g L}^{-1}$ ,  $2.00 \text{ g L}^{-1}$ );  $S_4$  (14.90%, 6.28%) and  $S_5$  (20.64%, 2.69%), respectively. The sludge and sediments were stored in refrigeration at  $4^\circ\text{C}$  in plastic containers.

### Culture media

The basal mineral medium used in the sulfate reduction and methanogenic bioassays contained (in  $\text{mg L}^{-1}$ ):  $\text{NH}_4\text{Cl}$  (280);  $\text{KH}_2\text{PO}_4$  (195);  $\text{MgSO}_4$  (49);  $\text{CaCl}_2$  (10);  $\text{NaHCO}_3$  (3000); yeast extract (10); and  $1 \text{ mL L}^{-1}$  of trace element solution. The trace element solution contained (in  $\text{mg L}^{-1}$ ):  $\text{H}_3\text{BO}_3$  (50),  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  (2,000),  $\text{ZnCl}_2$  (50),  $\text{MnCl}_2$  (32),  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  (50),  $\text{AlCl}_3$  (50),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (2,000),  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  (50),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (44),  $\text{NaSeO}_3 \cdot 5\text{H}_2\text{O}$  (100), EDTA (1,000), resazurin (200), and  $1 \text{ mL L}^{-1}$  of HCl 36% [6]. The pH of the basal mineral medium was adjusted to 7.1–7.3 with HCl and NaOH, as required.

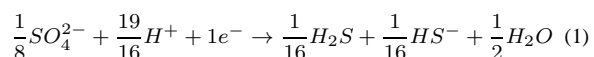
### Chemicals

Sodium sulfate (100% purity) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Ammonium and iron

(III) sulfate, sodium acetate and ethanol (96%) were obtained from Laboratorios Químicos H.V.O. (Quito, Ecuador). Sulfuric acid (95 - 97 %) and peptone were obtained from Merck KGaA (Darmstadt, Germany). DMP (N,N-dimethyl-p-fenildiamine oxalate) (> 99%) was obtained from Acros Organics (Geel, Belgium). Zinc chloride (97.1%) was obtained from J. T. Baker (Zedelgem, Belgium). Lactic acid (88 - 92%) was obtained from Riedel-de Haën (Seelze, Alemania). N<sub>2</sub> gas was delivered from AGA Ecuador (Guayaquil, Ecuador). All the chemicals were used in the condition they were received.

### Batch microbial bioassays

Batch microbial bioassays were conducted in duplicates using glass serum flasks (160 mL) with butyl rubbers stoppers and aluminum crimp seals. The headspace was flushed with N<sub>2</sub> 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 the case of the sulfate reducing activity bioassays, each flask was supplemented with 100 ml basal mineral medium, 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> as sodium sulfate, 10% v/v of microbial inoculum and the desired organic substrate concentration. The organic substrates evaluated were acetate (2.5 g COD L<sup>-1</sup>), lactate (2.5 and 25 g COD L<sup>-1</sup>), ethanol (2.5 and 25 g COD L<sup>-1</sup>) and peptone (2.5 and 25 g L<sup>-1</sup>). In the methanogenic activity bioassays, each flask was supplemented with 50 ml basal mineral medium, 2.14 g acetate-COD L<sup>-1</sup> and 10% v/v of microbial inoculum. The reduction of sulfate to sulfide was periodically monitored by measuring the S<sup>2-</sup> concentration in aqueous phase and the methane generation was monitored during 5 days according to the protocol described in analytical methods. The maximum specific sulfide generation (mg S<sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup>) and methanogenic (mg CH<sub>4</sub>-COD g VSS<sup>-1</sup> d<sup>-1</sup>) activities were calculated from the slope of sulfide production and substrate concentration, and cumulative methane production and biomass concentration; respectively, versus time (d), as the mean value of duplicate assays. The maximum specific sulfate reduction activity was expressed in mg SO<sub>4</sub><sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup>. The sulfate concentration consumed was calculated based on the following microbial reduction reaction responsible of the generation of H<sub>2</sub>S and HS<sup>-</sup> proposed by Metcalf and Eddy [7].



### Analytic methods

Total dissolved sulfide was analyzed colorimetrically by the methylene blue method at a wavelength of 670 nm [8, 9].

Methane generated during the anaerobic bioassays was determined by the liquid displacement method with serum flasks [10]. Total suspended solids (TSS) and volatile

suspended solids (VSS) were determined according to Standard Methods for Examination of Water and Wastewater [11].

### Results and discussion

In this study, different organic substrates were evaluated in batch bioassays as electron donors during the biogenic generation of sulfide by a mix culture of SRB present in anaerobic sludge and sediments from different sources. Figure 1 represents an illustrative example of the time course of sulfide production with the use of acetate (2.5 g COD L<sup>-1</sup>) as substrate in the presence of 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> in an abiotic control (absence of microorganisms) and in the treatment bioassay with the sludge of the facultative lagoon of the WWTP in Cuenca as microbial inoculum. There was practically no sulfide production in the abiotic control, while in the treatment bioassay the production of S<sup>2-</sup> gradually increased with incubation time. In fact, in the treatment, a maximum of 35.96 mg S<sup>2-</sup> L<sup>-1</sup> were achieved after 45 days; whereas in the control, a sulfide concentration of 0.43 mg L<sup>-1</sup> was obtained in the same period of time. The same trend was observed in each of the microbial inocula and substrates evaluated in this study; the sulfide concentration increased in the treatment bioassays with incubation time and in the abiotic controls it remained constant and it was negligible. These results show that SRB present in the inocula evaluated used the substrates (acetate, lactate, ethanol, and peptone) as electron donors in a greater or lesser extent to support microbial sulfate reduction.

Hydrogen sulfide is a weak acid with dissociation constants Ka<sub>1</sub> and Ka<sub>2</sub> of 9.6 x 10<sup>-8</sup> and 9.3 x 10<sup>-14</sup>, respectively [12]. Therefore at the working pH value of 7.1 - 7.3 evaluated in this study, the concentration ratio of the predominant species H<sub>2</sub>S : HS<sup>-</sup> was calculated to be 1 M : 0.96 M, practically [HS<sup>-</sup>] : [H<sub>2</sub>S]. These results are consistent with the sulfate reduction proposed by Metcalf and Eddy (Eq. 1) [7] that shows an equimolar relationship between the generated H<sub>2</sub>S and HS<sup>-</sup> during the microbial sulfate reduction. Moreover, based on the Henry constant for H<sub>2</sub>S at 25°C (1 x 10<sup>-1</sup> mol L<sup>-1</sup> atm<sup>-1</sup>) [13], the concentration ratio of H<sub>2</sub>S (ac) / H<sub>2</sub>S (g) is 2.45; namely, for each mol of H<sub>2</sub>S in the gaseous phase exists 2.45 mol of H<sub>2</sub>S in aqueous phase, meaning that approximately 70% of the produced biogenic sulfide is in aqueous phase. In fact, in this study in the presence of 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> and 2.5 g acetate-COD L<sup>-1</sup>, the theoretical production of sulfide was calculated to be 666.7 mg S<sup>2-</sup> L<sup>-1</sup>. However, only 463.3 mg S<sup>2-</sup> L<sup>-1</sup> were detected in aqueous phase in the bioassays conducted with the sediments of the artificial lagoon, so presumably the other 203.4 mg S<sup>2-</sup> L<sup>-1</sup> were in gaseous phase, demonstrating that, in fact, approximately 70% of the total sulfide was present in aqueous solution.

Figure 2 illustrates the time course of the sulfide production in the presence of 2.5 g acetate-COD L<sup>-1</sup> and 4000

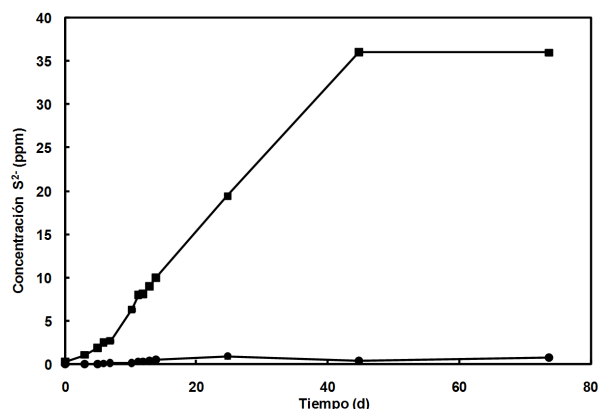


Figure 1: Time course of the sulfide production with 2.5 g acetate-COD L<sup>-1</sup> and 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> with 10% v/v of the anaerobic sludge of the facultative lagoon of the WWTP in Cuenca (L<sub>1</sub>). Legend: (○) abiotic control (acetate + sulfate); (■) treatment (acetate + sulfate + microorganism). Error bars represent the standard deviations of the bioassays performed in duplicates.

mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> for the different anaerobic sludge and sediments evaluated in this study. Among the microbial inocula tested, the anaerobic sediments of the artificial lagoon showed the highest sulfide production, 463.25 mg S<sup>2-</sup> L<sup>-1</sup> after 56 days of treatment. The final cumulative sulfide production, as well as the maximum specific sulfide production and sulfate reduction activities for the different anaerobic sludge and sediments evaluated in this study are presented in Table 1. The maximum specific sulfide production and sulfate reduction activities with the use of acetate as substrate varied between 0.25 and 8.74 mg S<sup>2-</sup> g acetate<sup>-1</sup> d<sup>-1</sup>, and 1.51 and 52.43 mg SO<sub>4</sub><sup>2-</sup> g acetate<sup>-1</sup> d<sup>-1</sup>, respectively. Based on these results, it can be concluded that the maximum specific sulfide generation or sulfate reduction activities of the sediments of the artificial lagoon is 18 times higher than the one of the sludge of the facultative lagoon in Cuenca, 15 and 35 times higher than the ones of the hydrocyclone sludge and discharge sludge of the WWTP in Quito, respectively.

These results are comparable with literature studies. Manoues *et al.* found maximum specific sulfate reduction activities up to 8.5 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> d<sup>-1</sup> with 1.92 g acetate-COD L<sup>-1</sup> and 3000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> with the sediments of Devils Lake in North Dakota [4]. 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 33 times higher than the one obtained by Manoues under similar conditions. These results indicate that acetate and the sediments of the artificial lagoon were highly efficient for the microbial sulfate reduction and are excellent candidates for the biotreatment of acid mine drainage in continuous systems.

Lactate was also evaluated in this study as electron donor during the microbial sulfate reduction. The highest maximum specific sulfate reduction activity was obtained with the sludge of the natural lagoon in the Orellana

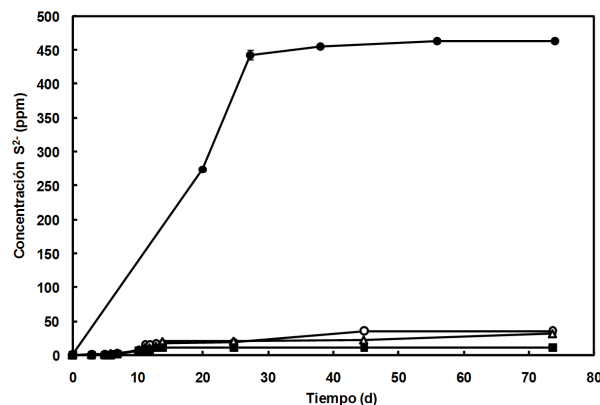


Figure 2: Time course of the sulfide production with 2.5 g acetate-COD L<sup>-1</sup> and 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> with 10% v/v of microbial inocula from different sources in the bioassays treatments (acetate + sulfate + microorganism). Legend: (○) sediments of the facultative lagoon of the WWTP in Cuenca (L<sub>1</sub>); (Δ) hydrocyclone sludge of a WWTP in Quito (L<sub>2</sub>); (■) discharge sludge of a WWTP in Quito (L<sub>3</sub>); (●) sediments of the artificial lagoon of the USFQ (L<sub>4</sub>). Error bars represent the standard deviations of the bioassays performed in duplicates.

Province (Table 1). Practically all other inocula evaluated (with exception of the discharge sludge of the WWTP in Quito) present maximum specific sulfide production or sulfate reduction activities half the value obtained with the sediments of the natural lagoon which were 4.16 mg S<sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup> and 24.97 mg SO<sub>4</sub><sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup>, respectively and those activities constitute only 50% of the maximum specific sulfide generation activity of the sediments of the artificial lagoon in the presence of acetate.

In recent studies, Oyekola and coworkers demonstrated the efficiency of lactate as substrate for the biological sulfate reduction [14]. Lactate concentration of 2.5 g lactate-COD L<sup>-1</sup> and sulfate concentrations between 1000 and 10,000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> were provided. The maximum specific sulfate reduction activity obtained was of 864 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> d<sup>-1</sup> [14]. Studies conducted by Celis *et al.* also evaluated the use of lactate as substrate in the biogenic sulfate reduction [15]. Lactate and sulfate were provided in concentrations of 1 g COD L<sup>-1</sup> and 1500 mg L<sup>-1</sup>, respectively and a maximum specific sulfate reduction activity of 830 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> d<sup>-1</sup> was obtained [15]. The sulfate reducing activity reported in the mentioned studies are one order of magnitude higher than the one obtained in this research work (65 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> d<sup>-1</sup> or 24.97 mg SO<sub>4</sub><sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup>) with the sediments of the natural lagoon. This difference suggests that the microbial consortium present in the sediments of the natural lagoon do not have a microbial lactate oxidizing activity as efficient as in the case of the mentioned studies.

Ethanol was also evaluated as substrate in the microbial sulfate reduction. The highest maximum specific sulfate reduction and sulfide generation activities achieved were 5.28 mg SO<sub>4</sub><sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup> and 0.88 mg S<sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup>, respectively; and were obtained with the sediments of the artificial lagoon (Table 1). In

Substrate	Concentration g COD L <sup>-1</sup>	Anaerobic sludge and sediments	Final S <sup>2-</sup> production mg S <sup>2-</sup> L <sup>-1</sup>	Sulfide production activity mg S <sup>2-</sup> g substrate <sup>-1</sup> d <sup>-1</sup>	Sulfide reduction activity mg SO <sub>4</sub> <sup>2-</sup> g substrate <sup>-1</sup> d <sup>-1</sup>
Acetate	2.5	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	35.96	0.48	2.88
		L2: Hydrocyclone sludge of a WWTP in Quito	31.99	0.60	3.58
		L3: Discharge sludge of a WWTP in Quito	11.64	0.25	1.51
		L4: Sediments of the artificial lagoon of the USFQ	463.25	8.74	52.43
Lactate	2.5	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	125.33	1.73	10.39
		L2: Hydrocyclone sludge of a WWTP in Quito	171.50	2.42	14.51
		L3: Discharge sludge of a WWTP in Quito	22.15	0.14	0.81
		L4: Sediments of the artificial lagoon of the USFQ	74.40	1.63	9.78
		L5: Sediments of the natural lagoon in the Orellana Province	44.88	0.99	5.96
	25	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	20.61	0.23	1.37
		L2: Hydrocyclone sludge of a WWTP in Quito	184.21	3.33	19.99
		L3: Discharge sludge of a WWTP in Quito	21.78	0.43	2.59
		L4: Sediments of the artificial lagoon of the USFQ	96.11	2.89	17.35
		L5: Sediments of the natural lagoon in the Orellana Province	44.88	0.99	5.96
Peptone	2.5	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	77.04	1.45	8.71
		L2: Hydrocyclone sludge of a WWTP in Quito	15.66	0.24	1.42
		L3: Discharge sludge of a WWTP in Quito	4.93	0.21	1.27
		L4: Sediments of the artificial lagoon of the USFQ	27.13	0.34	2.06
	25	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	6.43	0.013	0.08
		L2: Hydrocyclone sludge of a WWTP in Quito	15.72	0.023	0.14
		L3: Discharge sludge of a WWTP in Quito	19.19	0.036	0.22
		L4: Sediments of the artificial lagoon of the USFQ	12.54	0.024	0.15
Ethanol	2.5	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	16.78	0.24	1.46
		L2: Hydrocyclone sludge of a WWTP in Quito	20.48	0.29	1.75
		L3: Discharge sludge of a WWTP in Quito	1.20	0.14	0.84
		L4: Sediments of the artificial lagoon of the USFQ	22.40	0.88	5.28
	25	L1: Sediments of the facultative lagoon of a WWTP in Cuenca	49.94	0.048	0.288
		L2: Hydrocyclone sludge of a WWTP in Quito	1.70	0.003	0.020
		L3: Discharge sludge of a WWTP in Quito	0.36	0.001	0.004
		L4: Sediments of the artificial lagoon of the USFQ	1.34	0.007	0.042

**Table 1: Final cumulative sulfide production and maximum specific sulfide production and sulfate reduction activities in the presence of 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> with 2.5 and 25 g COD L<sup>-1</sup> of each substrate for the different anaerobic sludge and sediments evaluated in batch bioassays.**

the case of the sediments of the facultative lagoon in Cuenca and of the hydrocyclone of the WWTP in Quito, the maximum specific sulfate reduction or sulfide generation activities are between 3 and 4 times lower than

the ones obtained with the sediments of the artificial lagoon. In contrast, the discharge sludge of the WWTP in Quito presents a maximum specific activity six times lower than the highest activity obtained. However, the

Sludge and sediments	mmol CH <sub>4</sub> · L <sup>-1</sup>	mg CH <sub>4</sub> -COD g VSS <sup>-1</sup> d <sup>-1</sup>
L1: Sediments of the facultative lagoon of a WWTP in Cuenca	6.79	270.48
L2: Hydrocyclone sludge of a WWTP in Quito	4.99	208.23
L3: Discharge sludge of a WWTP in Quito	9.17	373.53
L4: Sediments of the artificial lagoon of the USFQ	2.19	48.85

**Table 2: Methane production and maximum specific methane generation activities with 2.5 g acetate-COD L<sup>-1</sup> for the different anaerobic sludge and sediments.**

maximum specific sulfide generation of the sediments of the artificial lagoon in the presence of ethanol is one order of magnitude lower than the one obtained with the same microbial inoculum with the use of acetate.

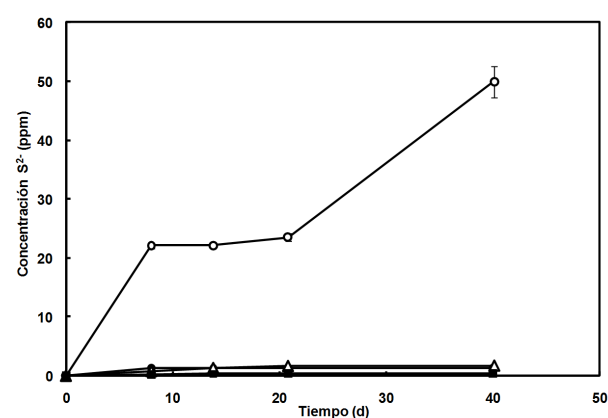
Previous studies conducted by Sierra-Álvarez *et al.* reported positive results in the removal of heavy metals with the use of ethanol as substrate in bioreactors packed with sulfate reducing bacteria [2]. In the study ethanol and sulfate were provided in concentrations of 0.9 g COD L<sup>-1</sup> and 700 mg L<sup>-1</sup>, respectively. The maximum biogenic activity obtained was of 409 mg S<sup>2-</sup> g VSS<sup>-1</sup> d<sup>-1</sup> together with heavy metal removal efficiencies exceeding 99.2% [2]. In this research work, using ethanol as substrate with the best inoculum evaluated (sediments of the artificial lagoon), the maximum biogenic activities achieved were of 0.18 mg S<sup>2-</sup> g VSS<sup>-1</sup> d<sup>-1</sup> which are considerably lower than the one reported by Sierra-Álvarez *et al.* indicating that ethanol was not an efficient substrate for the SRB present in the sediments of the artificial lagoon.

In this study, the use of peptone was also evaluated as substrate during the microbial sulfate reduction. The highest maximum specific sulfide generation activity obtained was 1.45 mg S<sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup> with the sediments of the facultative lagoon of the WWTP in Cuenca (Table 1). For all microbial inocula evaluated, the biogenic activities are similar averaging between 0.2 and 0.3 mg S<sup>2-</sup> g substrate<sup>-1</sup> d<sup>-1</sup> and they are six times lower than the ones obtained with the sediments of the facultative lagoon. The highest maximum specific sulfide generation activity in the presence of peptone is 3 times lower than the one in which acetate is used as electron donor with the same inoculum, demonstrating, once again, the superior efficiency of acetate as substrate. Miyazato and coworkers illustrated the existence of SRB in an activated sludge cultured with peptone with a biogenic activities between 7.2 and 19.2 mg SO<sub>4</sub><sup>2-</sup> g<sup>-1</sup> d<sup>-1</sup> which is comparable with the results obtained in this research work with the sediments of the facultative lagoon of the WWTP in Cuenca [16].

For all organic substrate evaluated in this study, lagoon sediments registered higher biogenic activities than the anaerobic sludge from the wastewater treatment plants. A possible explanation for this phenomenon is that during wastewater treatment the growth of methanogenic microorganisms is stimulated, debilitating sulfate reducing bacteria. In contrast, in lagoons, natural processes occur in which the sulfate reducing bacteria dominate because they are the strongest species.

The effect of the substrate concentration during the microbial sulfate reduction was also evaluated in this study. Lactate, peptone, and ethanol in concentrations 10 times higher than 2.5 g COD L<sup>-1</sup> were studied under the same experimental conditions described previously. Figure 3 represents an illustrative example of the time course of the sulfide generation in the presence of 25 g ethanol-COD L<sup>-1</sup>. The results with 25 g COD L<sup>-1</sup> of lactate and peptone with different microbial inocula are similar to those reported in Figure 3 in terms of sulfide generation monitoring (no figures reported). The maximum specific sulfide generation and sulfate reduction activities are shown in Table 1. It is important to note that in general, the maximum specific sulfide generation and sulfate reduction activities together with the final cumulative sulfide production were greater when a lower substrate concentration (2.5 g COD L<sup>-1</sup>) was provided in comparison with higher substrate concentrations (25 g COD L<sup>-1</sup>). For instance, in the case of the hydrocyclone sludge of the WWTP in Quito, the sulfide production rate is two orders of magnitude higher with 2.5 g ethanol-COD L<sup>-1</sup> as compared with 25 g ethanol-COD L<sup>-1</sup>. This fact can be attributed to a microbial inhibition when substrate is provided in excess as in the case of the bioassays conducted with 25 g substrate-COD L<sup>-1</sup>.

Among all substrates evaluated in this study, acetate was found to be the best electron donor for the microbial



**Figure 3: Time course of the sulfide production with 25 g ethanol-COD L<sup>-1</sup> and 4000 mg SO<sub>4</sub><sup>2-</sup> L<sup>-1</sup> with 10% v/v of microbial inoculum from different sources in treatments bioassays (ethanol + sulfate + microorganisms). Legend: (○) sediments of the facultative lagoon of the WWTP in Cuenca (L<sub>1</sub>); (Δ) hydrocyclone sludge from a WWTP in Quito (L<sub>2</sub>); (■) discharge sludge from a WWTP in Quito (L<sub>3</sub>); (●) sediments of the artificial lagoon of the USFQ (L<sub>4</sub>). Error bars represent the standard deviations of the bioassays performed in duplicates**



sulfate reduction with 4000 mg  $\text{SO}_4^{2-} \text{L}^{-1}$ . In the presence of 2.5 g acetate-COD  $\text{L}^{-1}$ , SRB present in the sediments of the artificial lagoon registered the highest cumulative sulfide production, maximum specific sulfide generation and sulfate reduction activities, 463.35 mg  $\text{S}^{2-} \text{L}^{-1}$ , 8.74 mg  $\text{S}^{2-} \text{g acetate}^{-1} \text{d}^{-1}$  and 52.43 mg  $\text{SO}_4^{2-} \text{g acetate}^{-1} \text{d}^{-1}$ , respectively. Those activities are twice the value of the most representative maximum specific biogenic activity obtained with the use of the sediments of the natural lagoon in the Amazonian region with 2.5 g lactate-COD  $\text{L}^{-1}$ .

In the anaerobic microbial inocula evaluated in this study, different bacterial consortiums are expected to be present; even more, microbial competition for the organic substrate is very likely to take place. Therefore, the possible competence between methanogenic and sulfate reducing microorganisms was also studied in batch bioassays. Table 2 summarizes the methanogenic activities of microorganisms present in the sludge and sediments evaluated in this study. From the results obtained, it can be concluded that the methane generation follows an opposite trend to the sulfide production. The sediments of the artificial lagoon which presented the highest specific sulfide generation activity, registered the lowest maximum specific methane generation activity in the presence of 2.5 g acetate-COD  $\text{L}^{-1}$ . On the other hand, the discharge sludge of the WWTP in Quito shows the highest methanogenic activity and the lowest sulfidogenic activity under the same experimental conditions. Moreover, the maximum specific sulfide generation activity of the sediments of the artificial lagoon is 35 times higher than the one of the discharge sludge of the WWTP in Quito. In the case of the methane generation, the methanogenic activity of the discharge sludge is 8 times higher than the one of the sediments of the artificial lagoon. These results demonstrate that indeed a substrate competence exists between SRB and methanogens; however, in the presence of acetate and sulfate, the growth of SRB was stimulated while the activity of methanogenic microorganisms was inhibited.

These results are consistent with literature studies regarding microbial competition. For instance, Kristjansson and coworkers demonstrated a kinetic competence between methanogenic and sulfate reducing microorganisms with the latter ones showing a greater affinity for acetate when the substrate was not provided in excess [17]. These authors calculated the Monod constant ( $K_s$ ) which were lower for SRB than for methanogenic microorganisms illustrating a greater affinity of the SRB towards acetate as substrate [17]. Moreover, Lowe *et al.* mentioned that in sedimentary ecosystems, the addition of acetate inhibits the methanogenesis [18]. In fact, a significant diminish in the methane production was obtained when acetate was provided, leading to the conclusion that carbon dioxide and hydrogen were the dominant precursors of methane in that environment [18].

## Conclusions

Acetate was the most efficient electron donor for the microbial sulfate reduction with the sediments of the artificial lagoon as microbial inoculum. The use of lactate resulted in the second more efficient donor; however, the maximum specific sulfide production was only 50% of that obtained with acetate. The use of ethanol and peptone as substrates did not present a significant efficiency in the biogenic sulfide activities compared with the ones obtained with the use of acetate or lactate during the microbial sulfate reduction. Microbial competence between sulfate reducing bacteria and methanogens for the organic substrate was demonstrated, in fact in the presence of acetate a clear trend was observed, the highest the sulfate reduction, the lowest the methane production. Finally, the use of acetate with the sediments of the artificial lagoon present a great potential for the biotreatment of acid mine drainage characterized by elevated concentrations of heavy metals, sulfates and acidity.

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