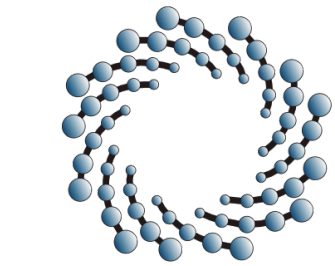

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# INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A. C.

**POSGRADO EN CIENCIAS AMBIENTALES**

# Sulfate reduction at acidic conditions: from the sediment to the reactor

Tesis que presenta

**Tonatiuh Moreno Perlín**

Para obtener el grado de

**Doctor en Ciencias Ambientales**

Director de la tesis

**Dra. María de Lourdes Berenice Celis García**

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# Constancia de aprobación de la tesis

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# Créditos Institucionales

Esta tesis fue elaborada en los Laboratorios de la División de Ciencias Ambientales del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección de la Dra. María de Lourdes Berenice Celis García. Durante la realización del trabajo el autor recibió una beca académica del Consejo Nacional de Ciencia y Tecnología (CONACYT-490710).

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DOCTOR EN CIENCIAS AMBIENTALES

sustentó el C.

**Tonatiuh Moreno Perlín**

sobre la Tesis intitulada:

***Sulfate reduction at acidic conditions: from the sediment to the reactor***

que se desarrolló bajo la dirección de

**Dra. María de Lourdes Berenice Celis García**

El Jurado, después de deliberar, determinó

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*Para Rasheed y Aisha, mis grandes tesoros.*

# Prefacio

*”No sigas el camino de los sabios,*
*busca lo que ellos buscaron”.*

*Matsuo Bashô*

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## Resumen

El drenaje ácido de mina es el flujo de agua contaminada con metales pesados, sulfato y ácido proveniente de algunas acitvidades mineras. Debido a sus características fisicoquímicas, el drenaje ácido de mina es tóxico, recalcitrante y difícil de tratar; actualmente, no existe ningún método que cumpla con las necesidades económicas y ambientales requeridas para su tratamiento. Una opción prometedora para llevar a cabo esta acción es el empleo de reactores sulfurogénicos. Sin embargo, los reactores sulfidogénicos cuentan con dos principales limitaciones para su uso: sensibilidad al estrés por ácido y la baja eficiencia para mineralizar la materia orgánica debido a la acumulación de acetato. Ambas limitaciones son consecuencia de las características fisiológicas de los microorganismos sulfato reductores, los cuales son los principales actores en los reactores sulfidogénicos. El empleo de comunidades microbianas es una opción factible para aumentar la resistencia al ácido y aumentar la eficiencia de degradación de la materia orgánica en los reactores sulfurogénicos; a través de la acción de diferentes gremios microbianos. Desafortunadamente existe una brecha en el conocimiento sobre como funcionan las comunidades sulfato reductoras en condiciones de estrés ácido y la relación que existe entre la composición de dichas comunidades y su funcionamiento. El objetivo de este trabajo fue investigar acerca de la relación que existe entre la composición microbiana y el desempeño de una comunidad sulfato reductora en condiciones de estrés ocasionado por pH ácido. Para alcanzar esta meta, se analizaron los cambios en la composición, la estructura y las características funcionales de una comunidad sulfato reductora durante su enriquecimiento a partir de una muestra ambiental, en su aclimatación al pH ácido y en su

aplicación en un reactor inverso de lecho fluidizado para el tratamiento de agua ácida sintética. Los análisis microbiológicos (PCR-DGGE y secuenciación masiva) indicaron que la composición de la comunidad cambió a lo largo del proceso; además, el análisis de los metabolitos extracelulares indicaron que la estructura de la comunidad también fue modificada, dando paso a la actividad fermentadora durante la mineralización del substrato. No obstante, las características funcionales de la comunidad, tales como las tasas y las eficiencias del consumo del donador de electrones y de la sulfato reducción, permanecieron constantes a lo largo de los experimentos. Empero, se encontró con un límite en el cual la presión ejercida por el estrés fue muy grande y la actividad microbiana se detuvo por completo. La estabilidad funcional del sistema puede ser explicada parcialmente por la alta redundancia funcional que se encontró en la predicción de perfiles funcionales de la comunidad. Mas aún, el colapso de la actividades microbianas parecieron relacionarse con limitaciones termodinámicas dentro de los sistemas estudiados. Este estudio demuestra que existe una estabilidad funcional de una comunidad sulfato reductora en condiciones de estrés por ácido a la vez que hace un llamado para evaluar las limitaciones biológicas inherentes a los microorganismos durante su aplicación en bioreactores.

**Moreno-Perlín, Tonatiuh (2019). Sulfate Reduction at Acidic Conditions: from the sediment to the reactor. Doctoral Thesis. Instituto Potosino de Investigación Científica y Tecnológica. México.**


## Abstract

Acid mine drainage is water polluted with heavy metals, sulfate, and acid as the result of some mining activities. Due its physicochemical characteristics, acid mine drainage is toxic, recalcitrant and difficult to treat. Currently, there is not a method that can fulfill economical and environmental needs to treat acid mine drainage. Off-line sulfidogenic bioreactors are a promising technology to treat acid mine drainage; however, it fails to stand acid stress and to completely oxidize organic matter, being acetate the most common byproduct. These limitations are a consequence of the physiological characteristics of sulfate reducing prokaryotes, the microorganisms responsible for the microbial activity in the sulfidogenic bioreactors. Acid resistance and substrate complete oxidation can be achieved in a community, where the activity of sulfate reducing prokaryotes can be enhance by the involvement of different microbial guilds. Unfortunately there is a lack in the understanding of how sulfate reducing communities work and the effect that the microbial composition has in its performance under acidic stress conditions.

The aim of this work was to investigate the effect that microbial composition has in the performance of a sulfate reducing community at different conditions of acidic stress. To achieve our goal, we analyzed the changes of the microbial composition, structure and functional traits in a sulfate reducing community, during its enrichment from an environmental sample to its acclimation to acidic pH and its final application to treat synthetic acidic wastewater in a down-flow fluidized bed reactor.

Molecular tools (PCR-DGGE and high-throughput sequencing) indicated that the community composition and structure changed along the path. Furthermore, analyses of the metabolic byproducts indicated a change in the structure of the community and the involvement of fermenting bacteria in the mineralization of the substrate. However,

functional traits of the community such as electron donor removal and sulfate reduction rates and efficiencies were remained constant across the experiments, until the conditions were too harsh and all microbial activity halted. The stability of the community traits can be partially explained by a high redundancy in the predicted functional profiles. Moreover, collapse of the community activity appeared to be related to thermodynamical constrains and physiological barriers.

This study demonstrates the functional stability of sulfate reducing communities at acidic stress conditions; yet, it also alerts of the inherent biological limitations that microorganisms have and urges for its consideration in the design of engineered biotechnological systems.

# 1. Theoretical Framework

Acid mine drainage is water polluted with heavy metals, sulfate, and acid as the result of some mining activities. Due to its physicochemical characteristics, acid mine drainage is toxic, recalcitrant and difficult to treat (Johnson and Hallberg, 2005). For example, it has been reported AMD with a pH of -3.6, metal concentration of 200 g/L and sulfate concentration of 760 g/L (Nordstrom and Alpers, 1999). There have been developed many treatment options for acid mine drainage, but none of the current methods is able to fulfill the environmental and economical needs. One promising option to treat acid mine drainage is the use of biological sulfidogenic processes in off-line reactors (Gopi Kiran, Pakshirajan, and Das, 2017). Sulfidogenic processes depend on the activity of prokaryotes called sulfate reducers, for their ability to reduce sulfate to sulfide in order to obtain energy for their growth and maintenance.

The main limitation of suldifogenic reactors is that they cannot withstand low pH, such as the pH found in acid mine drainage. Many efforts are carried away to develop sulfidogenic processes at low pH. Some of the efforts are focusing on finding sulfate reducers resistant or tolerant to acid pH ($< 5$) (Sánchez-Andrea et al., 2015). However, the main efforts are being made into looking for the appropriate conditions to enable the sulfidogenic activity in microbial communities at low pH (Gopi Kiran, Pakshirajan, and Das, 2017).

In this work we evaluated the performance of a sulfate reducing community since its retrieval from an environmental sample to its application in a lab-scale reactor paying close attention to the community traits of interest and its structure. Our main interest was to evaluate the effect that acidic stress has in the community performance and structure across a pH gradient from 6 to 3 in batch enrichments, and at different operation conditions in a lab-scale reactor fed with synthetic acidic wastewater (pH 3).

## 1.1 Acid Mine Drainage: generation, consequences and treatment

Acid mine drainage (AMD) is the result of the accelerated oxidation of sulfur minerals such as pyrite ($FeS_2$) promoted for both physicochemical and biological processes. The general equation of AMD production caused by environmental factors involves oxygen ($O_2$) from the air and water from the rain ($H_2O$); at pH ≈3 the stoichiometry is (Akcil and Koldas, 2006):

$$\mathrm{FeS_2} + \frac{15}{8}\,\mathrm{O_2} + \frac{13}{2}\,\mathrm{Fe^{3+}} + \frac{17}{4}\,\mathrm{H_2O} \longrightarrow \frac{15}{2}\,\mathrm{Fe^{2+}} + 2\,\mathrm{{SO_4}^{2-}} + \frac{17}{2}\,\mathrm{H^{+}} \tag{1.1}$$

Despite the fact that physicochemical oxidation of pyrite is limited to a very specific pH range (Johnson and Hallberg, 2005), microbial activity can enhance it. Microbial oxidation of pyrite is independent of oxygen and is resumed in the following reaction (Nordstrom, 2011):

$$\mathrm{FeS_2} + 14\,\mathrm{Fe^{3+}} + \mathrm{H_2O} \longrightarrow 15\,\mathrm{Fe^{2+}} + 2\,\mathrm{{SO_4}^{2-}} + 16\,\mathrm{H^{+}} \tag{1.2}$$

Evidently, both process of AMD generation can occur at the same time and magnify each other (Akcil and Koldas, 2006). The exact mechanism of AMD generation is site-dependant and varies according to the meteorological, geological, and biological conditions of the site. This makes predicting the risk of AMD a complicated task (Morin and Hutt, 1998). Although in most cases AMD is actively prevented, it usually occurs in abandoned mining sites and mine tailings (Johnson and Hallberg, 2005). The effects that AMD has in the environment are mild to severe according to its characteristics (Morin and Hutt, 1998).

### 1.1.1 AMD pollution

AMD can become a major issue in the affected areas since it causes acidification and elevates the concentration of sulfate, iron, and soluble metals in the ground water and

surface water receiving it (Sheoran, Sheoran, and Choudhary, 2010); accordingly, this areas have a decrease in its natural pH and they are polluted with metals and/or metaloids associated to the AMD (Klein et al., 2013). In addition to its toxicity, AMD also affects the biogeochemical cycles of sulfur and carbon. The modification of the carbon cycle is a consequence of the acidification of the media, which promotes the gasification of dissolved carbonates, releasing $CO_2$ to the atmosphere as in the following reaction:

$$\mathrm{HCO_3}^- + \mathrm{H}^+ \longrightarrow \mathrm{CO_2} + \mathrm{H_2O} \tag{1.3}$$

A study performed in the Coal Region in Pennsylvania, USA, calculated that the acidification of the watersheds in the area has caused the release of $\approx$3.1 Tg of carbon to the atmosphere for over the last century (Raymond and Oh, 2009). Besides its negative effects towards the environment, AMD also promotes bio-corrosion damaging man-made metallic structures near polluted sites (Hamilton, 1998; Klein et al., 2013).

### 1.1.2 AMD Treatment

Due the mobile nature of AMD it is difficult to calculate the extent of its impact. Nevertheless, it is known that AMD affects the soil and water in contact with it (Hudson-Edwards, Jamieson, and Lottermoser, 2011; Johnson and Hallberg, 2005). In order to mitigate the negative effects of AMD, many technological solutions have been developed. Currently, physicochemical processes such as neutralization and precipitation using hydroxides and/or carbonates are the most commonly used techniques to remediate AMD (Akcil and Koldas, 2006). The use of this kind of approximation has two main disadvantages: i) partially eliminates the pollutants, since sulfate remains in the media and ii) generates a toxic and unstable waste that still has to be disposed of (Johnson and Hallberg, 2005).

There are other options for AMD treatment such as aerobic wetlands, permeable reactive barriers, and off-line biological sulfidogenic reactors (Simate and Ndlovu, 2014). Sulfidogenic processes consist in the use of microorganisms to produce sul-

fide (Hudson-Edwards, Jamieson, and Lottermoser, 2011), details are reviewed in the next section.

## 1.2 Biological AMD treatment

Sulfidogenic processes are biotechnological processes based on the activity of sulfate reducing prokaryotes (SRP), that produce sulfide as a byproduct of their metabolism. According to the following reaction (Rabus, Hansen, and Widdel, 2013):

$$2\,\mathrm{CH_2O} + {\mathrm{SO_4}}^{2-} \longrightarrow \mathrm{H_2S} + 2\,\mathrm{HCO_3}^{-} \tag{1.4}$$

Sulfidogenic procceses take advantage of the sulfide produced by SRP through Reaction 1.4 and use it to precipitate metals ($M^{2+}$), present in the media, as metal sulfides (MS), as in the next reaction:

$$\mathrm{H_2S} + \mathrm{M}^{2+} \longrightarrow \mathrm{MS}\downarrow + 2\,\mathrm{H}^{+} \tag{1.5}$$

The combination of Reactions 1.4 and 1.5 has the potential to neutralize the pH, through alkalinity $HCO_3^-$ production and eliminate organic matter ($CH_2O$), sulfate ($SO_4^{2+}$) and metals ($M^{2+}$) in a single process; effectively treating all the possible sources of pollution. Furthermore, since metal sulfides can be selectively precipitated (Lewis, 2010), metals of interest could be recovered, converting the process in a form of bioleaching (Jameson et al., 2010). That is the reason why sulfidogenic processes are so appealing and have been a focus of interest in the past two decades (Lens et al., 1998a).

### 1.2.1 Sulfidogenic processes limitations

Despite the potential benefits, sulfidogenic processes are not widely used, and physicochemical treatment of AMD is usually preferred (Sánchez-Andrea et al., 2016). The

reason behind this is the fact that almost all know SRP have low tolerance to acidic pH (Koschorreck, 2008) therefore they become inactive at the conditions that AMD provide (i.e. pH $\approx$2) (Sánchez-Andrea et al., 2014). The decrease of the activity of SRP in sulfidogenic processes displays as a decrease in the sulfide production (Jong and Parry, 2003) and substrate accumulation, specially acetate (Lens et al., 1998b).

Obviously, AMD could be neutralized prior the contact with SRP, but this would reduce the efficiency of the process since metal sulfide precipitation is pH dependant and favored at pH $\lesssim$ 4 (Lewis, 2010) and would increase the operational cost adding a step to the process. Accordingly, there is a growing interest in biological sulfide production at acidic conditions (Sánchez-Andrea et al., 2016). Since the current drawbacks of the sulfidogenic processes are inherent to the nature and limitations of SRP, there has been an increase in the study of the effects of acidic pH to neutrophiles (Baker-Austin and Dopson, 2007) and in SRP in general (Rabus et al., 2015).

## 1.3 Sulfate reducing prokaryotes

As mentioned before, SRP is a guild assambled by anaerobes that share the common trait of using the non-assimilative reduction of sulfate (as in Reaction 1.4) for energy production during their catabolism. SRP are philogenetically diverse and can be found in four bacterial phyla: Proteobacteria, Nitrospirae, Firmicutes, and Thermodesulfobacteria and in two archeal phyla: Euryarcheota and Crenarcheota (Müller et al., 2015). Most SRP are mesophilic and neutrophiles, although some SRP are thermophilic and a few acidophilic SRP have been described, belonging to the Firmicutes phylum (Alazard et al., 2010).

SRP can be found in different environments, they naturally occur in marine, estuarine, freshwater, terrestrial habitats. They also form part of the gut microbiome of animals (including humans) and industrial wastewaters (Rabus et al., 2015). This wide range of distribution across contrasting environments within the biosphere is the result of the exceptional metabolic flexibility that SPR possess.

### 1.3.1 SRP metabolic capabilities and Bioenergetics

In energetic terms sulfate reduction is a poor energy yielding metabolism. For example, acetate oxidation via sulfate reduction has a standard Gibbs free energy ($\Delta G^\circ$) of -56 kJ/mole, whilst the same process yields -844 kJ/mole of energy through aerobic respiration (Rabus, Hansen, and Widdel, 2013). Even compared to other anaerobic metabolisms, sulfate reduction is still a limiting process and other types of metabolism such as denitrification or iron reduction produce more energy. Thus, most of the energy produced by SRP is used for cellular maintenance ($f_s^\circ$= 0.08) leading to low growth rates ($\mu$ = 0.5 $d^{-1}$) (Rittmann and McCarty, 2001), these limitations are specially severe for acetate dependant sulfate reducers (Montoya et al., 2012).

It should be noticed that reaction 1.4 is the general form for heterotrophic sulfate reduction, in which organic matter ($CH_2O$) is both, the electron donor and the carbon source; however, some SRP are known for being autotrophs and use hydrogen ($H_2$) as energy source and carbon dioxide ($CO_2$) as carbon source (Liamleam and Annachhatre, 2007).

SRP exhibit a wide variety of metabolic traits; many SRP are able to perform other metabolic pathways to gain energy from the environment. Some of them are able to use other sulfur compounds such as sulfite ($SO_3^{2-}$), thiosulfate ($S_2O_3^{2-}$) or elemental sulfur ($S^0$). Besides sulfur compounds, nitrate ($NO_3^-$), nitrite ($NO_2^-$), fumarate ($C_4H_3O_3^-$) and arsenate ($AsO_4^{3-}$) can also be used by some SRP for their catabolism (Rabus, Hansen, and Widdel, 2013).

### 1.3.2 Acetate oxidation via sulfate reduction

An important feature of the sulfate reducer guild metabolism is that there are two types of SRP; those who can degradate acetate commonly known as complete sulfate reducers (cSRP) and those who produce acetate as a byproduct of their metabolism, known as incomplete sulfate reducers (iSRP) (Hamilton, 1998; Rabus, Hansen, and Widdel, 2013. Furthermore, cSRP can be divided in those who are able to completely oxidize complex substrates, either directly or using acetate as an extracellular intermediary (Imhoff-Stuckle and Pfennig, 1983; Laanbroek et al., 1984),

and those that use acetate as their exclusive carbon and energy source (Rabus, Hansen, and Widdel, 2013; Schauder et al., 1986).

A practical implication of these metabolic differences is that the lack of cSRP in a reactor leads to acetate accumulation (Celis et al., 2013). Moreover, if sulfate is a limiting resource, iSRP will ultimately out-compete cSRP in the presence of complex substrates (Liamleam and Annachhatre, 2007; Rabus, Hansen, and Widdel, 2013).

### 1.3.3 Sulfate reducing prokaryotes at acidic conditions

In general, microorganisms have two types of mechanisms to respond to acidic stress: a natural resistence mechanism that is constituvely present and an inducible mechanism that requires an external stimuli to be active (Liu et al., 2015). As would be expected, the inducible mechanism requires energy and time to be activated, that leads to the general conclusion that a gradual increase in the stressful condition enables the activity of neutrophilic microorganisms at low pH (Baker-Austin and Dopson, 2007; Goodson and Rowbury, 1989; Leyer, Wang, and Johnson, 1995). This phenomena is called acclimation (Leroi, Bennett, and Lenski, 1994).

Some SRP have been isolated from acidic envrionments, such as the moderately acidophilic *Desulfosporosinus acidiphilus* which was isolated from a mining site at Chessy-Les-Mines (Beaujolais, France) and was found to grow optimally at pH 5.2, with a lower limit at pH 3.6 (Alazard et al., 2010). Another novel isolate of a moderately acidophile is *Thermodesulfobium narugense Na82 T* with a pH optimal for growth at 5.5-6.0 and a pH minimum at 4.0, it was isolated from a hot spring (Mori et al., 2003). Isolation of *Desulfosporosinus acididurans M1 T* (pH opt 5.5; pH min 3.8) from White River (pH 3.2) sediment in Montserrat was recently reported (Sánchez-Andrea et al., 2015).

Despite the limited succes in the isolation of acidophilic SRP, uncultured SRP have been repeatedly recognized as members of microbial communities thriving in acid mine drainage sediments with environmental conditions such as pH 2–3, and sulfate, iron, and arsenic concentrations of 29.5, 6.8, and 0.638 g/L respectively (Giloteaux et al., 2013; Martins et al., 2010; Moreau, Zierenberg, and Banfield, 2010; Sánchez-Andrea et al., 2011).

From *in situ* studies we have learn that a sulfate reduction at acidic conditions

benefits from summer warmer temperatures (Gyure et al., 1990; Herlihy and Mills, 1985; Jakobsen and Postma, 1999; Praharaj and Fortin, 2004; Sánchez-Andrea et al., 2011), and from the presence of a matrix to colonize and produce biofilm (Fortin, Davis, and Beveridge, 1996; Jorgensen, 1977). Besides these factors, the nature and concentration of the electron donor is also of great relevance (Church et al., 2007; Gyure et al., 1990; Praharaj and Fortin, 2004; Wielinga et al., 1999).

## 1.4 Scope of this research

One key question that has not been fully addressed about sulfate reduction at acidic conditions is what mechanisms enable this, apparently improbable, feature in an community context. The simplest explanation for sulfate reduction at acidic conditions is that there are truly acidophilic or acidtolerant SRP (Koschorreck, 2008; Sánchez-Andrea et al., 2015); which are rare and difficult to find and isolate (Müller et al., 2015; Rinke et al., 2013). In this scenario, the sulfate reducing activity would depend almost entirely on the activity of these rare microorganisms and their abundance in the community would increase at acidic conditions, whereas the relative abundance of other SRP will decrease as the environment turns acidic.

Another probable explanation is that different SRP are capable to respond to acidic conditions at different extent, by adjusting their physiological responses to the acidic stress (Moreno-Perlin, 2014). In which case, the relative abundance of any SRP would be determined by their ability to adapt to acidic stress (Liu et al., 2015), but their activity would remain constant in the condition where they are capable of survival.

Finally, it is plausible that the activity of SRP at acidic conditions is an emergent property of the communities and is the result of the interaction of all the members present in a community at a particular set of environmental variables (Konopka, 2009). This would mean that the sulfate reducing guild depends on the activity of other microbial guilds to survive; which means that their relative abundance would be in equilibrium with other guilds that facilitate their survival (Kimura, Hallberg, and Johnson, 2006; Rowe et al., 2007).

### 1.4.1 Objectives of this research

The main objective of this work was to analyze the role that the community composition and its structure had in the performance of a sulfate reducing community across a gradient of acidic stress, during the degradation of volatile fatty acids (i.e. lactate). We hypothesized that any change in the structure or composition of the community would be reflected in a change on its performance. To evaluate the performance of the community we focused on the sulfate and electron donor removal efficiencies and rates, as they are key parameters for the development of biotechnological processes to treat acid mine drainage. We also paid close attention to the biomass production rates and the overall energy flux within the system as a proxy to evaluate the stress of the community.

To achieve our goals, we used an indigenous community recovered from a sediment with a history of long exposure to acid mine drainage and enriched the sulfate reducing prokaryotes (Chapter 3). Once we had enriched the community with sulfate reducers, we acclimated them to acidic pH decreasing the pH of the system in which they were enriched from 6 to 4, one unit at the time (Chapter 4). Finally, we tested the ability of the sulfate reducing community to perform sulfate reduction in a down-flow fluidized bed reactor fed with acidic waste water at pH 3 in continuous conditions (Chapter 5).

# 2. General Methodology

To complete the objective of this work, environmental samples were taken from a sediment historically exposed to acid mine drainage (AMD). We had previously characterized the sampling site and we knew that we could find acidotoletant SRP (Moreno-Perlin et.al., 2019). We used the sediment to inoculate a reactor fed with a modified anaerobic minimal medium (Stams et al., 1993) supplemented with different concentrations of acetate and lactate. The reactor was operated in a fed-batch regime at pH 5 to promote the enrichment and selection of acidotolerant SRP. We kept a daily monitoring of the reactor to evaluate the sulfate reducing activity and the degradation of the electron donor (Chapter 3).

Once we observed that the reactor had entered a stable state and the consumption of sulfate was coupled to the mineralization of the electron donor we decided to separate the biomass from the sediment. There were two practical reasons to look for this separation: 1) the sediment was rich in sulfate (Moreno-Perlin et. al., 2019), which obscured the sulfate consumption and 2) the ultimate part of the work was intended to be the application of the sulfate reducing community (SRCo) in a fluidized bed reactor, which would require the attachment of the SRCo to a carrier material. We tried different approaches to separate the sediment, but eventually, agitation resulted as the more successful method (Chapter 3).

Once we had enough biomass of the SRCo free of sediment, we used the acclimation technique to promote the sulfate reducing activity at acidic pH (Moreno-Perlin, 2014). We were particularly interested in the changes of the community composition and performance during this stage of the experiments, so we set-up an experiment where the acclimation was carried out in tenth replicates of the same SRCo. We decreased one unit of pH at the start of each phase and in the end of each pH value, we mixed together all the biomass present in all the samples to avoid the drifting of the

communities as the experiment moved forward; ensuring that each of the replicates was a replicate for the pH being studied and not for the initial conditions (Chapter 4).

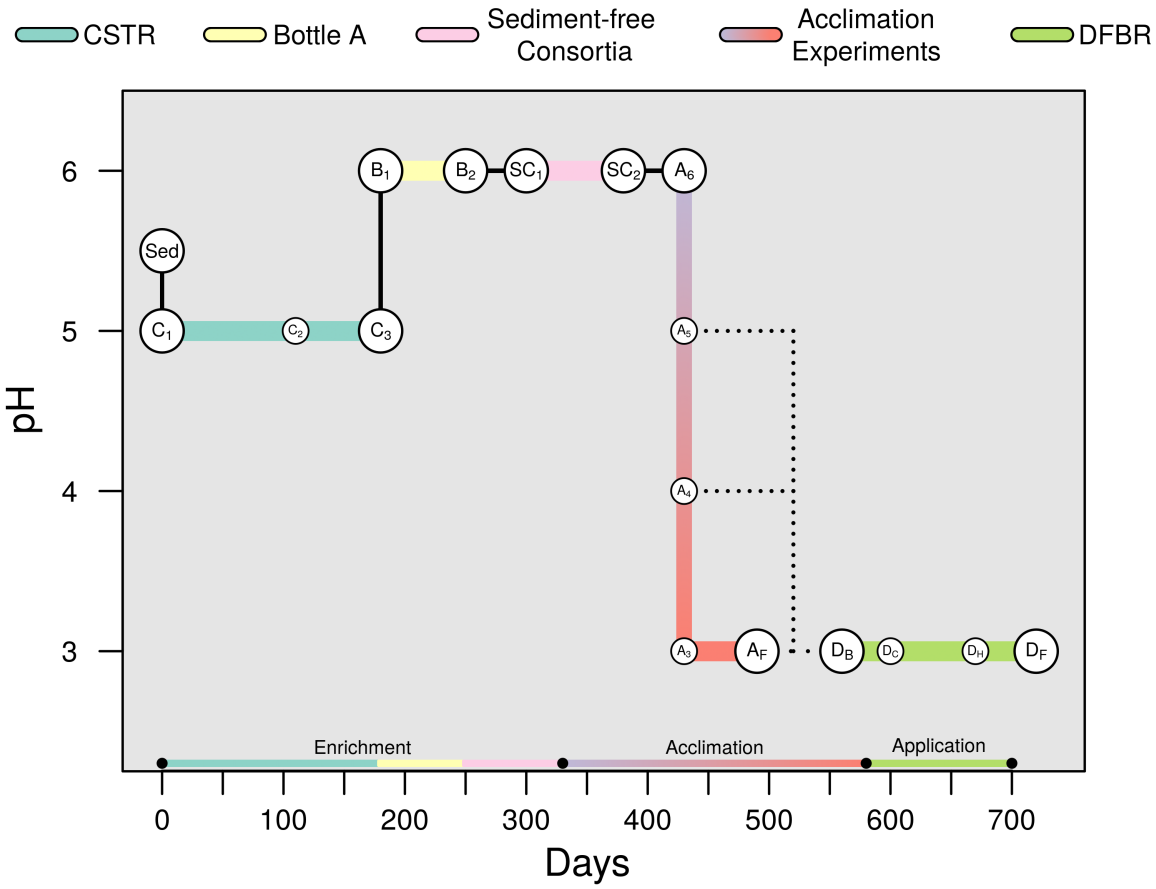


Figure 2.1. Path that followed the communities from the sediment (Sed) to their application in a down-flow fluidized bed reactor (DFBR). Each point represent a stage in their development; in all the stages we measured relevant traits of the community (see text for further details). The pH represents the initial pH of the media during each stage.

Finally, we used the acclimated SRCo to treat synthetic acidic waste water (pH = 3.02) using lactate as sole carbon and energy source in a down-flow fluidized bed reactor (Chapter 5). We decreased the hydraulic retention time to increase the acidic stress over the SRCo.

During all the experiments we measured the traits of the community such as rates and efficiencies of sulfate reduction and electron donor removal in order to asses the

performance of the community. An overview of the path that the SRCo followed from the sediment to the down-flow fluidized bed reactor can be seen in Figure 2.1.

## 2.1 Sampling

A total of five samples of 500 g each were taken from sediments historically exposed to the leachates from the Guaxcamá mine located in Villa Juárez, San Luis Potosí, México (Figure 2.2). The location of the sampling site is 22°10' 53.2" N and 100°16' 30.2" W. Temperature, pH, dissolved oxygen, conductivity, oxidation-reduction potential (ORP) and total dissolved solids in the water column were measured *in situ* using a multi parameter probe (6920-V2, YSI Incorporated, USA). At each sampling point, the upper 5 cm of the sediment were collected from five points along the stream (Figure 2.2), placed in sterile plastic containers and refrigerated at 4°C. Individual samples were mixed together into a single bulk sample. The bulk sample was sieved to 4 mm, homogenized, and used to inoculate the a continuous stirred tank reactor.

Figure 2.2. Sampling site, Villa de Juárez, San Luis Potosí, México

## 2.2 Reactors

### 2.2.1 CSTR Reactor

In Chapter 3, we used a stirred tank (Figure 2.3) operated in fed-batch mode for ≈ 191 days to enrich SRP present in the sediment. The tank had a capacity of 3 L, and was operated at a working volume of 2.4 L. It had baffles and was constantly stirred at 120 RPM to promote the detachment of the biomass from the sediment. Online detectors for pH, ORP, and temperature were used to control the system. Temperature was adjusted at 30±3°C using a thermal jacket. The pH was controlled at 5 using HCl 0.52 N. The reactor was inoculated in a ≈1:8 v/v proportion of sediment:media.

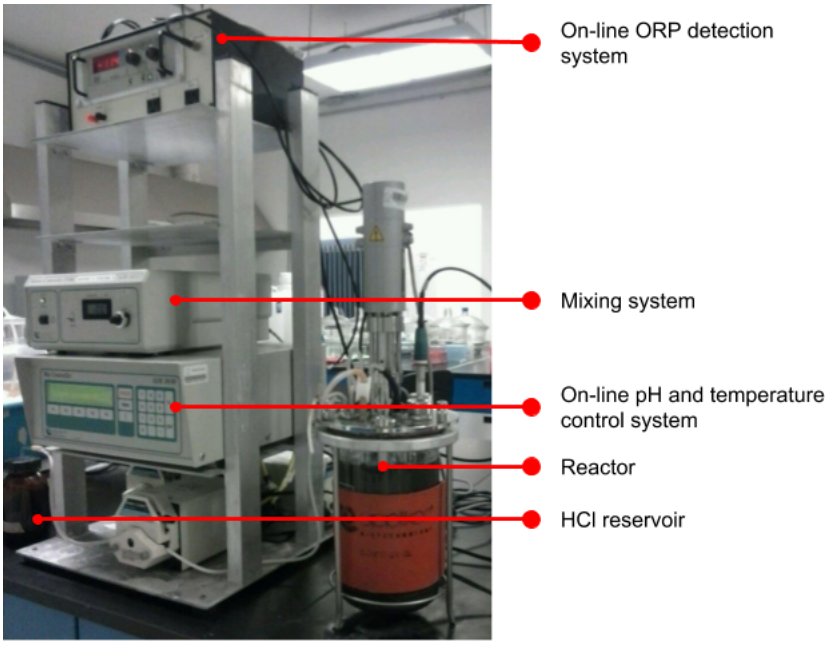


Figure 2.3. Continuous stirred tank reactor (CSTR) used for the enrichment of the communities present in the sediment (Chapter 3).

### 2.2.2 DFBR Reactor

The down-flow fluidized bed reactor (Figure 2.4) consisted of a transparent acrilyc column with a conical bottom and a total volume of 1.7 L. The reactor was operated

at room temperature (22°C). A small sedimentation tank (0.5 L) was coupled after the DFBR to prevent biomass loss. The affluent was supplied using a peristaltic pump which was connected to the re-circulation flow. The expansion of the bed was adjusted at 0.5 L (33% of the working volume of the reactor) varying the recirculation flow. 200 mL of low density poly propylene (diameter 0.6-0.8 mm) was used as carrier material.

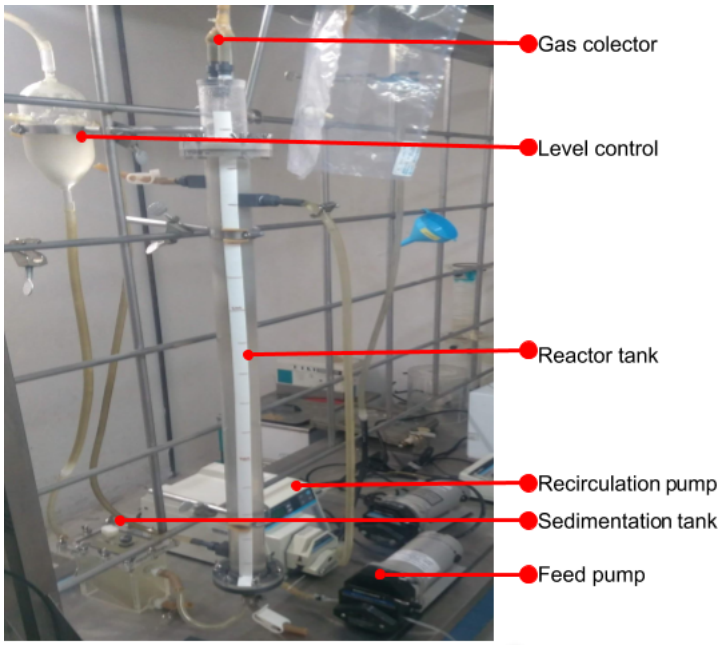


Figure 2.4. Down-flow fluidized bed reactor (DFBR) used in this work for the treatment of a synthetic acidic wastewater

## 2.3 Analytic Methods

### 2.3.1 Media

The growth medium used in all the experiments contained: 5 mM $NaH_2PO_4$, 4.5 mM $NH_4Cl$, 4 mM NaCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 g/L of yeast extract and 1 mL/L of trace elements (50 mM HCl, 1 mM $H_3BO_3$, 0.5 mM $MnCl_2$, 7.5 mM $FeCl_2$, 0.5 mM $CoCl_2$, 0.1 mM $NiCl_2$ and 0.5 mM $ZnCl_2$). The medium was amended with different

concentrations of lactate, acetate, and sulfate according to the experimental design. The pH of the media was neutralized using NaOH 0.1N when needed.

### 2.3.2 VFA and Sulfate

Acetate, lactate, and butyrate were quantified using a capillary electrophoresis equipment (Agilent 1600 A) fitted with a diode array detector following the method described by Soga and Ross (Soga and Ross, 1999).

### 2.3.3 Sulfide

Dissolved sulfide concentration was measured with the spectrophotometric method (Cord-Ruwisch, 1985), which takes into account the soluble $H_2S$ and $HS^-$ species. All the measures of sulfide were performed by triplicated avoiding agitation between sampling events.

### 2.3.4 VSS

Volatile, total, and fixed suspended solids were meassured by gravimetry according to standard methods (APHA, 2012) using 10 mL samples from the enrichment bottles (Chapter 4) and 20 mL samples from the tank reactor (Chapter 3).

### 2.3.5 Sulfate reduction activity assays

We used serological bottles of 120 mL to perform the sulfidogenic activity assays (Chapter 3 and Chapter 5). The bottles were fed with the media previously described and inoculated with 5 mL of biomass, unless stated the contrary. Inoculated bottles were hermetically sealed with rubber stoppers and aluminum crimps, and the headspace was purged for 2 min with $N_2/CO_2$ (80/20) gas mixture. Incubation was carried out under mesophilic conditions ($31\pm1^{\circ}$C) in the absence of light and without agitation, all the activity assays were performed by triplicate.

### 2.3.6 Calculations of the rates

We used the spline method Kimball, 1976 using the `splinefun` function in R version 3.4.2 (R Core Team and R Development Core Team, 2005) to obtain the spline function representing each curve. In this way we could easily interpolate the function and calculate the first derivate and hence the rate at any desired point of the curve and find the maximum rate of consumption/production with accuracy.

### 2.3.7 Calculations of the efficiencies

For an easier interpretation of the data, we converted the molar concentration of the substrates and products into electron milliequivalents ($e^-$ mEq) using the following equivalences: 1 mmol of lactate equals 12 $e^-$ mEq, 1 mmol of acetate corresponds to 8 $e^-$ mEq, 1 mmol of butyrate to 14 $e^-$ mEq, and 1 mmol of sulfate equals 8 $e^-$ mEq (Rittmann and McCarty, 2001). The efficiencies were calculated simply dividing the difference between the concentration of $e^-$ mEq at the end of the experiments ($C_f$) and the initial concentrations between the initial concentration ($C_i$) (i.e. $(C_i - C_f)/C_f$). Using this approach we were able to calculate the rate and efficiency of the electron donor including its byproducts (i.e. butyrate and acetate).

## 2.4 Bacteria community characterization

For community characterization, we extracted DNA from 0.3 g of sample using the MoBio PowerSoil DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's protocol for cells hard to lysate; all DNA extracts were stored at -20 °C until use. Samples from pooling events (Chapter 4) and DFBR reactor (Chapter 5) were composed of three different extraction of the same sample.

### 2.4.1 PCR-DGGE *16S RNA* gene

We used DGGE gels as a cost-effective approach to evaluate the change in the community during the acclimation to acidic pH (Chapter 4). PCR amplifications were

performed in a C1000 Thermal Cycler (BioRad, Hercules, CA, USA) in 25 µl 1x PCR buffer containing 200 µM of each dNTP, 0.2 µM of each primer, 2.5 mM MgCl2, 1.0 U of Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 15 ng of template DNA.

For the first round of 16S rRNA gene amplification, PCR primers were 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The second round was performed to add the GC clmap to the sequences using 357F-GC (5'-CTT ACG GGA GGC AGC AG-3') which contained a GC clamp attached to the 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3'). The first round cycling program was: initial denaturation at 94°C for 10 min, followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 49°C for 45 s and extension at 72°C for 2 min; the final extension was at 72°C for 7 min. The second round cycling program was: initial denaturation at 94°C for 10 min, followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 49°C for 45 s and extension at 72°C for 2 min; the final extension was at 72°C for 7 min.

DGGE was performed with a denaturing gradient of 30 to 60% denaturants (being 100% denaturant a mix of 7 M urea and 40% formamide) in a 6 (wt/vol) polyacrilamide gel. Electrophoretic conditions were: 0.5X TAE buffer at 60°C, 200 V for 10 minutes, following by 16.5 h at 85 V at the same temperature. All DDGE gels were silver-stained.

Analysis of the bacterial community in DGGE gel profiles was performed with the aid of GelQuant Express Analysis Software (Invitrogen, Carlsbad, CA, USA) according to the provider's instructions to quantify the relative abundance and molecular weight of the bands observed. At least five bands from each lane were excised and stored at -20°C in 30 µL of ultrapure water. After the statistical analyses, the DNA of excised bands, that were related to variables studied, was re-amplified using 357F (without GC tail) and 907R primers. Amplicons were purified using the Wizard SV gel and PCR Clean-Up system (Promega, Madison, WI, USA) and sent to the Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental (San Luis Potosí, Mexico) for sequencing. Sequences from bands were aligned and classified using the online SINA aligner (Pruesse et al., 2012), the sequences obtained from bands in Chapter 5 were deposited in the GenBank nucleotide sequence database under accession numbers MH165171- MH165173.

### 2.4.2 High-throughput sequencing

Samples of DNA from the pool bottles (Chapter 4), each one representing the whole community of the inoculum and after the acclimation to pH 6, 5, 4, were shipped to RTL Genomics (Research and Testing Laboratory, Lubbock, TX, United States) and samples of DNA from the DFBR (Chapter 5) were shipped to Zymo Research (Irvine, CA, USA) for amplification of the V4 hypervariable region of 16S rRNA gene and sequencing using the same primers and protocols.

Raw reads from both laboratories were processed using the dada2 (v.1.6.0) pipeline (v.1.8) (Callahan et al., 2016), using R version 3.4.2 (R Core Team and R Development Core Team, 2005). Forward and reverse read pairs were trimmed, filtered (minimum length of 200 nucleotides) and paired. Chimeric sequences also were removed using the same pipeline. To assign the taxonomy to the sequences we used the SILVA SSU Ref NR 132 database (Yilmaz et al., 2014) as reference.

### 2.4.3 Statistical analyses

We used R package vegan (Oksanen et al., 2017), for ordination analyses (RDA and NMDS), rarefaction curves, and diversity indexes calculation. We also used R package agricolae (Mendiburu, 2016) for HSD Tukey' test. We used R package phyloseq (McMurdie and Holmes, 2013) for handling the high-throughput sequencing data. Plots were made with the aid of viridis (Garnier, 2018) and RColorBrewer (Neuwirth, 2014) packages.

# 3. Selection and enrichment of sulfate reducing prokaryotes from an environmental sample

## Abstract

The objective of this part of the work was to enrich a sulfidogenic community from a sediment historically exposed to acid mine drainage. For this purpose we inoculated $\approx$ 0.3 L of sediment in a completely stirred tank reactor with 2.4 L of working volume and fed it with a minimal mineral media containing different concentrations of lactate and acetate at anaerobic conditions. During the 191 days of fed-batch operation, we changed the lactate:acetate ratios in order to maximize the biomass production. We obtained a biomass that produced 20.6 mg of VSS per liter per day. Once we had enough biomass we sought to separate the biomass from the soil particles using different cultivation strategies. At the end we obtained ca. 2.5 gVSS/L of a sediment-free community that consumed acetate and lactate at pH 5 and used sulfate as main electron sink. The results indicated that the cultivation techniques were suitable for our objectives albeit rather slow.

## 3.1 Introduction

The first aim of our work was to enrich a sulfate reducing community (SRC) for the forthcoming experiments. The successful development and application of sulfidogenic technologies, particularly for the treatment of acid mine drainage (AMD), requires biomass with certain particularities, such as (Bijmans et al., 2010):

- Resistant to acidic pH
- High efficiency in electron donor consumption
- High rates of sulfate reduction
- Sufficient growth

But finding a community with all these characteristics is hard by many reasons. Foremost because practically all known sulfate reducing prokaryotes (SRP) are neutrophils (Rabus et al., 2015). Furthermore, organic acids (such as lactate and acetate) which are among the most common substrates for SRP are toxic even at mildly acidic conditions: e.g. acetate is toxic at pH $<$4.8 and lactate at pH $<$3.1, because below this pH these molecules are in their protonated form and they can enter the cell and disrupt the respiratory chain (Baker-Austin and Dopson, 2007).

Despite all these difficulties, there has been a lot of work done in sulfidogenic reactors at low pH (for a extensive review see Gopi Kiran, Pakshirajan, and Das, 2017; Sánchez-Andrea et al., 2014). Currently, the most frequent pitfalls found in sulfidogenic processes are acetate accumulation (Celis et al., 2013) and a decline in the sulfate reduction activity as the pH decreases (Gallegos-Garcia et al., 2009); indicating that we still have failed to find a way to keep the activity of SRP, specially from the 'complete' sulfate reducers (cSRP) (see Section 1.3.2), at acidic pH.

Considering the prevailing limitations of the sulfidogenic processes at low pH, we explored for the best option to obtain an inoculum for our experiments of sulfate reducing communities at low pH. Although many works have been made using wide variety of inoculum sources, most of the work has been made using anaerobic granular sludge (Kaksonen, Riekkola-Vanhanen, and Puhakka, 2003; La et al., 2003; Montoya et al., 2013) and sediments from acidic environments (Koschorreck, Wendt-Potthoff, and Geller, 2003; Meier, Piva, and Fortin, 2012).

Besides the source of the inoculum, we also had to decide the electron donor

and carbon source to feed our microbial community; since SRP are known for growing in a variety of substrates (see Section 1.3.1), we had a lot of options (Liamleam and Annachhatre, 2007). The nature and bioavailability of the substrate fed to the experiments is a major fact determining the possible trophic networks: complex substrates can build complex networks involving several guilds (Werner et al., 2011). Conversely, simple substrates reduce the number of guilds that can act, reducing the complexity of the community structure. For example, acetate is a very limiting energy source and can only be used by a specific set of microorganisms such as SRP and methanogens (Montoya et al., 2012). Considering this, we wanted to use a simple and ready-to-use carbon source, which has been proved to promote the activity of SRB (Zhao, Wang, and Ren, 2010). There are roughly three types of simple substrates for sulfate reduction that have been extensively used: $H_2/CO_2$, simple alcohols (e.g ethanol and glycerol) and volatile fatty acids (-VFA- such as acetate, lactate, and propionate). Each one of these substrates bears specific challenges for successful application in sulfidogenic technologies.

The use of hydrogen ($H_2$) as electron donor and carbon dioxide ($CO_2$) as carbon source for sulfidogenic processes at low pH is one way to completely avoid the problems caused by acetate accumulation and substrate toxicity. Previous studies have successfuly used $H_2/CO_2$ at pH $<$4 in sediments (Meier, Piva, and Fortin, 2012) and at pH = 4 in a membrane reactor (Bijmans et al., 2010). However, the use of hydrogen as electron donor is very expensive, which makes these substrates a unlikely option for its use in industrial applications.

Another way to partially avoid the substrate toxicity is to use organic alcohols as electron donors and carbon sources. Since alcohols are non-polar compounds they are not ionized at any pH, hence they are not toxic. Many studies have used this approach using glycerol (Ñancucheo and Johnson, 2014), and ethanol (Kaksonen, Franzmann, and Puhakka, 2004) at pH as low as 3. Unlike hydrogen, glycerol and ethanol are cheap and are expected to have an increase in their generation during the production of biofuels (Yazdani and Gonzalez, 2007). However, the main disadvantage of these kind of substrate is their low efficiency, because they promote the growth of incomplete SRP, producing large amounts of acetate (Kimura, Hallberg, and Johnson, 2006).

Finally, we could use a volatile fatty acid, such as lactate or acetate. In practice, the use of only acetate as electron donor at acidic conditions is very limited, be-

cause its toxicity and the low energy yield it provides (Liamleam and Annachhatre, 2007). However, acetate can be used in combination with lactate in order to promote its consumption (Celis et al., 2013). Many successful experiments have been done using lactate as sole electron donor in both enrichments (Sánchez-Andrea et al., 2013) and reactors (Zhang, Wang, and Han, 2016) at acidic conditions. Nevertheless, lactate is also an expensive substrate and has a high value but not as high as $H_2$ (Cubas-Cano et al., 2018).

Considering the possible options, we chose to use an environmental sample with a history of previous contact to acid mine drainage as inoculum, and we chose lactic acid in combination with acetic acid as electron donors and carbon sources. Therefore, the objective of this first part of the study was to enrich a sulfidogenic community from an environmental sample. Our main interest was that such a community should have the potential to mineralize lactate and acetate and grow at mildly acidic conditions.

## 3.2 Materials and methods

### 3.2.1 Experimental outline

To achieve our objectives we sampled a sediment historically impacted by acid mine drainage to obtain a sample of a microbial community used to thrive at acidic conditions. Afterward, we operated a completely stirred tank reactor (CSTR) to promote the growth of SRP from the sediment. Finally, we used agitation to promote the separation of the biomass from the sediment particles.

#### Inoculum, media and reactor configuration

The sampling site and sampling details can be found in Section 2.1. A fraction (300 mL) of a composite sample of the sediment was used to inoculate a 3 L tank reactor. We fed the reactor with 2.1 L of anaerobic minimum media (Section 2.3.1). The reactor was maintained at 30°C using a thermal jacket and at pH = 5 using HCl (0.52 N) dosed by an online controller (details on the reactor instrumentation can be found in Section 2.2.1). The reactor was operated in a fed-batch regime, at the beginning

of each batch we adjusted the concentration of the electron donor according to the results observed during the previous batch. Table 3.1 indicates the moles of acetate and lactate added to each batch. Samples for activity assays were taken from the bulk of the reactor and centrifuged at 3000 rpm for 15 minutes to separate the media from the suspended particles. Activity assays were performed according to the details described in Section 2.3.5).

Table 3.1. Concentration of acetate and lactate at the beginning of each batch in the CSTR.

| **Batch** | **Days** | **Acetate (mmol)** | **Lactate (mmol)** |
|---|---|---|---|
| I | 3 | 0.0 | 0.0 |
| II | 7 | 10.0 | 0.0 |
| III | 14 | 27.0 | 3.0 |
| IV | 38 | 27.0 | 3.0 |
| V* | 48 | 20.0 | 5.0 |
| VI | 46 | 0.0 | 15.0 |
| VII | 15 | 0.0 | 15.0 |

* from day 28 the pH control stopped

**Sediment elimination**

After Batch VII, we used agitation to separate the biomass from the sediment. For this purpose, the solids from the reactor were splited and placed into three bottles of 1 L ($\approx$ 200 mL of solids in each bottle). These bottles were fed with the same media as the reactor (Section 2.3.1, without sulfate) and 15 mM of lactate as electron donor and C-source. Low density polypropylene (100 mL) was added to two of the three bottles (bottles B1 and B2) and the other bottle (bottle A) was left without any support material. These bottles were incubated at 30$^\circ$C in horizontal position at constant agitation (100 RMP) in the absence of light during 20 days. After this period of time, we separated the supernatant from the bottle A and a fraction of the material from bottles B1 and B2 and we transfer them into another two bottles. 500 mL of the supernatant from bottle A was centrifuged at 3000 RPM and placed in a bottle labeled as C. The material from bottles B1 and B2 (25 mL from each bottle) was

placed in a bottle labeled as D. The media from bottles A and C was decanted and replaced with fresh media without sulfate. Since bottles D and E were sediment-free we fed them with the same media as bottles A/C but we added 15 mM of sulfate at day 5. Bottles A-E were sealed, and placed in a incubator for another 20 days. We measured sulfide, VFA, and VSS during the cultivation time in order to evaluate the performance of the planktonic biomass and the biomass attached to the support material.

### 3.2.2 Analytical methods

We measured sulfide by the Cord-Ruwisch method (Section 2.3.3), VFA, and sulfate were measured by the Soga and Ross method (Section 2.3.2), total, suspended and volatile solids were measured by gravimetric analyses (Section 2.3.4).

### 3.2.3 Calculations

Data from sulfide, sulfate, acetate and lactate was used to calculate the rates of substrate consumption/production using the cubic splines method described in Section 2.3.6.

## 3.3 Results

### 3.3.1 CSTR performance

At the start of each batch in the CSTR we changed the electron donor in the feed media according to Table 3.1 in order to promote the growth of the SRP and the production of sulfide. The results of the sulfide and VFA concentration (in terms of $e^-$ mEq) in the bulk of the reactor are presented in the Figure 3.1. As can be seen, acetate accumulated in the reactor, indicating that the rate at which it was consumed was significantly lower than the rate it was produced. Interestingly, lactate also accumulated in the system, even when it was fed at very low concentrations (for example 3 mmol during Batch III), but it promoted the consumption of acetate. This

phenomenon has been previously reported, but no conclusive explanation has been found (Celis et al., 2013). In our case, at the start of each new batch the acetate consumption increased even when it was previously stalled at the end of the previous one.

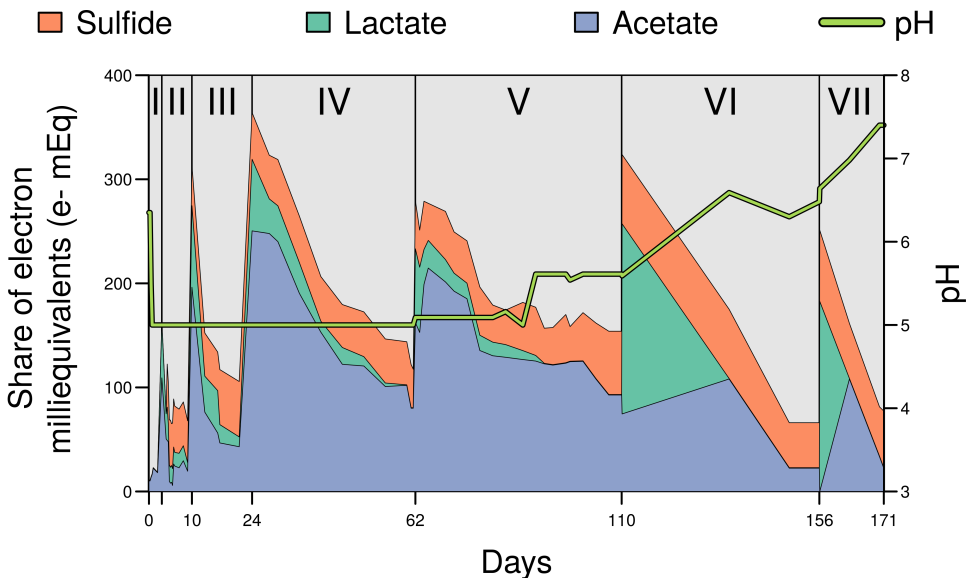


Figure 3.1. Performance of the consortia during the enrichment in a completely stirred tank reactor operated in fed-batch mode. Roman numerals correspond to different batches (Table 3.1).

### Sulfate reducing activity

Despite the consumption of lactate and acetate observed, we were not able to notice any significant increment in the concentration of dissolved sulfide in the reactor. So we took a sample of 100 mL from the mixed liquor at day 20 (third batch) and use it to analyze the sulfate reduction activity of the consortia (Section 2.3.5). This assay was repeated again almost 100 days after, at the beginning of Batch VI in order to compare the activity of the consortia. The results of these analyses are presented in Figure 3.2.

Figure 3.2 shows that the microorganism present in the bulk of the reactor were able to produce sulfide and consume acetate at mildly acidic conditions (initial pH =

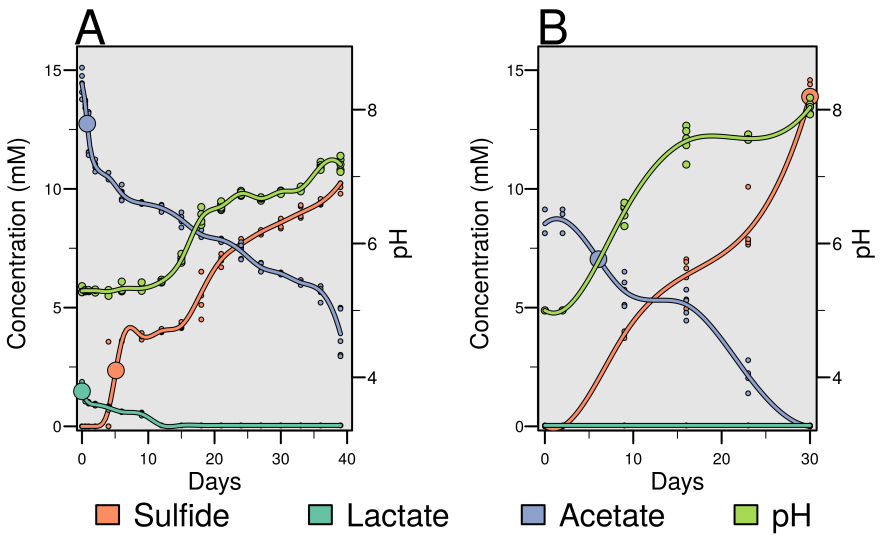


Figure 3.2. Sulfate reducing activity of the biomass present in the reactor at Batches III and VI.

5). The profile of the curve is similar for both samples and the rates are also alike: the sample from Batch III produced up to 1.61 mmol$H_2S$/L·d whereas the sample from Batch VI reached a maximum rate of 1.48 mmol$H_2S$/L· d (Figure 3.2 A and B, respectively). Since pH was not controlled during the activity assays, we had a glimpse of the magnitude of the impact that microbial activity had on the pH in our system. In the sample taken at Batch III the pH increased almost 2 units in 40 days when 15 mmol of acetate were consumed; in the case of the sample from Batch VI the pH elevated 3 units in 30 days after the consumption of 10 mmol of acetate.

A similar trend was observed in the reactor, since it was operated as a fed-batch reactor, the microbial activity increased the pH of the bulk. However, since we controlled the pH at 5 using HCl 0.5 N, we realized that increasingly bigger amounts of acid were required to maintain the pH at the fixed point after the third batch. Consequently, during the fourth batch we let the pH to raise from 5.0 to 5.5. At the end of the fourth batch we realized that the acid added to the reactor was almost 100 mL, which increased the working volume of the reactor almost 5% so we decided to stop the pH control afterward (Figure 3.1).

**Biomass production**

The main objective to cultivate the inoculum in the CSTR was to produce sulfidogenic biomass. Therefore we took samples from the bulk in the reactor at day 15 of the fifth batch. Another sample was taken 15 days after in order to measure the biomass that was produced in the CSTR in 15 days. The results of the biomass production are presented in Table 3.2.

Table 3.2. Biomass Production in reactor during Batch V.

| | $\mathbf{t}_0$ | $\mathbf{t}_1^*$ | $\Delta_g$ |
|---|---|---|---|
| gTSS $L^{-1}$ | 11.08 | 12.24 | 1.16 |
| gVSS $L^{-1}$ | 0.85 | 1.17 | 0.31 |
| gFSS $L^{-1}$ | 9.91 | 11.38 | 1.47 |

$^*$ 15 days after $T_0$

The results in Table 3.2 indicated that there was a high quantity of inorganic material in the bulk of the reactor, as the fixed suspended solids (FSS) were ten times higher than the organic fraction represented in the volatile suspended solids (VSS). Moreover, in the 15 days period between each sampling event the TSS augmented 10% from 11.08 g to 12.24 g, probably as a result of the shear forces disrupting sediment particles and suspending them. The volatile solids increased in 0.31 g in the same period of time which represented a 50% increment. Nevertheless, since we needed at least 2.0 gVSS/L we let the CSTR for 60 days more to achieve our goal.

### 3.3.2 Sediment elimination

As we moved forward past the biomass production stage we sought to eliminate (at least partially) the particles of sediment from the biomass. To achieve this we separated the solids from the CSTR in two and place one half in one bottle without any support material (Bottle A) and the other half in two bottles that contained 100 mL of a support material (Bottles B1 and B2). We followed the lactate, acetate, sulfate and sulfide in the bottles during 45 days to analyze the sulfidogenic activity. The results of these incubations are presented in the Figure 3.3 panels A for the sediment

alone and B as the average of bottles B1 and B2. In Figure 3.3 we can see that all bottles demonstrated sulfidogenic activity, but the ones without the support material (Figure 3.3C) had higher sulfide production but slightly lower substrate consumption compared to the other enrichments. After 45 days, we separated part of the support material from bottles B1 and B2 and placed it on a new bottle with fresh media, including sulfate -bottle D. We also took 500 mL of the supernant from bottle A and centrifuged it to recover the biomass. We used the pellet of biomass from bottle A to inoculate another bottle called C. The bottle C was also fed with mineral media supplemented with sulfate. We incubated bottles C and D as before and followed VFA, sulfate, and sulfide. The results of the performance of these bottles are shown in Figure 3.3 panels C and D.

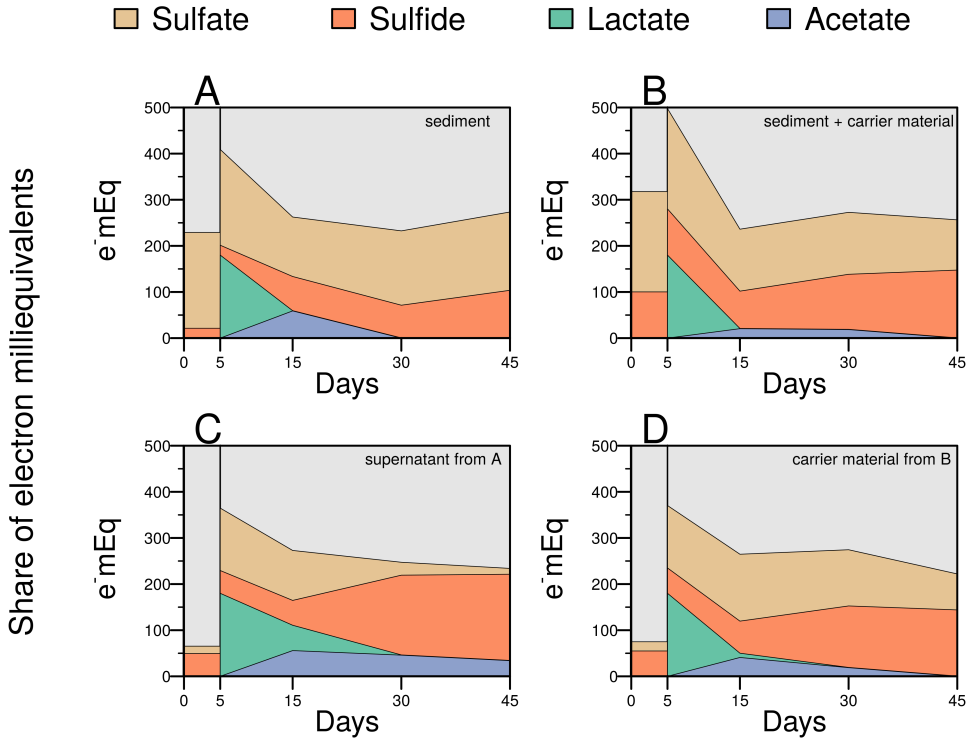


Figure 3.3. Performance observed in enrichments used to separate the biomass from the sediment.

Figure 3.3 panels C and D show that the strategy to separate the biomass from the sediment was successful, sulfate was practically absent in these bottles before we added it at day five. Moreover, both sources of inocula showed sulfidogenic

activity. Sulfidogenic activity in bottle C was high -almost 90% of the $e^-$mEq were used to reduce sulfate to sulfide at the end of the experiment; but the electron donor consumption was rather low compared to bottle inoculated with the carrier material (Figure 3.3D) and acetate was observed at the end of the experiment. Contrarily, the community in bottle D exhibited a high electron donor consumption but low sulfide production, which indicate that the electrons were diverted from sulfate reduction to other metabolic pathways, probably fermentation.

## 3.4 Discussion

Finding the right conditions for the enrichment of a sulfate reducing community (SRCo) at acidic pH is an arduous task, mainly because SRP are known neutrophiles and the acidic pH is a stress factor. In practical therms this means that if a neuthrophile (SRP in our case) is thriving in acidic conditions, its growth would be limited since it would be reallocating resources to resistance mechanisms instead of growing (Lund, Tramonti, and De Biase, 2014). To enrich a SRCo at pH 5 we sampled a sediment exposed to AMD which has been proved to had sulfidogenic activity (Moreno-Perlin, 2014) and we used CSTR operated in a fed-batch regime. This strategy has been used to promote high cell density cultures, since it allows a tight control of the cultivation conditions and a data feedback of the last batch, which makes easy to modify the conditions to improve the outcome (Korz et al., 1995).

Our first attempts were focused on developing a SRCo that could use preferentially acetate as substrate (Figure 3.1 Batchs I to IV), but we had a limited success, acetate was not completely oxidized and accumulated in the reactor. When we took a sample to quantify the sulfidogenic activity (Figure 3.2) we realized that we, indeed, had SRP able to oxidize acetate however their activity was somewhat limited in the CSTR.

Limited acetate oxidation in a sulfidogenic reactor at acidic conditions has been extensively documented (Gopi Kiran, Pakshirajan, and Das, 2017; Sánchez-Andrea et al., 2014) and some methods have been proposed to overcome it, such as the addition of lactate (Celis et al., 2013). We used this strategy and added lactate to the feed in a 1:10 proportion to acetate starting at Batch III. Lactate is a common substrate for SRP, it is not toxic at pH 5 and provides more $e^-$ mEq per mole than

acetate (12 instead of 8), thus it could be used as a co-substrate to favor the growth of a different set of SRP (Rabus, Hansen, and Widdel, 2013). However, we had to add it in small amounts to avoid the exclusive growth of incomplete SRP which would lead to an aggravation of the acetate accumulation problem (Montoya et al., 2013; Rabus et al., 2002).

Our strategy was successful during two batches (III and IV), but we realized that the increase of the microbial activity lead to an increase in the volume of acid required to maintain pH at 5, at the end of Batch IV the acid added represented almost 5% of the volume of the reactor. To compare the contribution of sulfate reduction activity (i.e. sulfide produced) and microbial activity (electron donor consumed) to the pH neutralization, we fitted a sigmoid function to the data obtained from activities assays were pH was not controlled. The results of this fit are presented in Figure 3.4 alongside a model representing the relationship observed between pH and microbial activity.

Figure 3.4 shows that the increase in pH is a consequence of both the sulfide production and the electron donor consumption, but also shows the difference between the impact that sulfide production and electron donor consumption have in the pH neutralization: the lag phase in the sulfide production is shorter for sulfide production (Figure 3.4A) than the lag phase for electron donor consumption (Figure 3.4B). This indicates that sulfide production neutralizes the pH in the system quicker than the electron donor consumption. This can be perfectly explained since sulfide production requires the oxidation (consumption) of the electron donor and implies the production of bicarbonate ($HCO_3^-$) besides sulfide ($H_2S$) as shown in Reaction 1.4:

$$2\,\mathrm{CH_2O} + {\mathrm{SO_4}}^{2-} \longrightarrow \mathrm{H_2S} + 2\,{\mathrm{HCO_3}}^{-} \tag{1.4}$$

In this case, both sulfide and bicarbonate contribute to alkalinity and neutralize the pH (Godoi et al., 2017). On the other hand, electron donor consumption does not necessarily implies bicarbonate production nor sulfide production (e.g. fermentation or biomass production). We tried two offline options to control pH: adding HCl 0.5 N and $FeCl_2$ 5 mM, FeCl was more successful for pH control (data not shown), reinforcing the fact that sulfide was the main force rising pH but its use would modify

completely the chemical environment of the reactor, hence the option was to let the pH to increase during the experiments rather than controlling it (Figure 3.2 V-VII).

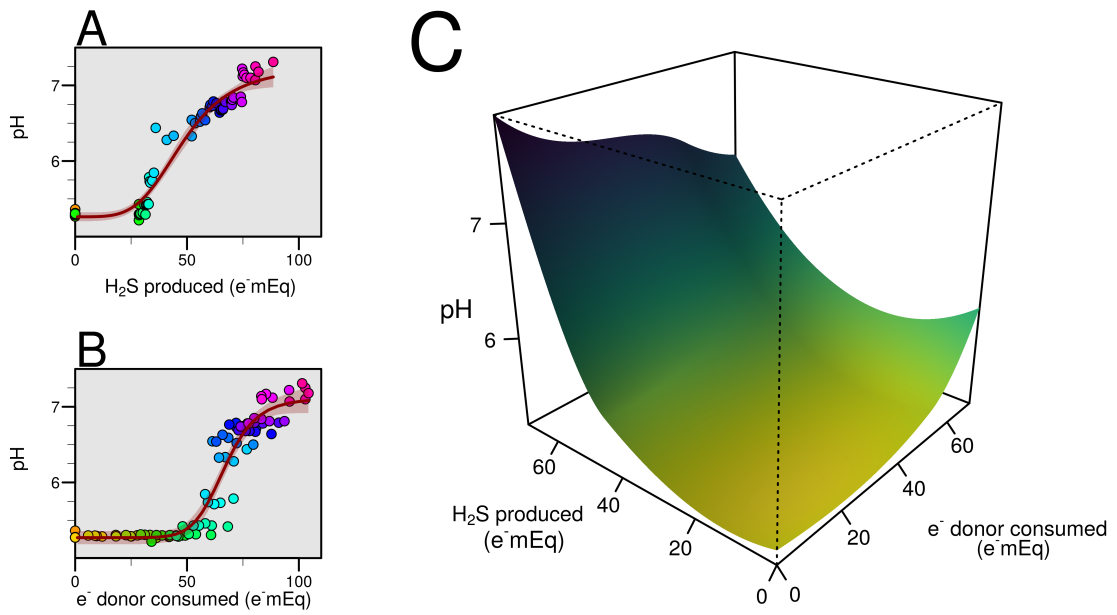


Figure 3.4. Influence of the microbial activity on the pH observed in activity assays. A) pH as function of sulfide produced. A) pH as function of electron donor consumed. C) pH as function of sulfide produced and electron donor consumed.

After seven batches in the CSTR we had enough biomass to perform the experiments planned, but firstly we wanted to remove most of the sediment particles from the biomass. We wanted to eliminate the sediment because it was made principally of calcium sulfate (gypsum) and had traces of metals that could interfere with the sulfur balances (Moreno-Perlin et al., 2019), making impossible to accurately calculate the sulfide produced and the sulfate consumed (as it did during the reactor operation). We tried two options to achieve this goal, one included a support material and the other did not. We observed that the microbial community that developed in the support material had a different activity that the one without it, the former was more efficient at electron donor consumption than the latter; however, the community found in the support material produced less sulfide than the one floating in the the media (3.3). SRP are known for their preference to grow within biofilms rather than as free-living cells (D'Acunto et al., 2011), in fact most of the reactor configurations used for sulfate reduction have either biofilms or were provided with a carrier material

(Sánchez-Andrea et al., 2014). Yet, the biomass that attached to the carrier material in our experiments of sediment elimination showed less sulfidogenic activity than the biomass found in the supernatant (Figure 3.3 panels C and D). It seemed that the biomass attached to the support material was metabolically more diverse than the planktonic one. It consumed the electron donor faster and used unidentified electron sinks besides sulfate (Figure 3.3D). However, since we were more interested in the sulfide production we preferred to keep our investigation using the biomass without the carrier material (Figure 3.3C).

## 3.5 Conclusion

The use of a completely stirred reactor under a fed-batch regime to produce sulfidogenic biomass at mildly acidic conditions proved to be a successful approach, even though it was slow. Despite the use of an inoculum with previous exposition to acidic conditions, biomass production was slow, probably due to the stress faced by the community. Unfortunately, biomass production is an overlooked result in many research studies of sulfate reduction at low pH, hence it is hard to compare the results we observed with other works. However, it should be considered as one key parameter to analyze the stress endured by the microbial communities.

At the end, we were able to obtain an acetate-consuming microbial community that could grow at mildly acidic conditions and used sulfate as main electron sink that could be used for the next stage of our investigation.

# 4. Acclimation of a sulfate reducing community to acidic pH


## Abstract

Sulfate-reduction is a viable option to treat metal containing effluents,which are generally acidic (pH $<$ 4). However, sulfate reducing processes are sensitive to low pH. We investigated the change of the community traits in a sulfate-reducing community subjected to aclimation to acidic pH, from pH 6 to 3. Substrate removal, sulfate reduction efficiency, and biomass yield remained constant through pH 6 to 4; at pH 3 the activity stopped. At pH 5 and 4, lactate was fermented to butyrate indicating a change in the structure of the community, but the sulfate consumption rate and efficiency was kept the same as at pH 6. Acetate dependent sulfate reduction was observed only at moderately acidic conditions (pH 5.0). Taxa of microorganisms involved in the sulfur cycle were identified in all the samples (*Acidithiobacillus, Desulfurella, Lentimicrobium, and Desulfumicrobium*). Acidic pH-stress induced changes in the taxonomic composition of the consortia and in the energy flow, but the efficiency and the rate of sulfate reduction remained constant through the experiment, indicating functional stability.



A version of this work was submitted to FEMS Microbial Ecology

## 4.1 Introduction

In the previous part of this work we enriched a sulfate reducing community (SRCo) at pH 5 using lactate as electron donor, the community operated in a two step process where lactate was firstly partially oxidized to acetate and afterward acetate was consumed (3.3). However the pH at wich our SRCo worked was still high considering that acid mine drainage usually has a pH below 3 (Johnson and Hallberg, 2005). Moreover, the ideal pH for sulfide selective precipitation of metals (as in Reaction 1.5)is also at acidic conditions (Ňancucheo and Johnson, 2012). To fulfill the main objective of this work we needed to adapt our SRCo to acidic pH. In a previous work (Moreno-Perlin, 2014), we observed that a gradual reduction in the pH is the better option to adapt a community to acidic pH, this method known as ”acclimation” has been used in many works to achieve microbial activity at stressful conditions, included sulfate reduction at low pH (Bijmans et al., 2010; Rabus et al., 2002).

Acclimation is a biological process that takes place when an organism, population or community is able to thrive at lethal conditions of a particular variable (e.g. temperature, pH or metal concentration) after previous exposure to sub-lethal conditions of the same variable (Wilson and Franklin, 2002). In the particular case of acid resistance, it has been observed that some neutrophilic Gram-negative bacteria have a better chance to survive at pH 3 if they were previously exposed to pH 4. This phenomenon is called ”acid habituation” (Koutsoumanis and Sofos, 2004; Lou and Yousef, 1997) and it is the result of gradual physiological changes, such as the activation of inducible acid resistance mechanisms (Section 1.3.3) (Beales, 2004).

The purpose of this work was to analyze if there was a change in the traits of a sulfate reducing community exposed to sequentially acidic conditions from pH 6 to 3. We were interested in functional traits, such as rates and efficiencies of substrate removal and in the composition and relative abundances of the members in the community.

## 4.2 Materials and methods

### 4.2.1 Experimental outline

To achieve our objectives, we used ten bottles of 1 L, each one representing an experimental unit, to perform an acclimation experiment exposing our previously enriched SRCo to sequentially acidic conditions from pH 6 to 3. Each bottle contained an initial biomass concentration of 0.2 gVSS/L and anaerobic minimum media (Section 2.3.1) supplemented with 15 mM of lactate and 0.2 g/L of yeast extract. Sulfate concentration was 22.5 mM to achieve a 1:1 ratio based on electron milliequivalents ($e^-$mEq) (i.e. 1.5 mmol of sulfate for each mmol of lactate). The bottles were sealed and incubated at 30°C without agitation in the absence of light until the electron donor and its by-products (i.e. acetate and butyrate) were completely consumed in at least six of the experimental units. At the end of each experiment, the culture medium of all experimental units was carefully separated by centrifugation at 1200 g for 10 minutes. All the biomass pellets were pooled together into a new single bottle and the volatile suspended solids of that bottle were quantified. This new "pool" bottle was used to re-inoculate another 10 experimental units in the same fashion as before but reducing the initial pH in one unit; this procedure allowed to sequentially expose the community to pH 6, 5, 4, and 3.

Experimental units were sampled every 5 days to evaluate their performance. Briefly, 3 mL aliquots were taken and used to determine pH, volatile fatty acids (VFA) and sulfate. Also, at the end of every pH level, a 50 mL sample was taken from each experimental unit to quantify VSS and for the 16S PCR-DGGE analysis. We also took a sample of the inoculum and pool communities from pH 6 to 4 for high-throughput sequencing.

### 4.2.2 Molecular analyses

PCR-DGGE fingerprinting technique was used to evaluate the differences in the consortia composition among different initial pH values and between replicates (details are presented in 2.4). Analysis of the bacterial community in DGGE gel profiles was performed with the aid of GelQuant Express Analysis Software (Invitrogen, Carlsbad,

CA, USA) according to the manufacturer's instructions.

### 4.2.3 Electron distribution and yield calculation

Electron distribution between the electron acceptor (sulfate) and cell synthesis was calculated as the observed fraction of electrons transferred from the electron donor to the electron acceptor ($f_e{}^\circ$) and the observed fraction of electrons from the electron donor utilized in cell synthesis ($f_s{}^\circ$) (Rittmann and McCarty, 2001). To calculate $f_e{}^\circ$we considered that sulfate used in cell synthesis was negligible (Rabus, Hansen, and Widdel, 2013) and converted sulfate to electron milliequivalents, denoted as $e^-$mEq (1 mmol $SO_4^{2-}$ = 8 $e^-$ mEq); then we calculated the fraction of electron milliequivalents of the electron acceptor used as follows: $f_e{}^\circ$= (final $SO_4^{2-}$ - initial $SO_4^{2-}$) / initial $SO_4^{2-}$ where initial and final $SO_4^{2-}$ are the initial and final $e^-$mEq of sulfate. To calculate $f_s{}^\circ$we used VSS as a proxy for biomass; thus, we divided the VSS produced ($VSS_{final}$ - $VSS_{initial}$) in terms of $e^-$mEq (20 $e^-$mEq $\approx$ 113 mgVSS) by the initial concentration of lactate also in terms of $e^-$mEq (1 mmol lactate = 12 $e^-$mEq).

### 4.2.4 Statistical analyses

Since we were interested in the maximum rates of consumption/production of all the substrates (i.e. lactate, acetate, butyrate, and sulfate) at the initial pH values, we calculated the cubic spline function for all the substrates at every initial pH value in each experimental unit and obtained their first derivative as function of time (Section 2.3.6) (Kimball, 1976). We used the maximum rates obtained from splines to conduct a one-way ANOVA in order to compare the effect of initial pH value on the maximum production/consumption rate of each substrate.

We used the intensity of the bands as a proxy for the abundance from DGGE gel profiles. We used these data to construct a sample-phylotype table and calculate the richness, diversity (as Shannon-Weaver index), evenness and dominance of the samples. Afterward, we performed a non-metric multidimensional scaling to analyze the relationship between the initial pH and the composition of the communities within the samples. Finally, we fitted a correlation between the ordination of the community and the variables of interest to examine if the communities composition was anyhow

correlated to the performance observed in the samples. We used R version 3.4.2 (R Core Team, 2017) to perform all statistical analysis; packages vegan (Oksanen et al., 2017) and agricolae (Mendiburu, 2016), were used to compute diversity indices, NMDS and fitting the performance to the community composition and Tukey's HSD tests, respectively. All statistical analyses were performed with RStudio software (version 1.1.383; RStudio Inc., Boston, MA, United States).

## 4.3 Results

### 4.3.1 Performance

Figure 4.1 illustrates the kinetics of the substrates followed during the experiments. It can be seen that lactate was incompletely oxidized to acetate in the first 20 days of the experiments, and the produced acetate was subsequently consumed. Depending on the pH more time was needed to degrade acetate: as the initial pH decreased more time was required for acetate consumption, despite the fact that lactate was consumed in less time. When the pH was reduced to 3, all the microbial activity stopped. This observation is in agreement with many previous reports pointing out that very low pH values ($\leq$ 3) are unfavorable for sulfate reduction (Sánchez-Andrea et al., 2014).

The comparison of the effect of initial pH value on the maximum production/ consumption rate of each substrate is presented in Figure 4.2 A-H. The statistical analysis of the results indicated that the initial pH only had a significant effect on the maximum consumption rate of lactate (Figure 4.2B, $p < .05$) among the pH values at which microbial activity was observed. The maximum rate of substrate consumption increased from 6.77 $\pm$ 4.81 at pH 6, to 16.4 $\pm$ 0.35 mmol Lac/gVSS$\cdot$ d at pH 4. On the contrary, acetate and sulfate (Figures 4.2C and 4.2A) were statistically consumed at the same maximum rates ($\approx$ 3.8 and $\approx$ 7.5 mM/gVSS$\cdot$ d, respectively) at pH 6, 5, and 4; also butyrate was produced and consumed at the same rate when it occurred (Figures 4.2D and 4.2H).

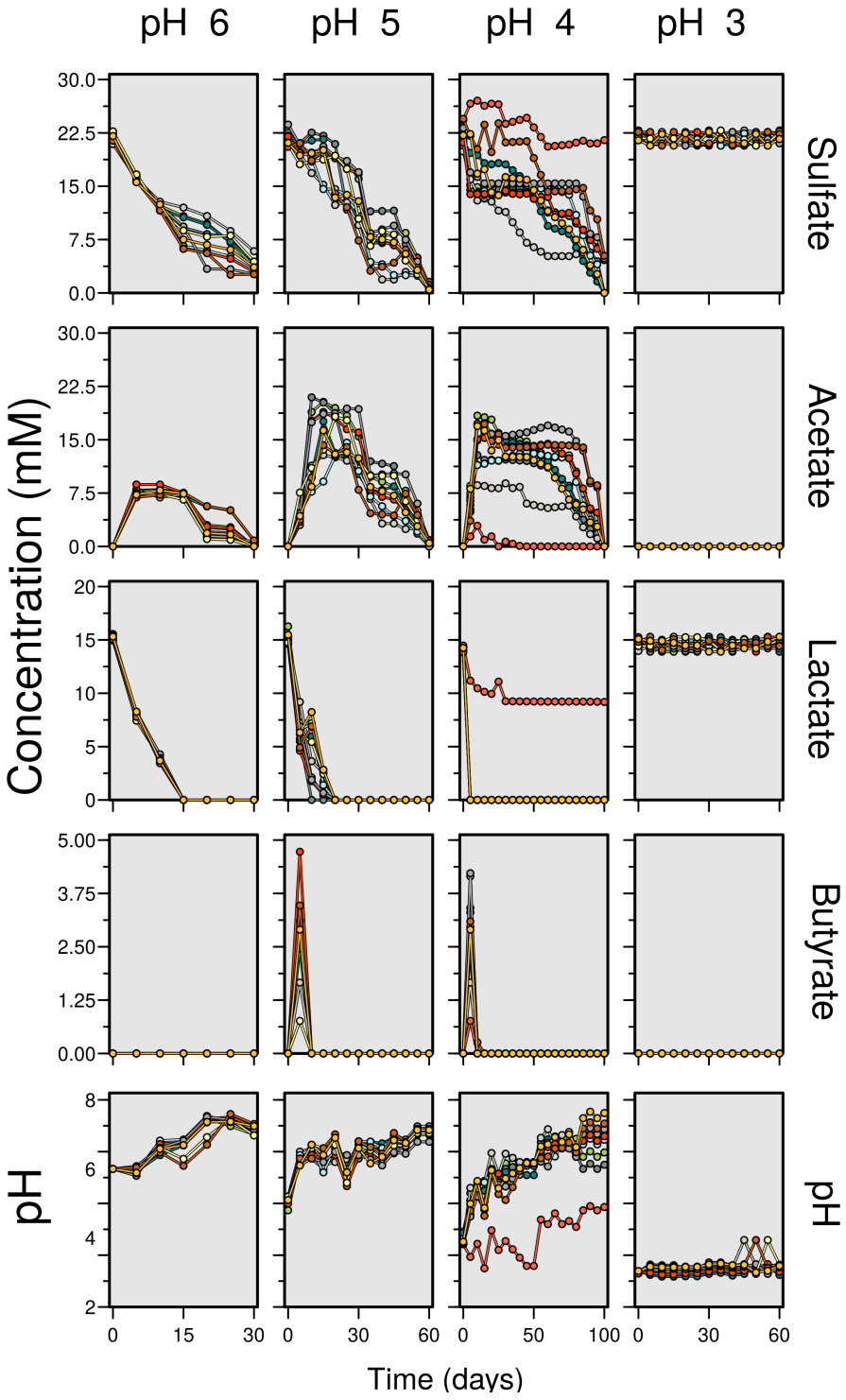


Figure 4.1. Kinetics of substrates and pH observed at different initial pH values during the acclimation of the microbial community to acidic pH. Note the difference in the scale in the x axis.

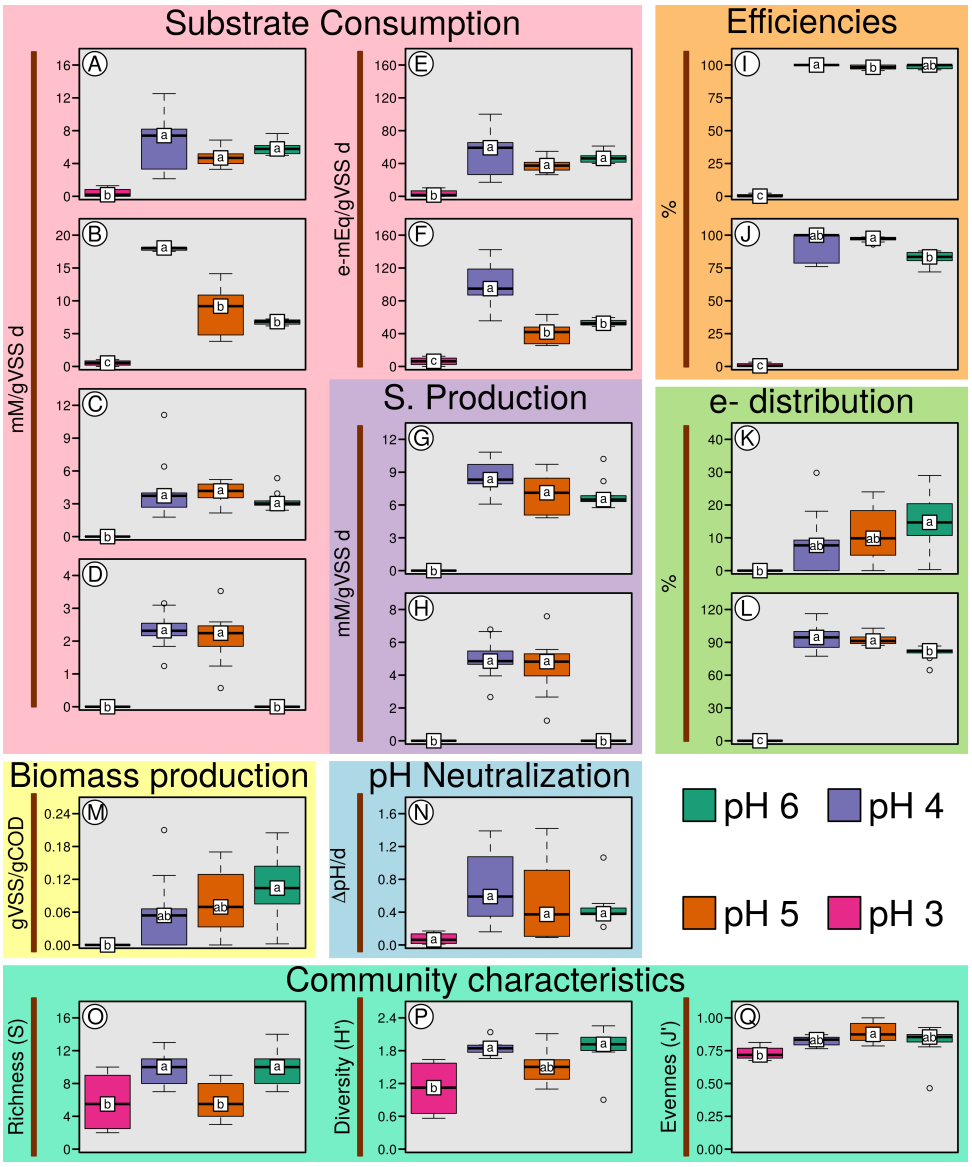


Figure 4.2. Traits of the community at all initial pH levels. A) Maximum sulfate consumption rate, B) Maximum lactate consumption rate, C) Maximum acetate consumption rate, D) Maximum butyrate consumption rate, E) Maximum electron-acceptor consumption rate, F) Maximum electron-donor consumption rate, G) Maximum acetate production rate, H) Maximum butyrate production rate, I) Electron donor removal efficiency, J) Electron acceptor removal efficiency, K) Fraction of the electron donor used in cell synthesis ($f_s^\circ$), L) Fraction of the electron donor used for energy production ($f_e^\circ$), M) pH neutralization ($\Delta$pH/d), N) Biomass production (gVSS/gCOD). Characteristics of the community at all initial pH values O) TR-F richness, P) TR-F diversity (Shannon-Wienner' Index) and Q) TR-F evenness (Pielous' Index).

As the stress caused by acidic pH and VFA toxicity, promotes the allocation of resources from growth to survival of microorganisms (Schimel, Balser, and Wallenstein, 2007), we were interested in the fate of electron equivalents in terms of the observed fraction of electron milliequivalents transferred from the electron donor to the electron acceptor ($f_e^\circ$) (i.e. sulfate) and in the observed fraction of electrons from the electron donor used in cell synthesis ($f_s^\circ$) (Rittmann and McCarty, 2001). Figures 4.2K-M, presents the comparison of the effect of the initial pH value on $f_s^\circ$, $f_e^\circ$and $Y_{obs}$ respectively.

Results indicate that there were no significant differences neither in the electron milliequivalents distribution between $f_e^\circ$and $f_e^\circ$, nor in the biomass yield across the pH gradient. Finally, the results of electron donor and acceptor removal efficiencies (Figures 4.2I and Figure 4.2J respectively) indicated that there was a significant difference in the sulfate removal efficiency among treatments ($p < .05$) , but the removal efficiency of the electron donor was the same. Finally, the maximum rate at which the pH was neutralized also was the same in the different treatments ($p < .05$).

Overall, Figure 4.2 shows that the SRCo was able to maintain almost all of its traits across the pH gradient. More importantly, as shown in Figure 4.3, the correlation between electron donor and acceptor (i.e. sulfate) consumption was >90% and the slope aproximated to 1, indicating that sulfate was the main electron sink in our experiments. Nonetheless, the correlation and the slope did change between different initial pH values. Indicating that at different pH values different electron acceptor-donor relations were observed. At pH 5 (Figure 4.3C) the slope and the correlation are 1, which suggest a perfect fit in the electron donor and acceptor (i.e. sulfate) consumption; differently, at pH 6 (Figure 4.3B) the correlation is 0.9, but the slope is 0.7 indicating that part of the electron donor was used in other processes (e.g. biomass production) instead of sulfate reduction.

One of the main reasons for the change of the relation between electron donor and sulfate consumption (Figure 4.3) was the butyrate production as a result of lactate-acetate fermentation, according to Reaction 4.1 (Duncan, Louis, and Flint, 2004):

$$4\,C_3H_5O_3^- + 2\,C_2H_3O_2^- + 2\,H_2O \longrightarrow 3\,C_4H_7O_2^- + 2\,H_2 + 4\,HCO_3^- + H^+ \qquad (4.1)$$

Reaction 4.1 indicates that the butyrate production was accompanied with acetate consumption,since acetate consumption has been proven to be a major challenge in sulfidogenic reactors at low pH and acetate (Celis et al., 2013).

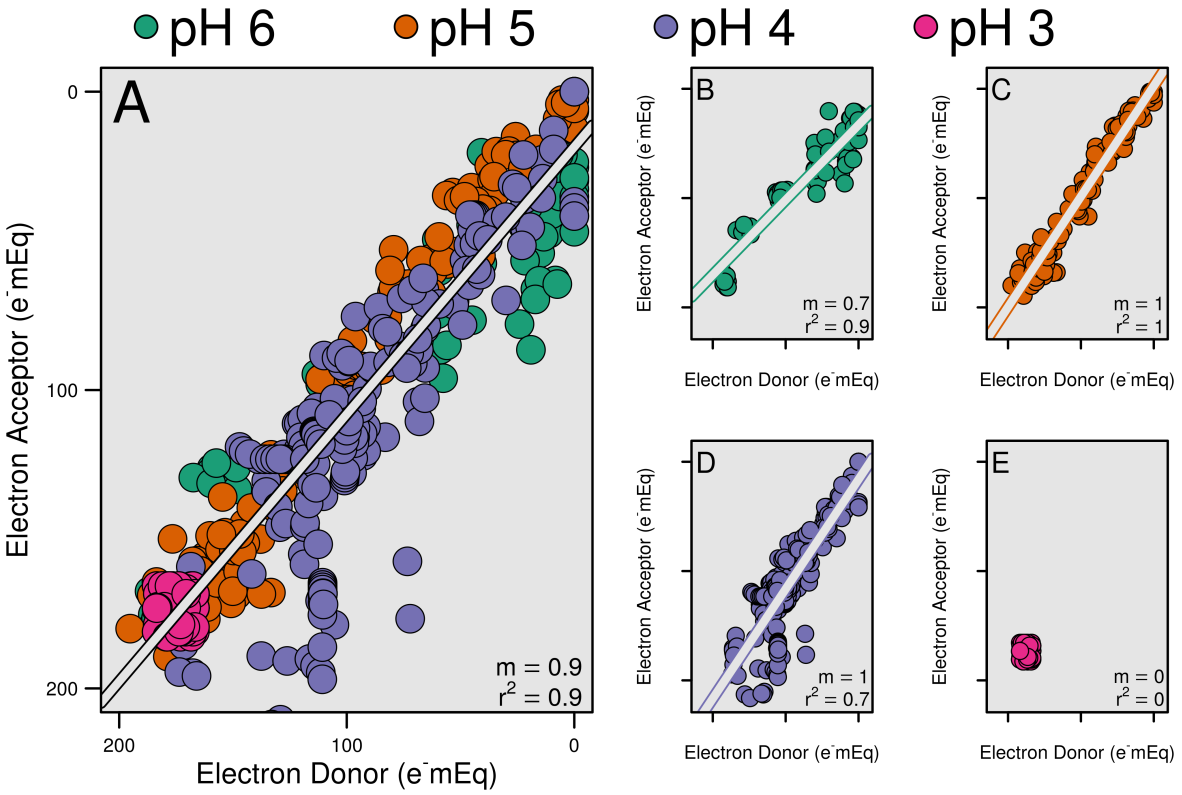


Figure 4.3. Correlation between the electron donor and sulfate consumption A) All pH, B) pH 6, C) pH 5, D) pH 4 and E) pH 3.

We were particularly interested in knowing at what pH acetate was consumed and whether acetate consumption was coupled to sulfate reduction or to fermentation. Therefore we used the results from splines to plot the consumption/production rates of acetate ($\Delta$Ace/ $\Delta$t) and butyrate ($\Delta$But/ $\Delta$t), and the consumption of sulfate ($\Delta$Sul/ $\Delta$t) and lactate ($\Delta$Lac/ $\Delta$t) as function of the pH of the system at each specific sampling point (Figure 4.4).

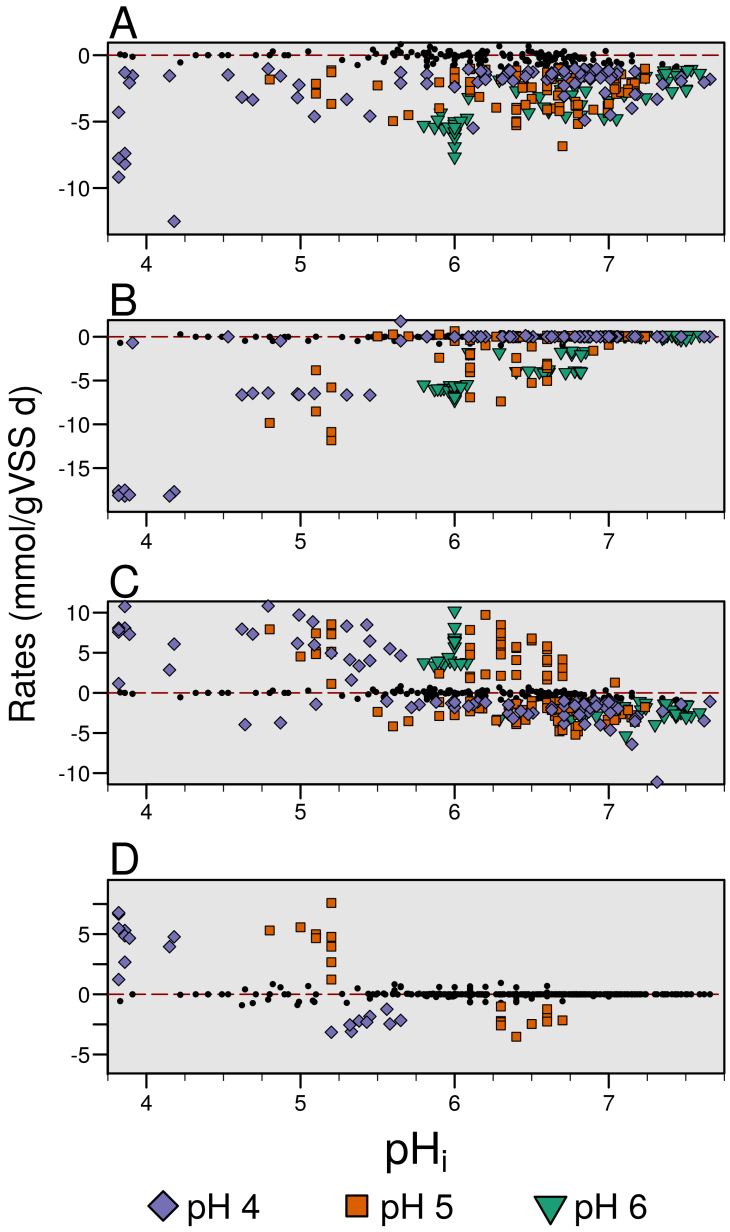


Figure 4.4. Production and consumption rates observed at different pH values. A) Sulfate, B) Lactate, C) Acetate and D) Butyrate. The red dotted line indicates the limit between production (above the line) and consumption (below the line) (points representing consumption/production below 1 mmol/gVSS·d are smaller for visualization purposes).

Figure 4.4 indicates that at the beginning of the experiments at pH 4 (rhomboid

points near pH 4 in Figure 4.4C), acetate was produced at a very fast rate ($\approx$ 8.97 mmol/gVSS· d), accompanied by the production of butyrate ($\approx$ 4.63 mmol/gVSS·d, Figure 4.4D) and the consumption of lactate ($\approx$ 17.27 mmol/gVSS·d Figure 4.4B) and sulfate ($\approx$ 9.15 mmol/gVSS·d Figure 4.4A). These rates indicated that although there was sulfate consumption at the begging of the experiment at pH 4, its rate of consumption was $\approx$ 55% slower than the consumption of lactate. The results also suggest that the acetate used for butyrate production was provided by incomplete oxidation of lactate through sulfate reduction, according to the stoichiometry of Reaction 4.1.

There is a dense cloud of points near zero for acetate in the range of pH from 6 to 7 (Figure 4.4D); these points represent mostly the lack of change in the acetate concentration that occurred between days 30 to 75 in the experiments initialized at pH 4 and some points between days 15 and 45 in the experiments initialized at pH 5 (Figure 4.1). According to these observations, we can infer that regardless that acetate consumption by cSRB was dependent on pH because it only happened at pH $>$5.5 (Figure 4.4C), time was another factor affecting the consumption of acetate, even at circumneutral pH.

### 4.3.2 Community

#### Acclimation seen by DGGE

To evaluate the taxonomic composition of the sulfate-reducing consortia across the pH gradient, we analyzed all samples taken at the end of each experiment through the commonly used 16S rRNA PCR-DGGE fingerprinting technique (Muyzer, Waal, and Uitterlinden, 1993) (Figure 4.5). The amplification of the 16S rRNA gene was successful in 34 out of 40 experimental units, it was more difficult to extract the genomic DNA from the experimental units at pH 3 compared to those at less extreme pH conditions (6, 5, or 4).

We also used band intensities to calculate some characteristics of the community such as richness, diversity (as Shannon Wiener' index) and evenness (as Pielous' index). Albeit the result indicated that the characteristics of the community did change across the pH gradient (Figure 4.2O-Q). It is important to note that the analysis is unbalanced and the treatment at pH 3 only has 4 replicates for these specific traits.

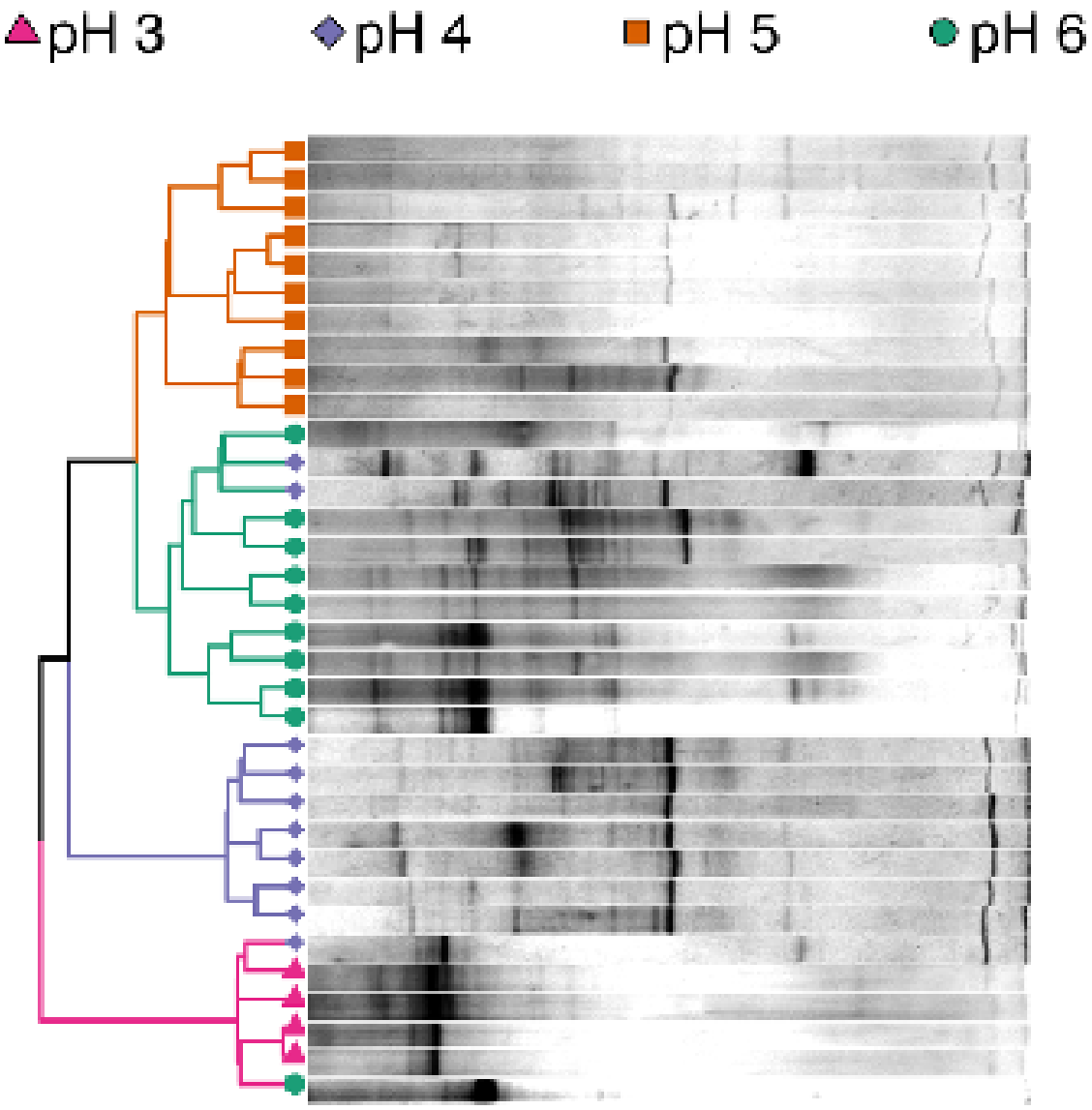


Figure 4.5. Hierarchical clustering of the DGGE profiles observed at different inital pH conditions during the acclimation to acidic pH.

DGGE profiles were used to perform a Non-metrical Multidimensional Scaling (NMDS) analysis (Ramette, 2007). Since our interest was to evaluate if there was any kind of correlation between the taxonomic composition and the function of the consortia, we fitted the relevant performance traits of the SRCo (presented in Figure 4.2A-N) to the NMDS ordination using the `envfit` function from the R package

vegan. The result of this analysis indicated that most of the community traits had a statistically significant correlation to the composition of the community, and only the maximum rate of butyrate consumption (-$r_{But}$), the fraction of electrons used for energy ($f_e^\circ$) and cell synthesis ($f_s^\circ$), and the biomass yield ($Y_{obs}$) were not correlated to the composition of the community (Table 4.1). The graphical representation of this analysis is shown in Figure 4.6.

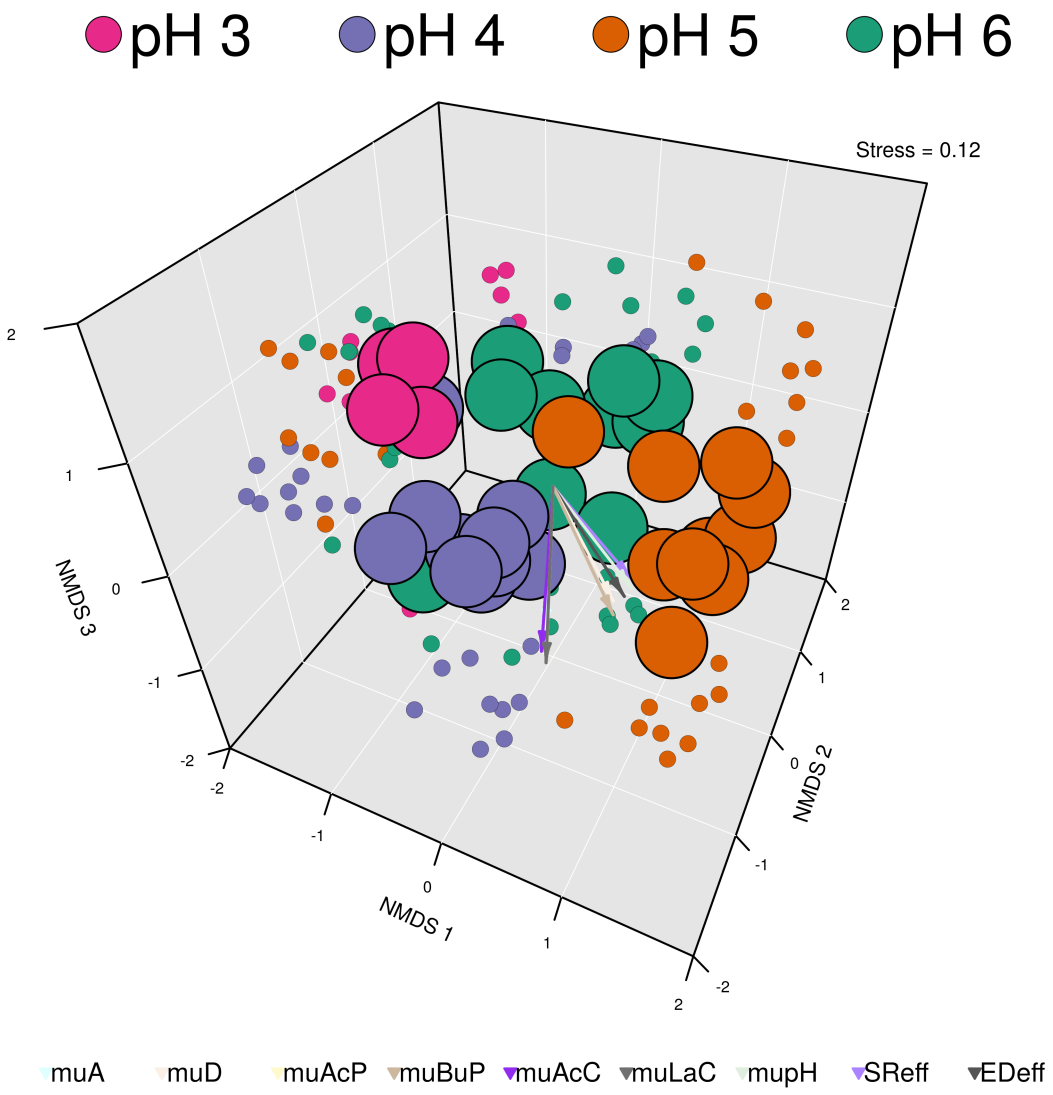


Figure 4.6. Ordination of DGGE results.

The NMDS ordination confirmed that the composition of the samples was closely

related, but they were only partially overlapped according to their initial pH (Figure 4.6), as it would be anticipated in an acclimation experiment where sterile conditions were maintained and each new step was a consequence of the previous stage. Altogheter, results indicate that the performance of the consortia was resilient, and was closely related to the composition of the consortia.

Table 4.1. Correlation ($r^2$) of the microbial community composition observed in DGGE profiles and the relevant variables of the communities observed in this study.

| **Variable** | **$r^2$** | **P** |
|---|---|---|
| Max. rate of e- acceptor consumption (-$r_{eD}$) | 0.28 | 0.016 |
| Max. rate of e- donor consumption (-$r_{eD}$) | 0.41 | 0.000 |
| Max. rate of acetate production ($r_{Ace}$) | 0.73 | 0.000 |
| Max. rate of butyrate production ($r_{But}$) | 0.53 | 0.000 |
| Max. rate of acetate consumption (-$r_{Ace}$) | 0.45 | 0.001 |
| Max. rate of lactate consumption (-$r_{Lac}$) | 0.73 | 0.000 |
| Max. rate of butyrate consumption (-$r_{But}$) | 0.11 | 0.301 |
| Max. rate of pH neutralization ($r_{pH}$) | 0.55 | 0.000 |
| Sulfate reduction Efficiency (%SR) | 0.45 | 0.000 |
| e- donor removal Efficiency (%ED) | 0.52 | 0.000 |
| Fraction of electrons used for energy ($f_e{}^\circ$) | 0.10 | 0.378 |
| Fraction of electrons used for synthesis ($f_s{}^\circ$) | 0.10 | 0.379 |
| Biomass yield ($Y_{obs}$) | 0.10 | 0.386 |

### Acclimation seen by high-throughput sequencing

We also used mass sequencing of the 16S rRNA gene to analyze the changes in the SRCo at the inoculation and at each pooling event, except for pH 3, since we did not obtain a SRCo at pH 3. The high-throughput sequencing yielded 119624 sequences. After processing them (i.e. filtering, denoising and removing chimeras -details in Section 2.4.2) 108194 sequences were kept; 34506, 38479, 13698, and 21511 of the sequences corresponded to samples from pH 4, 5, 6 and inoculum respectively (Table 4.2).

Table 4.2. DADA2 Pipeline results.

| Sample | Input | Filtered | Denoised | Tabled | Nonchimeric |
|---|---|---|---|---|---|
| pH4 | 34782 | 34659 | 34659 | 34659 | 34506 |
| pH5 | 44853 | 44829 | 44829 | 44829 | 38479 |
| pH6 | 15898 | 15892 | 15892 | 15892 | 13698 |
| Inoculum | 24091 | 24084 | 24084 | 24084 | 21511 |

The sequences were assigned into 509 different amplicon sequence variants (ASVs) using the SILVA database version 132 (Yilmaz et al., 2014). The percentage of successful taxa assignment is shown in Table 4.3). All ASVs belonged to Bacteria Kingdom, classified into 24 different Phyla and 36 Classes. Our analysis indicated that the Family rank was the highest rank at which we could describe our community with high accuracy (Table 4.3; at this rank we were able to identify 82 different Families, which accounted for $\approx$ 96% of the sequences. Thus, we selected the Family rank to perform the analysis of the results.

Table 4.3. Percentage of successful taxa assignment at different phylogenetic ranks.

| | Kingdom | Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|---|
| pH4 | 100 | 98.04 | 95.29 | 90.2 | 81.57 | 59.61 | 7.06 |
| pH5 | 100 | 95.38 | 90.38 | 86.15 | 77.69 | 56.54 | 4.23 |
| pH6 | 100 | 94.12 | 89.5 | 85.29 | 76.89 | 55.46 | 3.78 |
| Inoculum | 100 | 94.78 | 89.93 | 85.07 | 77.24 | 55.6 | 4.48 |
| *Total* | *100* | *96.07* | *92.34* | *87.23* | *78.59* | *56.78* | *5.5* |

Figure 4.7 shows the analysis of the alpha diversity within the samples analyzed via high-throughput sequencing. The results of the rarefaction indicates that the coverage of sequencing was incomplete in the sample from pH 6, but the rest of the curves reached the plateau (Figure 4.7B). We performed a hierarchical clustering of the samples with the data from the most abundant Families in the samples, the result shows that despite the diversity of Families in the samples, only the 25 more abundant Families represented more than the 90% of the sequences; moreover they

represented 93.9% of the sequences found at pH 5 and 93.3% at pH 6 (Figure 4.7A). Further, samples from pH 5 and pH 6 pools were very close together, whereas samples from pH 4 pool and inoculum were diferent among each other. This observation is also supported by the non-metrical multidimension scaling analysis (NMDS) (Figure 4.7C).

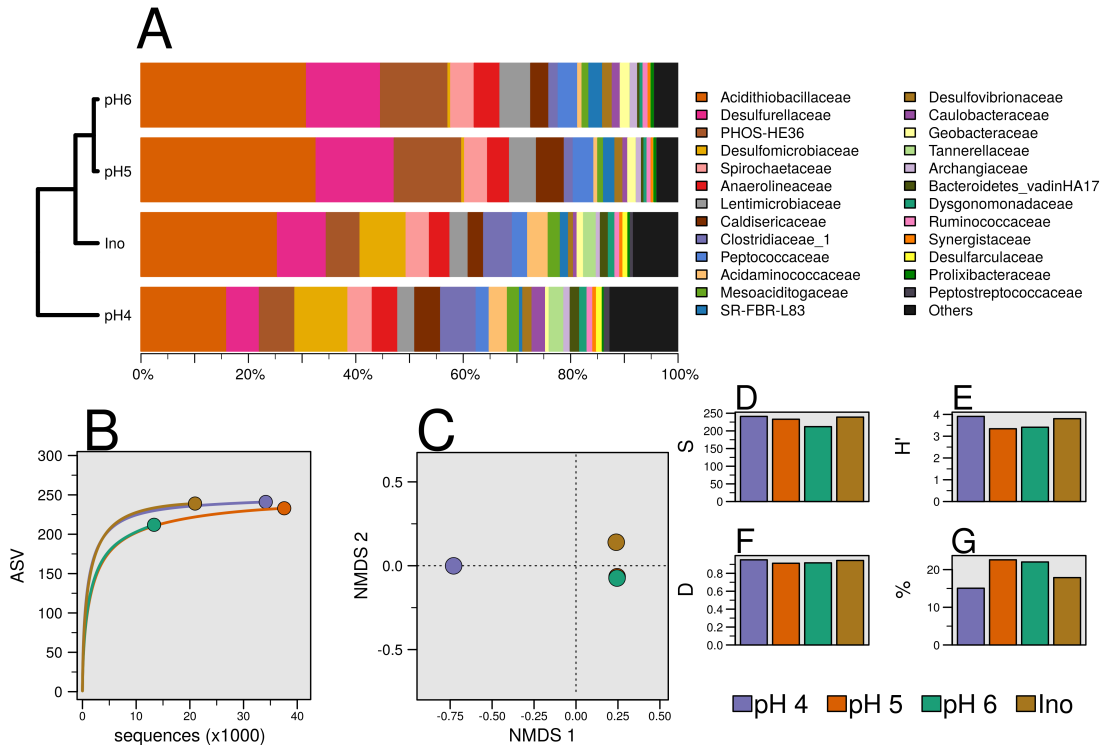


Figure 4.7. Results of the high-throughput sequencing of the 16S rRNA gene from the inoculum and pool samples of the acclimation experiments. A) Microbial composition of the samples at the Family Rank, only the 25 more abundant Families are shown. B) Rarefaction of the samples. C) Ordination of the samples. D) Richness of the samples as observed ASVs. E) Diversity of the samples as Shannon-Wienner' Index. F) Evenness of the samples as Simpson' Index. G) Dominance of the samples as the relative abundance of the most represented ASV in each sample.

Despite this difference in composition, all samples had a similar richness (Figure 4.7D), diversity (as Shannon-Wiener' Index Figure 4.7E) and evenness (as Simpson' Index Figure 4.7F). Yet, in samples from pH 6 and 5 we observed a stronger dominance of a single ASV, which represented ≈20% of the total of the sequences in each sample; in comparison to the other samples were the most represented ASV

only accounted for 10% at pH 4 and 17.23% at inoculum.

Since we were very interested in the composition of SRP in our sulfate reducing community, we looked for SRP using the sequences classified as Firmicutes or Proteobacteria- class Deltaproteobacteria from our ASVs in the GenBank database using the BLAST tool (details in Section 2.4.2). We found that 59 sequences matched (identity >97%) with 11 known sulfate reducing species. Further, we used the information of the relative abundance of the sequences to analyze the change of the abundance of the sulfate reduction guild in our SRCo during the acclimation process (Figure 4.8).

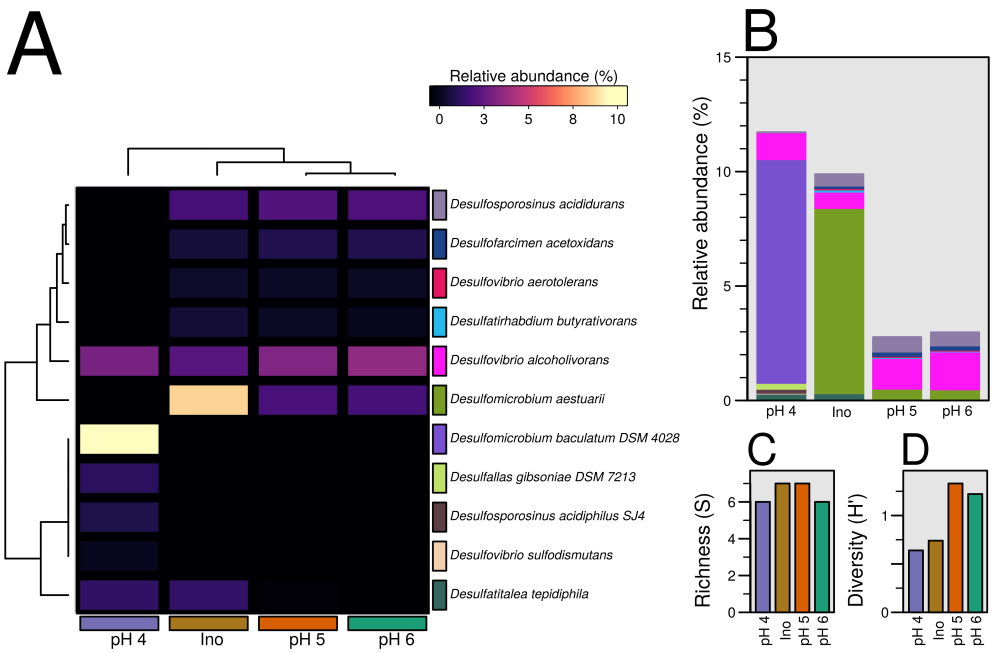


Figure 4.8. Change in the composition of the sulfate reducing bacteria guild across the pH gradient. A) Heatmap of the relative abundance of sulfate reducing bacteria at each sample. B) Comparison of the relative abundance of sulfate reducing bacteria at each sample. ASV (C) richness and diversity (D) of sulfate reducing bacteria at each sample.

Figure 4.8A shows that the composition of sulfate reducing bacteria (SRB) changed across the pH gradient, there is a remarkable difference in the group of SRB that were observed at pH >4 in comparison to those observed at pH 4. Only *Desulfovib-*

*rio alcoholivorans* was present in every sample, but at low abundance. Interestingly, the total fraction of ASVs that SRB represented in the samples was higher in the inoculum and in the pool pH 4 samples than in the samples of pool 6 and 5. The richness was 6 for the pH 4 pool and the inoculum and 7 for the pH 6 and 5 pools, nevertheless, the diversity was $\approx$0.75 for the pH 4 pool and the inoculum and $\approx$1.25 for the pH 6 and 5 pools. This is a consequence of the high dominance that *Desulfomicrobium baculatum* and *Desulfomicrobium aesturaii* had in the communities at pH 4 and in the inoculum respectively.

## 4.4 Discussion

Acclimation has been proven to be a very useful tool to overcome inhibitory conditions in biotechnological applications (Tian et al., 2018). Despite the lack of specific information regarding the resistance of SRB to acidic conditions, it is known that the mechanisms of acid resistance in bacteria require time and involve a shift in the regulation and expression of several genes (King et al., 2010; Liu et al., 2015). This study reveals an insight into the ecological processes that occur in sulfate-reducing consortia when they are exposed to sequentially acidic conditions using an ionizable substrate (lactate) as electron donor and carbon source.

Previous studies have documented sulfate reduction at acidic conditions (pH $\pm$ 3) using either sediments (Meier, Piva, and Fortin, 2012) or biofilm reactors (Ňancucheo and Johnson, 2012). However, in our study, sulfate reduction and lactate consumption stopped when the consortium was exposed to pH 3, after sequential exposure to pH 6, 5, and 4. Therefore the consortia acclimation to pH 3 was unsuccessful, most probably due to the lack of the shelter provided by the sediment matrix (previoulsy eliminated -Section 3.2.1), to cope with the stress caused by acidic pH (Krekeler, Teske, and Cypionka, 1998).

Our results indicate that the performance of the iSRB was not affected with the change of the initial pH value in the experiments at pH 6, 5, or 4, because the maximum substrate consumption rates of lactate and butyrate remained constant (Figure 4.2B). Moreover, iSRB were still active at pH as low as 4 (Figure 4.1). However, acetate dependent sulfate-reducers (i.e. cSRB) were affected in their kinetics, altough their maximum rate of consumption of acetate (Figure 4.2C) was constant accros the

pH gradient their lag phase increased from a few days at pH 6 to about two months at pH 4 (Figure 4.1). It is also important to highlight that even when the pH reached the optimal value for acetate consumption ($\approx$ 5.5), cSRB remained inactive until day 60 in the experiment at pH 4, despite the fact that pH reached a value of 5 since day 30 (Figure 4.1), suggesting that there could be physiological changes that may affect their activity after a short exposure to acidic pH (Goodson and Rowbury, 1989).

At optimal pH conditions acetate consuming SRB have high specific sulfate reduction rates and short doubling times (Rabus, Hansen, and Widdel, 2013), but in our experiments they faced several limitations that included low pH (pH 6-3) (Sánchez-Andrea et al., 2014), acetate toxicity (Beales, 2004), and competition with other acetate consumers (Montoya et al., 2012).

In addition to the rates of substrate consumption, there are other performance parameters that must be considered when developing any engineered system, such as biomass production. In this study, the observed biomass yield per electron donor ($Y_{obs}$), and the fraction of electrons used for cell synthesis ($f_s{}^\circ$) remained statistically constant through the pH gradient. Yet, we must consider that when we calculated $Y_{obs}$ and $f_s{}^\circ$we calculated, in fact, the biomass production of the community and not only that of SRB, which points out that the eventual growth of butyrate producers and other guilds could be helping to keep constant $Y_{obs}$ and $f_s{}^\circ$through the pH gradient.

Altogether, our results indicate that the acid stress faced by our SRCo modified its microbial composition (Figures 4.6 and 4.7). But the functioning of the system was constant through the pH gradient from pH 6 to 4 sulfate was the main electron sink (Figure 4.3) and most of the traits of the community were kept equal, except for those related to biomass synthesis (Figure 4.2). This was accomplished through a change in the energy flow inside the system, the production of butyrate from lactate-acetate fermentation. Butyrate is not a common by-product in sulfidogenic systems, however thermodynamically and stoichiometrically it is possible for some bacteria to produce butyrate from lactate and acetate (Duncan, Louis, and Flint, 2004). Moreover, there are previous evidences that stress can shift the carbon flow in microbial communities (Schimel, Balser, and Wallenstein, 2007).

Previous studies reported that acetate must be supplied or produced by cross-feeding interactions to obtain butyrate; indicating that acetate production and butyrate production are two different processes not necessarily performed by the same

microorganism (Bhat and Barker, 1947; Duncan, Louis, and Flint, 2004). In our experiments, acetate used for the production of butyrate could be produced by either lactate fermentation (Oyekola, Hille, and Harrison, 2009) or by incomplete oxidation of lactate through sulfate reduction. The evidence suggests that acetate for butyrate production was provided by iSRB, because in the experiments where butyrate was observed (pH 5 and 4, Figure 4.1) sulfate was consumed at the starting point of the experiments between days 0 and 5, in addition to the high rates of sulfate consumption and butyrate production in the first stages of the experiments (Figure 4.4). Moreover, the kinetic profiles (Figure 4.1) are in concordance to the thermodynamics and stoichiometry (Reaction 4.1) of butyrate production from acetate and lactate, where acetate is supplied by incomplete oxidation of lactate by iSRB.

The conversion of lactate to butyrate appears to be a fascinating case of cross-feeding where iSRB produce acetate that is used by fermenters to produce butyrate, which in turn is used by iSRB. Butyrate use by iSRB may be possible since the $H_2$ hypothetically produced (Reaction 4.1) was available to perform the incomplete degradation of butyrate, according to the following reaction (Liamleam and Annachhatre, 2007):

$$\mathrm{C_4H_7O_2^-} + 2\,\mathrm{H_2} + \mathrm{SO_4^{2-}} \longrightarrow 2\,\mathrm{C_2H_3O_2^-} + \mathrm{HS^-} + 4\,\mathrm{H_2O} \tag{4.2}$$

It is important to remark that butyrate production consumed lactate, which probably affected iSRB, but in compensation, iSRB were provided with butyrate and $H_2$, which are valuable sources of energy and richer than lactate (Liamleam and Annachhatre, 2007).

In previous reports when butyrate was present in sulfate-reducing consortia, SRB were outcompeted by syntrophic butyrate oxidizers in UASB reactors regardless of the sulfate concentration (Visser et al., 1993); conversely, it has been observed that in sediments with high sulfate availability, SRB are responsible for the butyrate turnover (Banat and Nedwell, 1983). The latter was also observed in our experiments, which may be a consequence of the previous treatment of the inoculum (Chapter 3). An interesting fact that remains uncertain is if butyrate producers are acid-tolerant or truly acidophilic because they were not observed at circumneutral conditions (pH 6) but were very active at pH 4. Therefore butyrate producers might

be either outcompeted by neutrophilic sulfate-reducing bacteria or inhibited by pH at neutral pH conditions.

According to the evidence, the mechanism of substrate uptake at pH 5 and 4 could have been as follows: First, lactate was consumed by iSRB to produce acetate; shortly after, butyrate producers, consumed lactate and acetate to produce butyrate and $H_2$. Later, both substrates (butyrate and $H_2$) were consumed by iSRB and acetate was formed. Finally, the acetate was eventually consumed by cSRB.

It is important to remark the fact that even though sequences belonging to known SRB represented ≈10% of the communities, sulfate was the main sink of carbon (Figure 4.3), indicating a high metabolic activity of the SRB with a deep impact in the performance of the community. A previous report in peatlands reported that a *Desulfosporosinus spp.* was able to sustain high sulfate reduction rates and was responsible for a most of sulfate reduction *in situ*, despite the fact that it represented less than 0.01% of the community composition based on 16S rRNA gene sequences (Pester et al., 2010). These observations suggest that some SRB might be keystone species that have a high impact in the community functioning regardless their abundance (Giller and O'Donovan, 2002). Interestingly enough, the composition of the SRB guild changed at different pH values (Figure 4.8) which could mean that their occupation range does not overlaps (Table 4.4).

Although many phylotypes were present at all pH values (Figures 4.7A and 4.8), the evidence indicates that the stress faced by our consortia had an impact at community level: 16S metagenomics results indicated that there was a turnover in less abundant phylotypes at the different levels of pH, but the community maintained a highly similar composition across the pH gradient. Suggesting that only those organisms that adapted or were fitted enough to thrive under acidic conditions survived or remained abundant. This was specially true for the sulfate reducing guild, there was a notorious change in the composition of SRB from the inoculum to pH 4 (Figure 4.8B). The ordination analysis indicated that the community performance was associated to its composition (Figure 4.6), but we were not able to correlate none of the diversity indexes to its performance (data not shown). This could be a problem with the resolution of the molecular tool used (Karczewski, Riss, and Meyer, 2017), an effect of species redundancy (Giller and O'Donovan, 2002), or a combination of both.

Table 4.4. Relevant metabolic characteristics of SRB found at the different initial pH values during the acclimation process.

| SRB | Acetate consumption | pH |
|---|---|---|
| *Desulfosporosinus acididurans* | No | 5.5 |
| *Desulfofarcimen acetoxidans* | Yes | 7.1 |
| *Desulfovibrio aerotolerans* | Yes (as carbon source) | 5.7 |
| *Desulfatirhabdium butyrativorans* | Yes (as carbon source) | 7.0 |
| *Desulfovibrio alcoholovorans* | No | 7.0 |
| *Desulfomicrobium aestuarii* | Yes (as carbon source) | 7.0 |
| *Desulfomicrobium baculatum* | No | 7.2 |
| *Desulfallas gibsoniae* | No | 6.9-7.2 |
| *Desulfosporosinus acidiphilus* | No | 5.2 |
| *Desulfovibrio sulfodismutans* | No | 6.9 |
| *Desulfatitalea tepidiphila* | Yes | 6.8-7.3 |

## 4.5 Conclusion

In this work we have shown that the SRCo was able to thrive at acidic conditions from pH 6 to 4, but it failed to survive at pH 3. This could indicate a natural limit of sulfate reduction at acidic conditions. Remarkably, the community used maintained its functionality and diversity through the pH gradient and traits of relevance for environmental engineering such as sulfate reduction rate and efficiency were kept high even at pH = 4.

Moreover, we observed that the activity of incomplete SRB was not limited by pH at acidic conditions. However, SRB faced competition for lactate with butyrate producing microorganisms. In contrast, complete SRB were inactive even at moderately low pH conditions ($\approx$ 5.0) and, in consequence, acetate accumulated despite the pH reached optimal values for its consumption (pH $>$ 5.0). These findings are relevant for the design of bioprocesses aimed to benefit from the sulfate reduction process at acidic conditions, in which the interactions of the microbial consortia are of utmost importance to determine the failure or success of the biological process.

# 5. Application of an acid-tolerant sulfate reducing community for the treatment of synthetic acidic wastewater


## Abstract

We used a down-flow fluidized bed reactor inoculated with a sulfate reducing community previously acclimated to acidic pH to treat acidic (pH 3.02) syntetic wastewater using lactate as sole electron donor. During the operation we decreased the hydraulic retention time (HRT) from 2 to 1 days. When the HRT was above 1.5, we observed average removal efficiencies of 90% of sulfate and electron donor. However, further decrease of the HRT to 1 day caused a system failure and the sulfidogenic activity was lost, probably due to a $H^+$ overloading. Molecular analyses indicate that although the community changed during the operation of the reactor (average Jaccard' distance of 0.7 between the inoculum samples and the final samples), the predicted functional profile was constant during all the process (average Jaccard' distance of 0.2).


## 5.1 Introduction

After the acclimation of the sulfate reducing community (SRCo) to acidic pH, its energy flow and biological composition changed. Nevertheless, most of the traits of

interest of the community such as electron donor and acceptor removal efficiencies, and rates remained constant through the pH gradient. At pH 4, the electron donor removal was above 80%, sulfate reduction efficiency reached 75%, biomass yield observed was 0.05 gVSS/gCOD, and the rates of lactate and sulfate were ≈6 mmol/gVSS·L and ≈5 mmol/gVSS·L respectively (Chapter 4).

To complete the work of this thesis we wanted to evaluate the performance of the SRCo in continuous conditions fed with an acidic inffluent (pH $\sim$ 3.0). For this purpose we selected a down-flow fluidized bed reactor (DFBR) to inoculate with the acclimated SRCo. The reason to select this reactor configuration is that it operates with a high recycle flow wich helps to dilute the inffluent concentrations. Moreover, since the biomass in the reactor is immobilized, it decouples the hydraulic retention time (HRT) from the sludge retention time, allowing to slow growing microorganism to establish. Finally, this configuration makes easier to separate metal precipitates from bulk media (Elliott, Ragusa, and Catcheside, 1998; Gallegos-Garcia et al., 2009; Villa-Gomez et al., 2011).

## 5.2 Methods

### 5.2.1 Inoculum, medium and reactor

The reactor (detailed description in Section 2.2.2) was fed with minimum anaerobic medium (Section 2.3.1) and operated at continuous conditions during 75 days. Before the continuous operation of the reactor, the reactor had a start-up stage that lasted 30 days, in which the DFBR was operated in a fed-batch mode. During this time, 4 g of VSS were inoculated to the reactor in three different events. In the first inoculation event (day -30), 2 gVSS of an acid-acclimated sulfate reducing community were inoculated; in the other two events, (days -25 and -15) 1 g VSS was inoculated per event. Once the reactor electron donor removal efficiency became stable and the duration of each batch was less than 3 days, we started the operation of the reactor in a continuous mode at the operational parameters shown in Table 5.1. During the continuous operation of the DFBR, the HRT was gradually decreased from 2 to 1 days to observe the effects that acidic stress had in the performance of the reactor. The pH inside the reactor was uncontrolled and depended completely of the micro-

bial activity. We monitored the pH of the reactor and its effluent on a daily basis and sampled the bulk, the effluent and the inffluent of the reactor every 3 days to quantify sulfate, and volatile fatty acids (VFA).

Table 5.1. Operational conditions for the sulfide production in the down-flow fluidized bed reactor.

| | **Start-up** | **Stage I** | **Stage II** | **Stage III** | **Stage IV** |
|---|---|---|---|---|---|
| Days | -30-0 | 1-30 | 31-40 | 41-60 | 61-75 |
| HRT (days) | - | 2.0-1.7 | 1.4 | 1.2 | 1.0 |
| Recirculation flow (mL/min) | 350 | 400 | 400 | 400 | 400 |
| pH (affluent) | 3.08* | 3.08 | 3.08 | 3.08 | 3.08 |
| OLR (mmol Lac/L·d) | - | 4.34±0.30 | 5.60±0.11 | 6.44±0.04 | 7.71 ±0.07 |
| SLR (mmol $SO_4^{2-}$/L·d) | - | 5.90±0.44 | 7.71±0.32 | 8.79±0.12 | 10.47±0.06 |
| C removal efficiency (%) | - | 86.82 | 89.40 | 79.85 | 33.94 |
| S removal efficiency (%) | - | 84.30 | 82.42 | 61.47 | 26.66 |
| pH (reactor) | 5.60±0.11 | 4.86±0.46 | 4.84±0.07 | 4.81±0.02 | 4.07±0.27 |
| pH (effluent) | 6.05±0.17 | 5.07±0.75 | 4.93±0.04 | 4.91±0.01 | 4.15±0.30 |

* the pH of the first batch was adjusted to 4.0 using NaOH 0.1 N.

### 5.2.2 Batch experiments

At the end of the DFBR operation we took a sample from the sedimentation tank and from the effluent in order to quantify the sulfidogenic activity of the samples. Activity assays were performed as described in Section 2.3.6 by triplicate. We used 0.2 gVSS $L^{-1}$ to inoculate the bottles and amended them with 7.5 mM of sulfate (negative control) or 7.5 mM of sulfate + 5 mM of lactate at pH 3 or 7.5 mM of sulfate + 5 mM of lactate at pH 6 (positive control).

### 5.2.3 Analytical methods

VFA and sulfate were quantified by capillary electrophoresis according to Section 2.3.2. pH and VSS were measured according to Section 2.3.4. Dissolved sulfide was determined using the protocol described in Section 2.3.3.

### 5.2.4 Molecular analysis

We performed high throughput sequencing targeting the V4 region of the 16S rRNA gene to evaluate the differences in the composition of the community at three different times of the reactor operation: at inoculation (T1, one sample per inoculation event), at the start of the continuous operation (T2) and at end of the experiment (T3). The samples corresponding to T2 and T3 were taken from the reactor tank (labeled as R), the biofilm in the carrier material (labeled as M) and the effluent (labeled as E). DNA was extracted according to Section 2.4, analysis of the sequences was according to Section 2.4.2. We used R version 3.4.2 (R Core Team, 2017) to compute diversity indexes and ordination analyses using the vegan package (Oksanen et al., 2017). We used the R package Tax4Fun2 (Wemheuer et al., 2018) to predict the functional profile of the samples based on their taxonomy determined by their 16S rRNA gene sequences, more than 90% of the sequences were used. All statistical analyses were performed with RStudio software (version 1.1.383; RStudio Inc., Boston, MA, United States).

## 5.3 Results

### 5.3.1 Reactor performance

The performance of the reactor during the operation at continuous conditions is summarized in Figure 5.1.

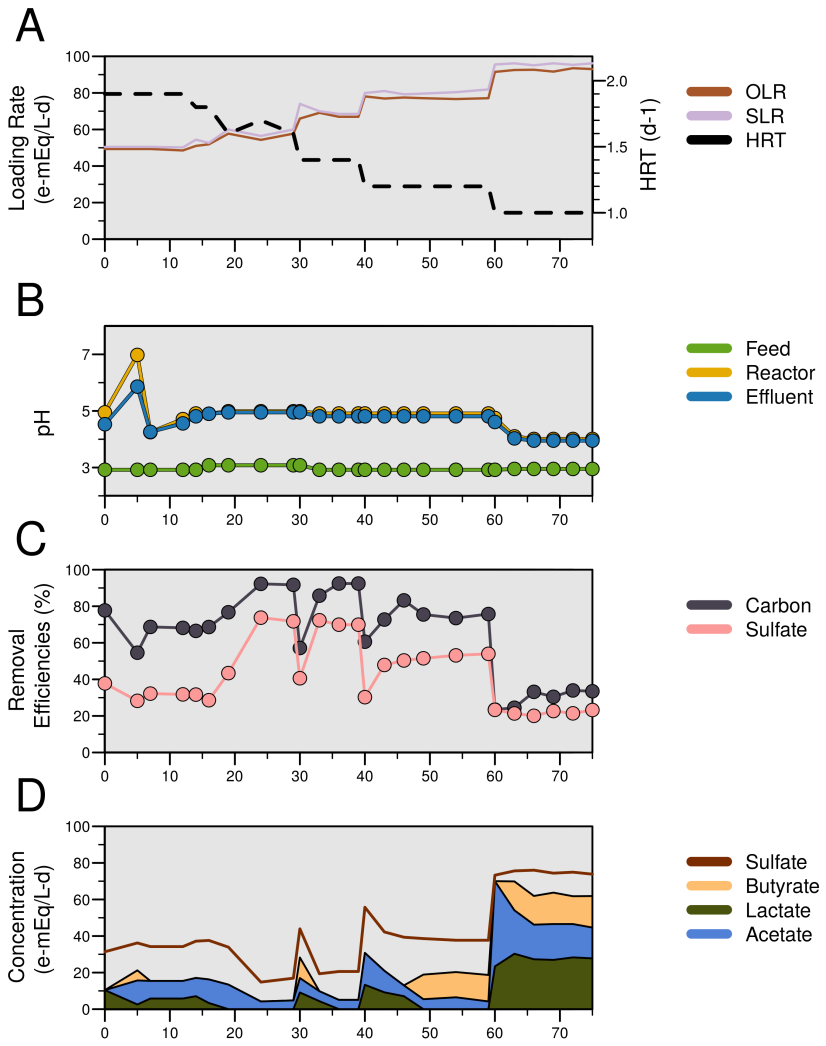


Figure 5.1. Reactor performance at different hydraulic retention time (HRT). A) Organic (OLR) and sulfur (SLR) loading rate and hydraulic retention time (HRT). B) pH in the feed, reactor and effluent. C) Percentage of removal efficiency of carbon and sulfate. D) Volatile fatty acids and sulfate profile in the effluent.

Figure 5.1 shows that the sulfate and carbon removal efficiencies were close to 80% when the HRT was between 2.0 and 1.4 d. But when the HRT decreased to 1 day, the performance of the reactor changed abruptly, reducing the efficiency of sulfate and carbon removal below 40% (Figure 5.1C). The analysis of the effluent (Figure 5.1D) reflects this change, during the operation at HRT $>$ 1.4 d, acetate was the main byproduct in the effluent; however, when the HRT was reduced to 1.2 d, almost 30% of the incoming lactate remained in the effluent, the sulfate removal efficiency also decreased to 20%, suggesting a serious loss in the sulfidogenic activity.

To test if there was sulfidogenic activity in the microbial community present in the reactor or in the effluent at the end of the experiment, we took a sample from the bulk and another from the effluent at the end of the experiment and evaluated their sulfidogenic activity at pH 6 and 3.

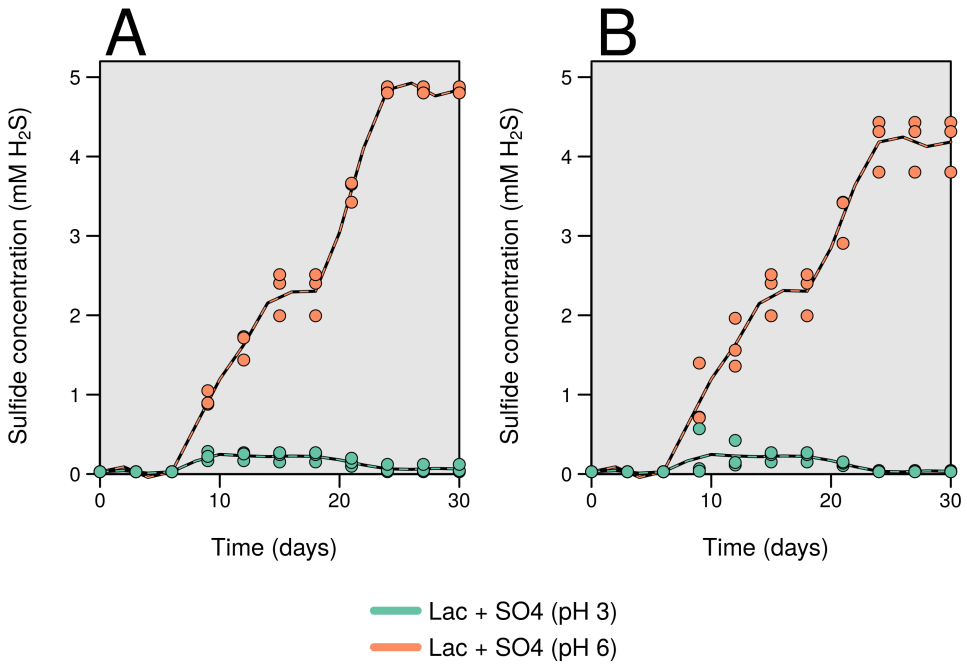


Figure 5.2. Sulfidogenic activity at different initial pH values observed in the samples taken from the bulk (A) and the effluent (B) of the DFBR at the end of the experiments.

The results of these analyses indicated that SRP were present in both the reactor (Figure 5.2A) and the effluent (Figure 5.2B); but they were only active at pH 6 condi-

tions, whereas at pH 3 they were inactive. Additionally, we did not find any statistical difference in the rate of sulfate reduction between the samples from the reactor and the effluent ($\approx$ 3.3 mmol $H_2S$/d·L) at initial pH = 6. These results indicate that SRP remained in the reactor until the very end of the experiment; yet, for some reason the sulfidogenic activity in the reactor dropped in the late phase of operation (Figure 5.1C and D) of the DFBR.

To further analyze the possible lost of functions in the community during the operation of the reactor, we used samples from the inoculation events, the beginning and the end of the reactor operation to evaluate the microbial composition and the predicted functional profile using the 16S rRNA gene marker sequences in combination with the Tax4Fun2 tool (Wemheuer et al., 2018). The general results of these analyses are presented in Figure 5.3 and a more detailed analysis can be seen in Figure 5.4.

The redundancy analysis of the microbial composition of the samples indicated that $\approx$40% of the variance in the composition could be explained by the time the samples were taken (Figure 5.3A). In general, the inoculum samples (Time 0) had higher abundance of sequences related to the Anaerolineae and Ignivibacteria clases; whilst sequences associated with the $\beta$- and $\epsilon$- proteobacteria classes were more associated with samples at the start of the continuous operation of the reactor (Time 1). Finally, sequences belonging to the $\alpha$-proteobacteria were more abundant at the end of the experiment (Time 2) (Figure 5.3A and Figure 5.4A).

The community showed differences between sampling times during the operation of the reactor and the average dissimilarity (measured as Jaccard' Index of dissimilarity) among the samples was 0.42. The data indicates that the changes in the composition of the community during the operation of the reactor were sequential: the composition of samples from T0 is more similar to the composition of the samples from T1 (average Jaccard' Index distance = 0.55) than to the samples from T2 (average Jaccard' Index distance = 0.70). Moreover, the change in the community was more drastic from T0 to T1 than the change from T1 to T2 (average Jaccard' Index distance = 0.47) (Figure 5.3B)

The turn over in the composition of the community, did not have a high impact in the predicted functional profiles of the samples, the average Jaccard' Index distance was $\approx$0.20 among all the samples. Contrarily to the results observed for the

community composition, the major change in the functional profiles occurred during the operation of the reactor from T1 to T2 (average Jaccard' Index distance = 0.20) (Figure 5.3D). Unlike the results from the microbial composition, the redundancy analysis of the functional profiles indicated that the majority (≈65%) of the variance in the predicted functions is correlated with the time of the operation. The highest scores in the ordination corresponded to the two-component system, ABC transporters, buntanoate metabolism, bacterial chemotaxis and biofilm formation (*P. aeruginosa*) KEGG' functions (Figure 5.3C).

Altogether, the results from 16S rRNA gene marker and the predicted functional profiles pointed out that even there was a change in the composition of the community during the operation of the reactor, the high level of functional redundancy of the microorganisms could potentially alleviate the species turnover. For example, the sulfur metabolism pathway was present in all samples (Figure 5.4B).

Since the community composition (Figure 5.3A) and function (Figure 5.3B and Figure 5.2) appeared to remain constant through the reactor operation, the reason for the sulfidogenic activity loss in the reactor remained unanswered. A plausible explanation for this could be overloading (i.e. inhibition by substrate), however, since the maximum concentration of lactate faced by the reactor was $\sim$7.7 mM (Table 5.1) and preliminary analysis (batch) were performed at concentrations as high as $\sim$ 15.0 mM of lactate (Figure 4.1) we ruled out this possibility. Another possible explanation is that the microorganisms in the reactor were unable to cope with the rate at which acid entered the system. In order to analyze this possibility, we calculated a simple mass balance in terms of $H^+$.

Briefly, we estimated the concentration of $H^+$ that had to be neutralized in order to keep the pH in the reactor at 4.8 (optimal condition observed in the reactor) in one day ($\Delta[H^+]$). To calculate this, we used the following balance:

$$\Delta[H^+] = [H^+]_{Reactor} + [H^+]_{Inffluent} - [H^+]_{Effluent} - [H^+]_{Neutralized} \qquad (5.1)$$

$[H^+]_{Reactor}$ was fixed as a constant (1.38 $e^{-02}$ mM $H^+$) and the $[H^+]_{Neutralized}$ was calculated using the data of pH change over time in preliminary analysis (Figure 4.2M) resulting in 0.2 mM $H^+$/gVSS·d.

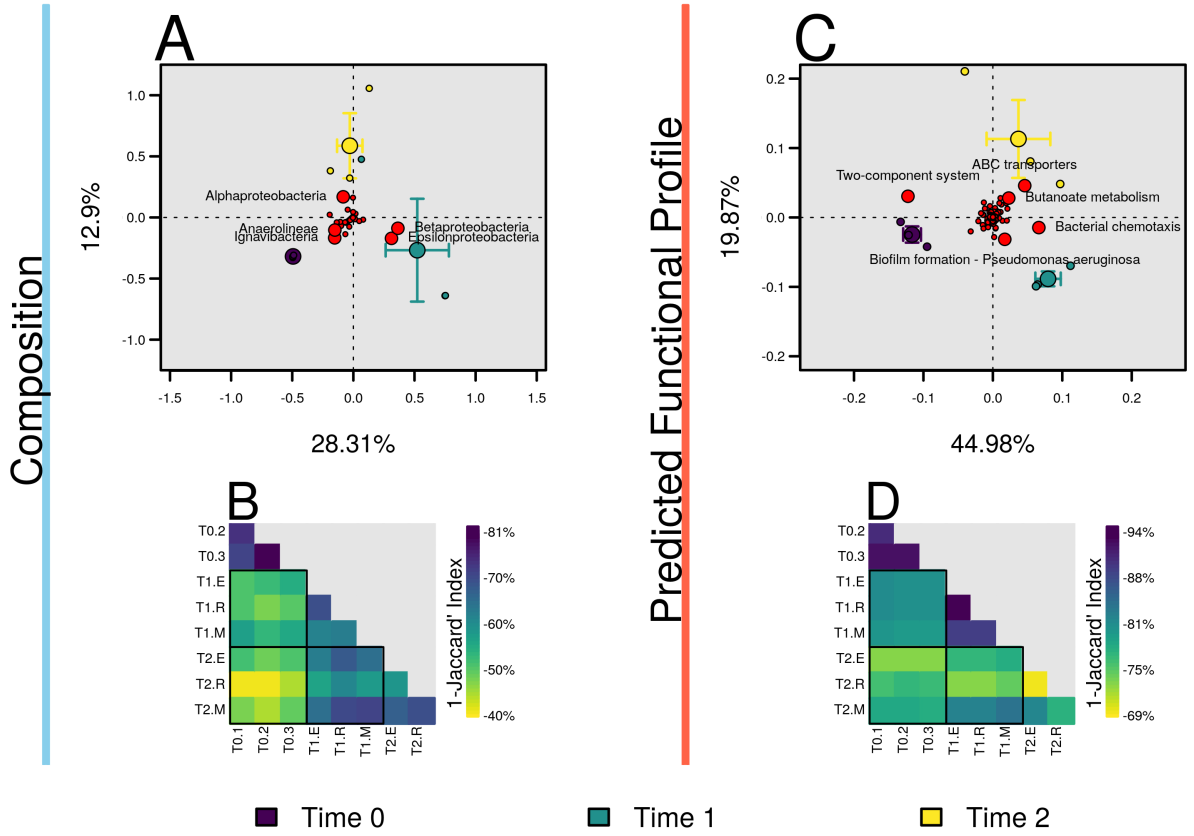


Figure 5.3. Observed microbial diversity and predicted functional diversity of samples taken from the DFBR at different times. Redundancy analysis (RDA) of the microbial classes observed (A) and predicted pathways (C) from the Kyoto Encyclopedia of Genes and Genomes (KEGG) in the samples. The highest 5 scores of classes and pathways are highlighted with bigger circles and their corresponding labels. Similarity of microbial composition (B) and predicted pathways (D) among the samples. Samples labeled as T0 correspond to the three inoculation events during the reactor start-up. T1 correspond to samples taken at the end of the start-up process. Samples labeled as T3 were taken at the end of the experiment. The letters R (reactor), M (carrier material) and E (effluent) correspond to different sampling points in the reactor.

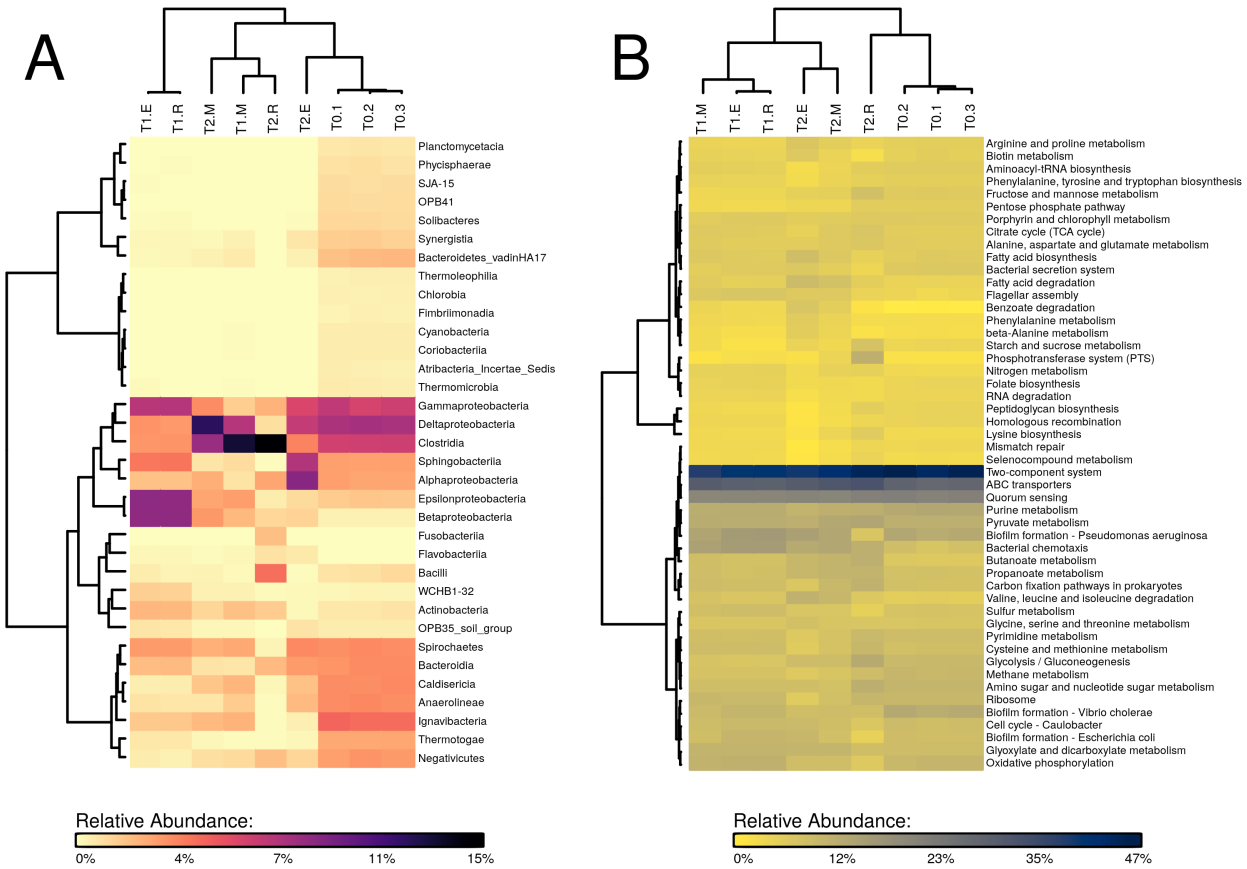


Figure 5.4. Changes in the community composition and relative abundance of predicted pathways observed in samples at different times. A) Relative abundance of the more represented classes (at least 0.1% in total) in the samples. B) Relative abundance of the 50 most abundant predicted pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG). T0 samples correspond to the three inoculation events during the reactor start-up. T1 correspond to samples taken at the end of the start-up process. Samples labeled as T2 were taken at the end of the experiment. The letters R (reactor), M (carrier material) and E (effluent) correspond to different sampling points in the reactor.

According to our balance, the pH in the reactor is a function of the HRT, since it determines the rate at which $H^+$ enters and exits the system (through $[H^+]_{Inffluent}$ and $[H^+]_{Effluent}$); and the biomass present in the reactor, since the rate of $H^+$ neutralization is a function of the grams of volatile suspended solids (VSS). Using this information we calculated the minimum HRT at which the reactor is expected to be at different pH values as function of different concentrations of biomass (Figure 5.5).

The response for the theoretical pH in the reactor indicates that the HRT is the most important factor to prevent the acidification of the reactor. According to this model, the minimum HRT that could support the stability of the reactor at pH = 4.8 was 1.52 d for the biomass we inoculated (4 gVSS) (Figure 5.5). Therefore, when we lowered the HRT below 1.5 days we inadvertently crossed a kinetic barrier.

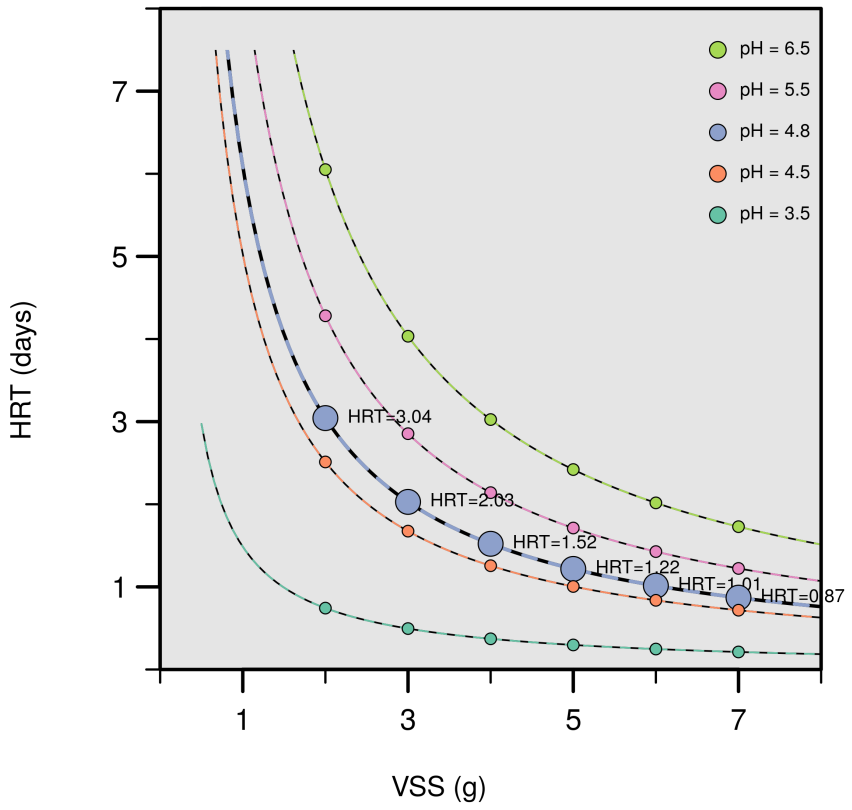


Figure 5.5. Estimation of biomass and hydraulic retention time in order to control the pH within the reactor bulk at selected values.

## 5.4 Discussion

In this study we evaluated the performance of a sulfate reducing community in a DFBR fed with acidic synthetic waste water at increasing stressful conditions driven by a decrease in the HRT. One of the main advantages of DFBR is that the recirculation flow dilutes the influent high concentrations of acid and generates a less harsh environment for SRM (Gopi Kiran, Pakshirajan, and Das, 2017). An additional perk of the recirculation flow is that it neutralizes the effect that the HRT may have in the shear forces and hydrodynamics of the reactor. Because the feed flow is hundreds of times lower than the recirculation flow, it means that HRT only controls the organic loading rate and the velocity of the recirculation flow is in charge of the hydrodynamics of the reactor.

We observed a maximum of sulfate and carbon removal efficiency of $\approx$ 90% for both substrates during days 20-40 (Figure 5.1) similar results were found in the studies mentioned before. As in many previous studies acetate was the main byproduct in the effluent, but in our case it represented less than 10% of the electron donor fed in comparison to 56% or 18$\pm$ observed in previous studies in fluidized bed reactors fed with ethanol (Gallegos-Garcia et al., 2009; Sahinkaya et al., 2007). Furthermore, it is important to consider that both studies used ethanol as carbon source, reducing the potential toxicity of the environment using a non-polar substrate (Liamleam and Annachhatre, 2007).

The results of pH neutralization (Figure 5.1B and Table 5.1) are comparable to previous reports. Sahinkaya et al. (Sahinkaya et al. (2007)), observed an increase in pH from 4.0 in the feed to 8.0 at HRT of 24 h. Kaksonen et al. (Kaksonen, Franzmann, and Puhakka (2003)), observed an increase form 2.5 to 7.5 of pH at HRT of 16 h. Although we could have let the pH in the reactor drift until it approximated neutral values (as it did in the batch previous experiments -Figure 4.1-) we wanted to investigate the performance of the SRCo at moderately acidic conditions, so we let the pH to remain at 4.8.

The analysis of the microbial composition in the reactor indicated that the community changed during the experiments (Figure 5.3B), but this change did not affect the predicted functionality (Figure 5.3D). It appeared that the reactor kept a core community composed mainly of microorganisms belonging to the *Clostridia* and $\alpha$- $\delta$-, and $\gamma$-

proteobacteria classes which were relatively abundant in all samples (Figure 5.4A), it is important to remark that SRP are represented within the Deltaproteobacteria and Clostridia classes (Müller et al., 2015).

Previous reports of microbial community dynamics showed that the composition and diversity of the community is trivial if there is a high level of redundancy within the community (Fernández et al., 1999; Wertz et al., 2007). However, general ecology theory hypothesizes that changes in the community composition could lead, eventually, to a change in the community function (Allison and Martiny, 2008). In that case, the species turnover observed from T1 to T2 (Figure 5.3A and B) would explain the loss of the sulfate reducing activity at the end of the experiment (Figure 5.1).

It is difficult to attribute the activity loss of the reactor at the end of the experiment to a single reason. Nevertheless, the fact that active sulfidogenic biomass remained in the reactor (Figure 5.2), and that 16S rRNA gene sequences were retrieved from the reactor and the carrier material (Figure 5.4) make difficult to impute the change of the function to a change in the microbial composition; at least completely. A more plausible cause for the failure of the system probably was $H^+$ 'overloading', which caused the reactor to shift from sulfate reduction to fermentation (Figure 5.5). This behavior was previously observed with the inoculum during the preliminary characterization in batch experiments. At the start of the experiments at pH 4, butyrate was produced by fermentation and sulfate reduction was neglected. However, once the pH reached an optimal level (above 4.80) sulfate reduction became again the main electron sink (Section 4.3). In the case of the reactor, the pH never could stabilize above 4.80 because it appears that $H^+$ ions kept entering the system in a higher rate that they were neutralized (Figure 5.5).

The proposed model for $H^+$ neutralization points out the relevance of microbial growth in the system. The increase of active biomass in the reactor from 2.0 to 3.0 gVSS would hypotetically allow the decrease of the HRT from 3.04 to 1.52 days (Figure 5.5). According to thermodynamics (Kleerebezem and Van Loosdrecht, 2010), the theoretical biomass yield expected for incomplete SRP in the system at the experimental conditions (10 mmol of lactate, 15mmol of sulfate, at 25°C, pH 4.86, and 0.05 mmol of ammonia as nitrogen source) per mol of lactate are determined by the next equation:

$$\begin{aligned} \mathrm{C_3H_5O_3^-} + 0.479\,\mathrm{SO_4^{2-}} + 0.021\,\mathrm{NH_4^+} \longrightarrow \\ 0.964\,\mathrm{C_2H_3O_2^-} + 0.964\,\mathrm{HCO_3^-} + 0.479\,\mathrm{HS^-} + 0.471\,\mathrm{H^+} \\ + 0.107\,\mathrm{CH_{1.8}O_{0.5}N_{0.2}} + 0.043\,\mathrm{H_2O} \end{aligned} \tag{5.2}$$

As for incomplete SRP, the equation (same conditions but with 10 mmol of acetate) becomes:

$$\begin{aligned} \mathrm{C_2H_3O_2^-} + 0.479\,\mathrm{SO_4^{2-}} + 0.015\,\mathrm{NH_4^+} + 0.502\,\mathrm{H^+} \longrightarrow \\ 0.946\,\mathrm{HCO_3^-} + 0.479\,\mathrm{HS^-} + 0.076\,\mathrm{CH_{1.8}O_{0.5}N_{0.2}} + 0.992\,\mathrm{H_2O} \end{aligned} \tag{5.3}$$

As can be seen, the biomass yield ($CH_{1.8}O_{0.5}N_{0.2}$) is around 0.1 mol of biomass per mol of carbon source. Indicating extremely harsh conditions for SRP, specially for the acetate-depending sulfate reducers. If we add this energy-limited scenario to the stress imposed by the acid and flow rate, it becomes evident why there are only a handful of previous reports of sulfidogenic reactors at acidic conditions.

## 5.5 Conclusions

The previously acclimated SRCo demonstrated to be appropriate for sulfate reduction in a down-fluidized bed reactor fed with acidic wastewater (pH = 3.02) in continuous conditions. Sequencing of the 16S rRNA gene and predicted function profiling indicated that the community changed its composition during the operation of the reactor, but the predicted function remained constant at least in 80%. Results indicates that the gradual decrease in the hydraulic retention time from 2 to 1 day collapsed the system. However, the reactor showed sulfate and lactate removal efficiencies comparable with those in the literature at HRT $>$ 1.6 days. The possible cause for the reactor collapse was the increase of $H^+$ beyond the capacity of neutralization exhibited for the SRCo.

This study makes an emphasis in the acidic stress that is faced by SRCo when

they are used to treat acidic wastewater. In a real life application, the acidic stress would coincide with high metal concentration, which would increase the level of stress upon the community. The successful development of sulfidogenic technologies for acid mine drainage treatment depends on the understanding of the microbial requirements to cope with such stressful conditions.

# 6. General discussion, conclusions and final remarks

## 6.1 General discussion

Successful development and application of sulfidogenic technologies for the treatment of acid mine drainage (AMD) requires the use of a sulfate reducing community able to fulfill certain particularities:

- Resistant to acidic pH
- High efficiency in electron donor consumption
- High rates of sulfate reduction

In this work, we wanted to analyze the role that the community composition and its structure plays in the efficiency of substrate consumption and in the rates of sulfate reduction across a gradient of acidic stress during the degradation of lactate. We hypothesized that the changes in the structure or the composition of the community would inherently change some of its performance traits. The results showed that the sulfate reducing and electron donor efficiencies and rates were constant across the pH gradient, regardless of the community structure or composition, up to a certain limit in both batch (Chapter 4) and continuous (Chapter 5) experiments.

Results showed that the sulfate reducing bacteria guild composition changed during the acclimation of the SRCo to acidic pH (Figure 4.8) indicating different acid resistance capabilities and different niche occupation at species level across the pH gradient. However if we look at the results in a broader phylogenetic scale (Figure 4.7), it is clear that these changes are obscured at the family level and the main

changes in the community occurred in other non-sulfate reducing families. The same observation was made in the DFRB, the main changes in the community composition occurred in non-sulfate reducing classes (Figure 5.3). This evidence supports the explanation that some SRP are capable to adapt to extremely harsh conditions until they met a physiological limit where their activity is no longer possible. For example, sequences related to the *Desulfosporosinus acididurans* specie were found in high abundance only at pH 6 and 5, but were not present at pH 4 (Figure 4.8). This would mean that even if we did have a truly acidophilic/acidotolerant SRP in the community, the functioning of the sulfate reducing guild relied in the activity of other non-sulfate reducing microorganisms, especially the fermenters guild.

It is possible to reconstruct, at least theoretically, the flow of energy and matter that was observed in this work with only a few microorganisms belonging to the Deltaproteobacteria and Clostridia classes. In this hypothetical scenario, an iSRP such as *Desulfosporosinus acidiphilus* (Clostridia) (Sánchez-Andrea et al., 2015) or *Desulfovibrio sp. TomC* (Deltaproteobacteria) (Karnachuk et al., 2015) would oxidize lactate into acetate. Lactate and acetate could be used by *Eubacterium hallu* (Clostridia) to produce butyrate and $H_2$ (Duncan, Louis, and Flint, 2004). Butyrate and $H_2$ produced by fermentation could be partially oxidized to acetate by a large variety of sulfate reducers and syntrophic bacteria (Deltaproteobacteria) (Liamleam and Annachhatre, 2007). Finally, residual acetate could be mineralized by *Desulfotomaculum spp.* (Clostridia) (Rabus, Hansen, and Widdel, 2013) or *Desulfobacter spp.* (Deltaproteobacteria) (Montoya et al., 2012) or used to produce butyrate again. However, the conditions at which the activities of the aforementioned microorganisms were observed are very different among each other, and have never been observed in a single system before.

We were never able to correlate the diversity (richness, diversity, or evenness) to any of the functional traits observed in the SRCo. In general, we observed a similar diversity across the different stages of the study. Yet, the PCR-DGGE analysis during the acclimation experiments (Chapter 4) made a case for a relationship between the community composition and some of its functional traits, such as electron donor removal rates (Table 4.1). These results in addition with the observations made in the predicted functional profiles (Chapter 5) also supports the hypothesis that the efficiency of sulfate reduction at acidic conditions is an emergent property of the system and it depends on the composition and the structure of the commu-

nity (Konopka, 2009; Zarraonaindia, Smith, and Gilbert, 2013), in consequence it requires a broader set of microorganisms besides the SRP to be achieved (Kimura, Hallberg, and Johnson, 2006; Rowe et al., 2007).

We must remark that our observations are limited by the molecular techniques used and the lack of replicates in the case of performance of the community in the reactor (Section 5). The limitations of the use of 16S rRNA gene for microbial ecology have been extensively reviewed and discussed (Poretsky et al., 2014). Despite these limitations, the use 16S gene amplicons as in fingerprinting or sequencing techniques remains as the basic tool to study microbial communities in a cost-effective way. Recent studies have proven that even old fashion techniques such as TRFLPs and DGGE gels still remain useful at some extent to analyze the composition of a community (De Vrieze et al., 2018) if proper considerations are taken into account.

Regardless of the success of the SRCo to face acidic conditions, we found a limit where all microbial activity stopped (pH = 3 for the acclimation experiments Chapter 4) or decayed (hydraulic retention time $<$ 1.5 d in the DFBR experiments -Chapter 5-). These limits highlighted the natural existence of energetic and physiological barriers beyond sulfate reducing activity is null. The energetic barrier is easy to point out. It is possible to calculate the general reaction for the oxidation of lactate in a two step procces where acetate is produced first (Kleerebezem and Van Loosdrecht (2010)) :

$$\begin{aligned} &\mathrm{C_3H_5O_3}^- + 1.382\,\mathrm{SO_4}^{2-} + 0.049\,\mathrm{NH_4}^+ + 0.471\,\mathrm{H}^+ \longrightarrow \\ &2.774\,\mathrm{HCO_3}^- + 1.382\,\mathrm{HS}^- + 0.226\,\mathrm{CH_{1.8}O_{0.5}N_{0.2}} + 0.0885\,\mathrm{H_2O} \end{aligned} \tag{6.1}$$

This reaction assumes that energy of maintenance is equal to 4.5 kJ/mol·h, efficiency of electron transfer is -3 $e^-$mol/h per mol of substrate (Kleerebezem and Van Loosdrecht, 2010). Thus the $\Delta$ G'$^\circ$of the incomplete SRP using lactate as C-source and electron donor is -475.78 kJ/mol. The $\Delta$ G'$^\circ$of the reaction is -500.37 kJ/mol. Reaction 6.1 shows that thermodynamically, at best 6.7% of the carbon present in lactate will be used for growth and most of the energy will be used in the catabolism. However, it is important to consider that the growth efficiency is linked to the nature and concentration of the electron donor; thus under limiting resource

concentration, like the conditions offered by AMD, growth efficiency will be smaller (Roller and Schmidt, 2015). It is also worth to mention that stress can reduce the biomass production diverting electrons from synthesis to maintenance energy (Pirt, 1982).

Besides the stress caused by acidity, we need to remember that AMD also has a high concentration of metals at lethal concentrations (Nordstrom, Blowes, and Ptacek, 2015), but in this study we did not addressed the effect that high metal concentration could have in the community used. The source of the inoculum was in contact with acidic water with 31 mS/cm of conductivity and pH of 3.59. The pore water of the sediment contained 25.25 mg/L of dissolved metals, mainly iron (9.57 mg/L) (Appendix A, Moreno-Perlin et al., 2019), so microorganisms were used to the presence of metals at concentrations used in synthetic AMD with low metal concentration (Kaksonen et al., 2004). Moreover, it has been observed that induced resistance to acid results in cross protection against other stress factors in some bacteria (Leyer and Johnson, 1993). Nevertheless is hard to guess what would be the effect of metal stress in the consortia.

Environmental engineering has been looking for answers in microbial ecology to improve their biotechnological processes for a long time now (McMahon, Martin, and Hugenholtz, 2007; Rittmann et al., 2006), but there has been a very limited success. In the case of AMD microbial ecology, many studies have been focusing on composition at low taxonomic levels trying to explain the differences in alpha diversity (Méndez-García et al., 2015), which is the common tendency of the majority of microbial ecology studies (Hugerth and Andersson, 2017).

However, a trait-based approach (Krause et al., 2014) focusing in the abundance and activity of functional groups of interest, instead of their taxonomic lineage (Louca et al., 2018), could be used as a more suitable framework to generate systematic principles to improve our knowledge of biotechnological processes. In these scenario, engeneering could benefit from the analysis of energy and matter flux between different functional groups and this knowledge could be used at the community level (Röling, Ferrer, and Golyshin, 2010).

## 6.2 Conclusions and final remarks

In this work we investigated the effect that acidic stress has in the structure and performance of a sulfate reducing community. We found a high redundancy of functions in the community that prevent the loss of most of the functional traits of the community, such as the rates and efficiencies of sulfate and electron donor removal; even when the composition and the structure of the community changed. Despite this functional stability, the community exhibited a breaking point of its capacity to endure acidic stress, after which the microbial activity halted in both continuous and batch experiments. This study makes a case for taking in consideration the inherent biological limitations of the microorganisms for properly engineered biological systems.

It is important to remark that the path to develop an acid resistant sulfate reducing community from an environmental sample was culture dependent and was time intensive: the acclimation took more than 300 days.

In the future, molecular analyses may be able to quickly evaluate and predict the potential of any inoculum or even design a microbial community *ad hoc* to fulfill a determined purpose. Yet, firstly we must establish the mechanistical basis for microbial community functioning and the physiological limits of the microorganisms within the communities. To do so, an experimental approach to microbial ecology with a strong orientation on ecosystem functioning would be more profitable for environmental engineering than the descriptive ecology approach commonly used in biotechnological studies.

# A. Appendix A

Table A.1. Elemental composition of the pore water from the sediment used in this study.

| Element | ppm | mM |
|---|---|---|
| Ca | 175.3 | 4.373 |
| Na | 40.4 | 1.758 |
| Mg | 39.0 | 1.604 |
| K | 47.1 | 1.205 |
| Si | 5.0 | 0.177 |
| Fe | 9.6 | 0.172 |
| Sr | 6.3 | 0.072 |
| Mn | 3.4 | 0.062 |
| Li | 0.4 | 0.057 |
| B | 0.6 | 0.055 |
| As | 3.8 | 0.050 |
| Zn | 0.7 | 0.011 |
| Al | 0.2 | 0.008 |
| P | 0.1 | 0.004 |
| Mo | 0.1 | 0.001 |
| Cr | 0.0 | 0.001 |

* Cu, Ni, V, Co, Cd, Ba, Be, Pb, Sb, Se, and Sn were not detected.

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