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Enrichment and application of a high-performance sulfate-reducing microbial community to treat acidic streams

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Staring at the blank page before you
Open up the dirty window
Let the sun illuminate the words that you cannot find
Reaching for something in the distance
So close you can almost taste it
Release your inhibitions
Feel the rain on your skin
No one else can feel it for you
Only you can let it in
No one else, no one else
Can speak the words on your lips
Drench yourself in words unspoken
Live your life with arms wide open
Today is where your book begins
The rest is still unwritten…..

Overview of this thesis

Chapter 1. General introduction	**18**
Chapter 2. In search of sulfate-reducing consortia able to degrade acetate under acidic conditions	**42**
Chapter 3. Acetotrophic sulfate-reducing consortia develop active biofilms on zeolite and glass beads in batch cultures at initial pH 3	**69**
Chapter 4. Continuous bioreactor performing acetotrophic sulfate reduction at pH 3.25 of extremely acidic stream (pH 1.7)	**103**
Chapter 5. General discussion, conclusions, and perspectives	**133**
References	**152**
Published papers	**171**

Table of figures

Chapter 1.

Figure 1.1 General description of AMD formation: occurs when sulfide minerals, typically pyrite (iron sulfide FeS_2), are exposed to oxygen and water; and acid mine formation reaction. Other minerals that could be present are: FeAsS, $CuFeS_2$ or PbS. Figure modified from Zhang et al. 2023., and created in Biorender.com …… **23**

Figure 1.2. Equilibrium species Eh-pH Diagram for Sulfur and Alkaline Sulfide Gold (Anderson et al., 2005)…… **25**

Figure 1.3. Total concentration of sulfide species in function of pH (Costa et al, 2016)…… **26**

Chapter 2.

Figure 2.1. Diagram of the experimental strategy followed to obtain the seven cultivable and reproducible consortia at acidic conditions (initial pH 3 or 4)…… **47**

Figure 2.2 Kinetic profiles of sulfide (●) and acetate (■) of the seven consortia, Consortium 1 (C1) to Consortium 7 (C7), from successive transfer one (T1) to successive transfer five (T5). C1 - C3 substrate lactate, initial pH 4; C4 - C6 substrate lactate, initial pH 3; C7 substrate glycerol, initial pH 3…… **52**

Figure 2.3. Kinetic profiles of sulfate (◆) and lactate (▲) of the seven consortia. Consortium 1 (C1) to Consortium 7 (C7), during successive transfer one (T1) to successive transfer five (T5). C1-C3: lactate (substrate), initial pH 4; C4-C6 lactate (substrate), initial pH 3; C7 glycerol (substrate), initial pH 3…… **53**

Figure 2.4. Kinetic profiles of pH (▼) of the seven consortia. Consortium 1 to Consortium 7 (C1-C7), during the successive transfer one (T1) to successive transfer five (T5). C1- C3: lactate (substrate), initial pH 4; C4 - C6 lactate (substrate), initial pH 3; C7 glycerol (substrate), initial pH 3…… **54**

Figure 2.5. Profiles of sulfide (●); sulfate (◆); lactate (▲) and acetate (■); optical density at 600 nm (**O**); and pH (▼) in the triplicate assays of the seven consortia (C1 - C7) after successive transfer 5. **58**

Figure 2.6. Sulfide production rates obtained at different initial pH values for each cultivable consortium…… **59**

Figure 2.7. Dendrogram based on relative abundances of the 21 OTUs, at the genus level, obtained from the seven consortia (C1-C7)…… **61**

Figure 2.8. Rarefaction curves of the seven consortia…… **63**

Figure 2.9. Principal component analysis (PCA) of the microbial communities of the seven consortia. C.1=Consortium 1, C.2=Consortium 2, C.3=Consortium 3, C.4=Consortium 4, C.5=Consortium 5, C.6=Consortium 6, and C.7= Consortium 7…… **64**

Chapter 3

Figure 3.1. Experimental set-up showing the sampling days. Arrows refer to samples for chemical analysis, and asterisks indicate that DNA was extracted from the biofilm and liquid, and stored at -20ºC…… **76**

Figure 3.2. Kinetic profiles of sulfate consumption, sulfide production, and pH in the experiments with A) Zeolite (ZEO); B) Glass beads (GB); and E) Biological control (BIO) without carrier material. Profiles of sulfate and pH in the abiotic experiments with C) Zeolite (ZEO/C) and D) Glass beads (GB/C)…… **80**

Figure 3.3. Kinetic profiles of the experiments with granular activated carbon. A) Glycerol consumption and intermediates production. B) Consumption of sulfate, production of sulfide and pH.

C) Concentration of glycerol and intermediates in the abiotic control. D) Concentration of sulfate and pH in the abiotic control…………………………………………………………………………………….. 81
Figure 3.4. Consumption of glycerol and formation of intermediaries with the different carrier materials and abiotic controls: A) Zeolite (ZEO); B) Glass beads (GB); C) Abiotic control of zeolite (ZEO/C), D) Abiotic control of glass beads (GB/C), and E) Biological control (BIO) without carrier material………..... 82
Figure 3.5. Scanning electron microscopy (SEM) images of zeolite (ZEO) and glass beads (GB). The arrows show the microorganisms attached to the surface of glass beads and zeolite…………… 85
Figure 3.6. A) Zeolite before the experiment. B) Zeolite after 25 days of experiment. C) Detail of zeolite at day 25. D)Glass beads before the experiment. E) Glass beads after 30 days of experiment. F) Detail of glass beads at day 30. Nikon D40X…………………………………………… 87
Figure 3.7. Heatmap of the microbial community at the family level in the experiment with glass beads. SOL denotes the biofilm community developed over the carrier material and LIQ the planktonic community, in the early (day 10), and late (day 30) stages. The community of the inoculum is also shown. The number after the period indicates the number of the replica………………………………… 89
Figure 3.8. Heatmap of the microbial community at the family level in the zeolite experiments. Samples from the biofilm are identified as SOL and those from the liquid phase as LIQ, at the early, (day 7), and late (day 25) stages of the experiments, in comparison with the inoculum. The number after the period indicates the number of the replica…………………………………………………. 90
Figure 3.9. Heatmap of the sulfur cycle microbial community at genus level with zeolite (ZEO, top panel A) and glass beads (GB, bottom panel B) in the different matrices liquid (LIQ) or solid (SOL) in the early stage, day 7 or 10, and in the late-stage, day 25 or 30. The community of the inoculum is also shown. The number after the period indicates the number of the replica…………………………… 91
Figure 3.10. A.- Non-metric multidimensional scaling (NMDS) plot and B.- Redundancy analysis (RDA) of the microbial communities obtained with zeolite (ZEO) or glass beads (GB) in the different matrices liquid (LIQ) or biofilm (SOL), sampled at 7, 10, 25, or 30 days………………………………… 93

Chapter 4

Figure 4.1. Schematic diagram of the anaerobic bioreactor. The arrows represent the direction of the gas flow. Image created in BioRender.com……………………………………... 110
Figure 4.2 Profiles of the reactor during set-up (Period I) and continuous operation (Periods II-IX). A) Outlet of byproducts as result of glycerol oxidation. B) Glycerol concentration in the inlet and outlet. C) Consumption efficiency (%) of glycerol and sulfate, D) Hydraulic retention time (HRT) E) pH values in the reactor and in the feed media (inlet). F) Proton rate in the fed media (inlet) and in the exit of the continuous reactor………………………………………………………………… 118
Figure 4.3. Loading and consumption rates A) Glycerol and sulfate average loading and consumption rates; B) and C) Box plots showing the sulfate and glycerol loading and consumption rates in each of the operational periods………………………………………………………………….. 119
Figure 4.4 A) Diversity indexes of planktonic and biofilm communities developed during continuous operation of the reactor from Period II (pH = 5) to Period VIII (pH = 3.25). And B) rarefaction curves of the planktonic and biofilm communities from amplicon sequence variants (ASVs), which provide a more precise resolution of microbial diversity by denoising raw amplicon data, resulting in distinct variants that capture finer taxonomic differences……………………………………………………………………………… 121
Figure 4.5 A) Diversity indexes of the planktonic (liquid) and biofilm (solid) communities during all the continuous operation. B) Diversity indexes at pH level…………………………………….. 123
Figure 4.6. A) Statistical analysis achieved by Multivariate Homogeneity of Groups Dispersions (Variances) from B) the community samples evaluated at the different pH values, and retrieved from the biofilm (solid) or planktonic (liquid) communities………… 125
Figure 4.7. 16S rRNA based community composition at the family level and relative abundances at the different periods when decreasing the pH from 5 (Period II) to 3.25 (Period VIII)……………… 126

Figure 4.8. a) Relative abundances of the SRM found in each sample at different pH values in the solid/biofilm and liquid/planktonic phases. b) relative abundances of each of the SRM at different pH values in the liquid and solid phase…………………………………………………………………. **131**

Chapter 5

Figure 5.1 General diagram of the development of this thesis………………………………… **135**

List of tables

Chapter 1.

Table 1.1 A summary of documented species belonging to moderately acidophilic sulfate-reducing microorganisms. Describing pH optimum, temperature range, source of inoculum and the consumption of acetate………………………………………………………………………………………….. **36**

Chapter 2.

Table 2.1. Initial pH, electron donor, and type of inoculum used to obtain the consortia…………………………………………………………………………………………… **23**

Table 2.2. Rates of acetate consumption (A) and sulfide production (B) of the seven cultivable consortia obtained in each one of the five successive transfers (T1 to T5)……………………………… **53**

Table 2.3. Rates of sulfide production and acetate consumption, percentage of substrate used to perform sulfate-reducing activity, and interval of favorable pH of the seven cultivable consortia……... **57**

Table 2.4. Diversity indices of the seven consortia. S) Richness; H) Shannon-Wiener index; D) Simpson index of dominance; and E) Evenness………………………………………………………….. **59**

Chapter 3

Table 3.1. Main characteristics of the support materials……………………………………… **71**

Table 3.2. Sulfide yield, percentage of substrate used for sulfate-reduction, sulfide production rate, and acetate consumption rate with the different carrier materials and in the biological control without carrier material……………………………...……………………………………… **81**

Table 3.3. Diversity indexes of the consortia developed on zeolite and glass beads. Richness (S), Shannon-Wiener index (H), Simpson index of dominance (D), and Evenness (E)……….. **85**

Chapter 4

Table 4.1. Performance of the continuous sulfate-reducing reactor under acidic conditions (controlled pH 5 to 3.25) ………………………………………………………………………………........................ **118**

Table 4.2. Sulfide yield, percentage of substrate used for sulfate-reduction, sulfide production rate, and acetate consumption rate with the different carrier materials and in the biological control without carrier material……………………………...……………………………………… **81**

Table 4.3. Diversity indexes of the consortia developed on zeolite and glass beads. Richness (S), Shannon-Wiener index (H), Simpson index of dominance (D), and Evenness (E)……….. **85**

Nohemi Graciela Campos Quevedo (2024). Enriquecimiento y aplicación de una comunidad sulfato-reductora de alto rendimiento para el tratamiento de corrientes ácidas. Tesis de Doctorado. División de Ciencias Ambientales, IPICYT, México.

Resumen

Las corrientes ácidas, como el drenaje ácido de minas (DAM) tienen un pH bajo (pH < 4) y altas concentraciones de metales y sulfatos. Los microorganismos sulfato-reductores (SR) pueden ayudar a remediar las corrientes ácidas. Un inconveniente de la sulfato-reducción es que algunos SR no oxidan completamente el sustrato a CO_2 y el ácido acético puede permanecer como subproducto, afectando la eficiencia del proceso.
Nuestro objetivo fue operar un reactor sulfidogénico continuo a pH ácido en condiciones sulfato-reductoras. Inicialmente, mediante transferencias sucesivas, variaciones en los donadores de electrones (lactato y glicerol), y niveles de pH (3 ó 4), conseguimos cultivar siete consorcios sulfidogénicos que metabolizaban eficazmente el acetato generado a partir de la oxidación incompleta del sustrato. Además, se evaluaron simultáneamente diversos materiales de soporte en el consorcio utilizando glicerol como donador de electrones a un pH inicial de 3 para facilitar el desarrollo de biopelícula sobre carbón activado granular (CAG), perlas de vidrio, y zeolita. En los tres casos se logró la formación exitosa de biopelículas sulfato-reductoras, aunque sólo las perlas de vidrio y la zeolita mostraron una degradación completa del acetato. Estos resultados sugieren que la zeolita favoreció a microorganismos sulfato-reductores capaces de oxidar completamente el glicerol y acetato en condiciones ácidas iniciales, lo que sirvió de base para la posterior inoculación del reactor continuo. Inoculamos un reactor continuo de biopelícula inmovilizada utilizando zeolita como material de soporte para tratar medios sintéticos extremadamente ácidos (pH 2.5 a 1.7) suplementados con glicerol como sustrato. Se evaluó la eficiencia de sulfato-reducción, los productos y su tolerancia al medio ácido en continuo variando y controlando el pH dentro del reactor de 5.0 – 3.0 en 159 días. El reactor alcanzó una eficiencia de sulfato-reducción en los periodos más ácidos de ~60%, y no se acumuló acetato/ácido acético. El análisis del gen 16S rRNA en muestras de los experimentos en continuo y en lote mostró que los SR alcanzaron una abundancia relativa mayor en las comunidades microbianas del reactor en continuo, comparado con los experimentos en lote. En condiciones continuas, los SR proliferaron hasta tener once taxones en comparación con los cultivos en lote. De éstos, los miembros que pertenecen a *Desulfofarcimen*, *Desulfatirhabdium*, y *Desulfobacter* pueden utilizar acetato para sulfato-reducción a pH ácido tan bajo como 3.0
Este resultado podría deberse a la preferencia de la comunidad microbiana por el pH ácido, resaltando la importancia de realizar un seguimiento de la comunidad microbiana a lo largo de los experimentos continuos y adquirir un mejor control y conocimiento del rendimiento del reactor. Este trabajo nos permitió describir la importancia de buscar comunidades acidófilas reproducibles que puedan oxidar completamente el acetato.

Nohemi Graciela Campos Quevedo (2024). Enrichment and application of a high-performer sulfate-reducing microbial community to treat acid streams. Ph.D. Thesis. Environmental Sciences Division, IPICYT, Mexico.

Abstract

Acidic streams, such as AMD, have low pH (pH < 4) and high metal and sulfate concentrations. SRM can help to remediate acidic streams. One drawback of sulfate reduction is that some SRM does not completely oxidize the substrate to CO_2, and acetic acid may remain as byproduct, affecting the process efficiency. To overcome these limitations, we aimed to operate a sulfidogenic continuous reactor at acidic pH under sulfate-reducing conditions. Initially, through successive transfers, variations in electron donors (lactate and glycerol), and pH levels (3 or 4), we managed to cultivate seven sulfate-reducing consortia that effectively metabolized acetate generated from the incomplete oxidation of the substrate. Furthermore, various carrier materials were tested simultaneously in the consortium using glycerol as the electron donor at a starting pH of 3 to facilitate biofilm development on GAC , glass beads, and zeolite. Sulfate-reducing biofilm formation was successful in the three cases but only glass beads and zeolite exhibited complete acetate degradation. These findings suggest that zeolite favored specific sulfate-reducing microorganisms capable of fully oxidizing glycerol under initial acidic conditions, which was the subsequent inoculation of the continuous reactor. We inoculated a continuous biofilm reactor with immobilized biomass using zeolite as the carrier material for treating extremely acidic synthetic media (pH 2.5 to 1.7) supplemented with glycerol as the electron donor. The sulfate-reduction efficiency, byproducts, and tolerance to continuous acidic media were evaluated, varying and controlling the pH inside the reactor from 5.0 – 3.0 in 159 days. The reactor reached a sulfate-reduction efficiency in the most acidic periods of ~60%, acetate/acetic acid did not accumulate. Analysis by 16S rRNA gene amplicon sequencing of samples from the continuous and batch experiments showed a higher relative abundance of SRM in the microbial communities of the continuous reactor compared with those of the batch experiments. In continuous conditions, sulfate-reducing proliferated to eleven taxa in comparison to batch cultures. From these, members belonging to *Desulfofarcimen*, *Desulfatirhabdium*, and *Desulfobacter* could use acetate for sulfate reduction at acidic pH as low as 3.0.
This result could be due to the microbial community's preference for acidic pH. It highlights the importance of tracking the microbial community throughout the continuous experiments and acquiring better control and knowledge of the reactor performance. This work allowed us to describe the importance of searching for acidophilic reproducible communities that can completely oxidize acetate.

Chapter 1

General introduction

1.1Introduction

Low annual precipitation and high evapotranspiration rates in arid and semi-arid zones result in water scarcity, presenting difficulties for agriculture and human settlements. To address the challenges related to water scarcity and sustainable agriculture in countries with limited water resources, such as Mexico, it is crucial to examine new methodologies. Specifically, by employing microorganisms such as sulfate-reducing communities for efficiently removing pollutants like sulfate, heavy metals, and emerging contaminants (Diao et al., 2023).

However, the use of treated wastewater in agriculture requires careful management to avoid adverse environmental outcomes (Sayyed-Hassan et al., 2020). Moreover, the reclamation and reuse of wastewater in agriculture requires the integration of advanced treatment technologies and proper monitoring (Fito and Van Hulle, 2021). Generally, in arid and semi-arid zones water is rich in minerals, including metals like iron, copper, and zinc, which are not degraded during the treatment but could be recovered and separated from the water using sulfate-reducing processes (Moreira, 2018). Some types of industrial wastewaters (papermill, electroplating, mining, etc.) are a significant environmental and health concern due to the acidity (pH 2.55-6.5), sulfate concentrations, and metal content (Kieu et al., 2011; Liu and Adanur, 2014; Megrelishvili et al., 2020; Zhang et al., 2022). Heavy metals like lead, chromium, arsenic, nickel, cadmium, and mercury have various harmful effects on human health, including nervous system damage, skin allergies, cancer risk, and kidney dysfunction (Peñaloza et al., 2023).

The available methods for wastewater treatment include physical and chemical procedures, in addition to various biological treatment approaches. The drawbacks associated with only using physical and chemical methods include their significant expenses, energy utilization, and the generation of chemical byproducts that could pose environmental risks (Shamaev et al., 2023).

Therefore, biological treatment of acidic wastewater offers several advantages, including efficient treatment, and low operation costs. Using sulfate-reducing microorganisms to recycle wastewater makes it possible to

efficiently purify the wastewater and transform metals into precipitates (Sahinkaya et al., 2015). Implementing sulfate-reducers to treat wastewater could contribute to sustainable agriculture; treated wastewater brings more nutrients to the soil compared to freshwater, such as nitrogen, phosphorus, potassium, and organic matter, acting as natural fertilizers and reducing the need for chemical ones (Rusănescu et al., 2022). Using sulfate-reduction processes offers different advantages like metal precipitation; for example: Ostermeyer et al., (2022) successfully treated industrial wastewater containing various metals such as arsenic, iron, thallium, zinc, nickel, antimony, cobalt, and cadmium, removing all these metals below discharge limits, demonstrating the effectiveness of sulfate-reducing bioreactors in metal precipitation. Also, Kumar and Pakshirajan, (2021) used anaerobic sulfate-reducing biomass to remove heavy metals from a multicomponent system at low pH, achieving 100% removal for Cd^{2+} and Cu^{2+}, 99% for Pb^{2+}, 98% for Zn^{2+}, 88.2% for Ni^{2+}, and 64% for Fe^{2+}.

Sulfate-reducing microorganisms (SRM) are highly specialized and use sulfate as final electron acceptor for energy metabolism; many can also use thiosulfate and sulfite as alternative electron acceptors, and few may use sulfur or nitrate. SRM, known for their diverse nature, are distinguished by their contribution to the biogeochemical processes in their respective habitats (Rabus et al., 2015). In order to optimize the selection of sulfate-reducing microorganisms for enhanced bioremediation, it is imperative to carefully consider the type of microorganisms present in the environment as well as their specific capabilities and characteristics. This understanding will facilitate the selection of microorganisms that best match the specific contaminants targeted, thereby ensuring their effective (Ayangbenro et al., 2018; Li et al., 2018a).

SRM are widely distributed in terrestrial, subterrestrial, and marine ecosystems and are capable of producing sulfide under a wide range of environmental conditions like: mine sediment (Moreno-Perlin et al., 2019; Sánchez-Andrea et al., 2012), bovine dairy manure (Pruden et al, 2006), wastewater sludge (Gallegos-Garcia et al., 2009), volcano mud (Slobodkina et al., 2024), geotermal field (Brito et al., 2014), etc.

Sulfate-reducing microbes play a crucial role in the production of sulfide and the precipitation of metals in harsh environments such as acid mine conditions. Understanding the biogeochemical processes in these environments, including the role of microbial communities, can the development of bioremediation and metal recovery methods. The percentage of organic matter consumed by sulfate reducers can vary depending on the specific conditions and substrates present however, a drawback of this process is the generation of sulfide, not pleasant odor, corrosive and toxic (Utgikar et al., 2002).

1.2 Acidic streams

Acidic streams are aqueous environments characterized by a diminished pH resulting from elevated concentrations of acidic substances (sulfur and nitrogen compounds). The pH spectrum spans from 0 to 14, wherein 7 denotes a state of neutrality. A pH value lower than 7 denotes acidic properties, in contrast, a pH value exceeding 7 signifies alkaline attributes. By definition, Acidic streams commonly exhibit a pH below 7 (Rowe et al., 2007).

Acidic streams, often a result of AMD, can have significant impacts on aquatic ecosystems. The abundance of mineral sulfides in the Earth's crust, such as pyrite (FeS_2), makes them a significant supply of sulfur, but their oxidation by lithotrophic prokaryotes can lead to the formation of AMD and other environmental issues (Byl et al., 2023). Sulfur-oxidizing bacteria play a key role in this process, contributing to the release of sulfur into the atmosphere and the formation of AMD. These bacteria can also have an adverse effect on the ecosystem by producing acidic or toxic byproducts (Rana et al, 2020). In addition, the role of microorganisms in the formation, dissolution, and transformation of secondary minerals in mine rock and AMD, including the mobilization of heavy metals, is crucial for understanding and managing the environmental impact of mining activities (Ortiz-castillo et al., 2021). Furthermore, the deep terrestrial subsurface harbors an active microbial community of sulfate-reducing and sulfide-oxidizing bacteria, mediating a sulfur cycle that can impact the safety of geological repositories (Bell et al., 2020).

Acidic streams are characterized by high conductivity and elevated concentrations of iron (Fe ≥0.5 g/L), aluminum, zinc, and copper (5-7mg/L) (Lounate et al., 2020; Shane et al., 2021; Vallero et al., 2004). The presence of

AMD can lead to a decrease in taxa richness and the disappearance of certain species, with the most severe conditions supporting only a limited number of very tolerant taxa (Svitok et al., 2014).

The presence of high sulfate concentrations in acid streams, often associated with industrial processes, poses a significant environmental concern (Runtti et al., 2018). These streams are a common byproduct of mining activities, particularly coal mining, and can have detrimental effects on water quality (Acharya and Kharel, 2020). Identifying the sources of sulfate contamination in water environments is crucial for addressing this issue, and stable isotopes have been proposed as a potential tool for this purpose (Wang and Zhang, 2019).

The formation of AMD is a significant environmental issue, with highly acidic and metal-laden streams threatening human health and ecosystems (Rambabu et al., 2020). AMD is primarily formed through the oxidation of sulfide minerals, such as pyrite, and is a common byproduct of coal mining, as seen in Figure 1.1 (Acharya and Kharel, 2020). In the context of abandoned coal mines in Shanxi, China, AMD has become a major problem, impacting water resources and local drinking water safety (Wang and Zhang, 2019). The use of renewable raw materials and developing greener mitigation solutions are highlighted as potential strategies for addressing AMD (Rambabu et al., 2020). Using renewable raw materials, such as biomass, can help in the treatment of AMD by providing organic matter for remediation processes, and reducing reliance on non-renewable resources like, wood chips, sawdust, bagasse, cocopeat, between others (Sekarjannah et al., 2023).

The recovery of AMD is indicative of the broader ecological restoration that can be achieved through effective remediation strategies. Moreover, remediation can alter the bacterial community structure in AMD-impacted streams, which is essential for restoring ecological balance. Changes in pH and metal concentrations due to remediation efforts can lead to shifts in microbial assemblages, although complete restoration to pre-impact conditions may not always be achieved (Bijmans et al., 2009b; Montoya et al., 2013). This highlights the complexity of ecological recovery and the need for ongoing monitoring and adaptive management.

Greener mitigation solutions, like bioremediation using SRM, offer an eco-friendly approach to AMD treatment by minimizing chemical usage and secondary contamination, thus promoting environmental sustainability. Highlighting that the recovery and reuse of valuable resources from AMD, and metal ions, can offset treatment costs and reduce environmental impact, making the process both economically viable and environmentally friendly (Yuan et al., 2022).

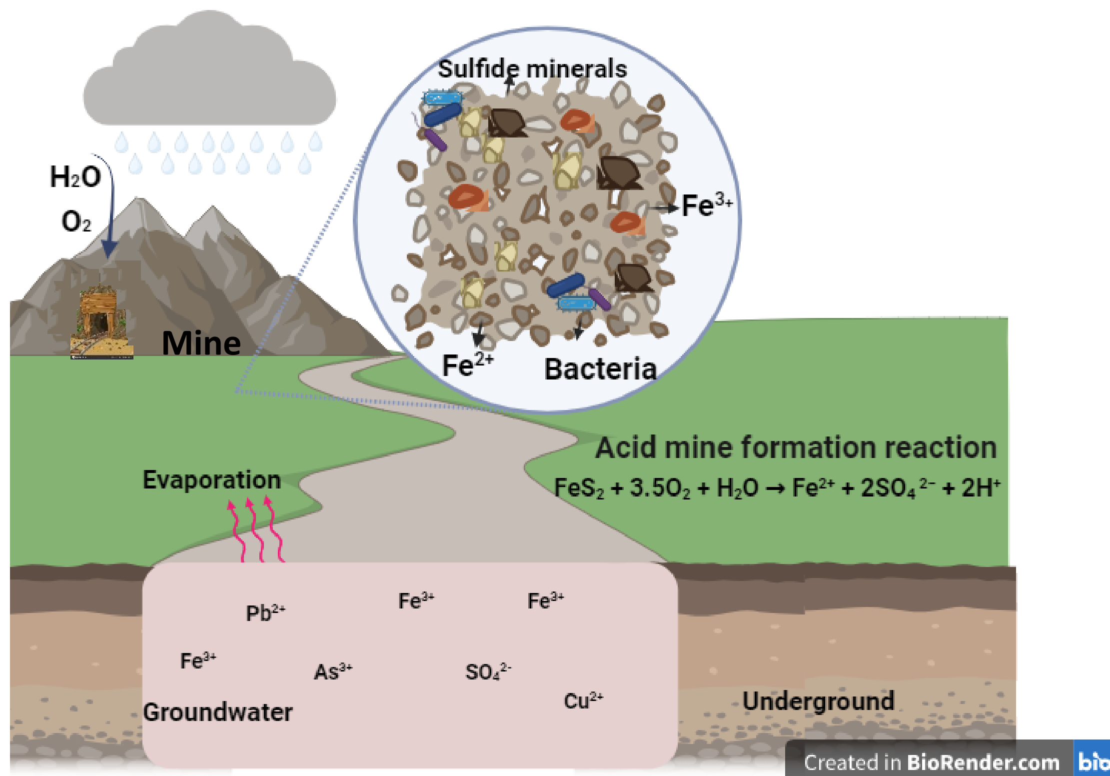


Figure 1.1. General description of AMD formation: occurs when sulfide minerals, typically pyrite (iron sulfide FeS_2), are exposed to oxygen and water; and acid mine formation reaction. Other minerals that could be present are: FeAsS, $CuFeS_2$ or PbS. Figure modified from Zhang et al. (2023), and created in Biorender.com

The use of SRM is considered a sustainable and eco-friendly approach due to its low cost and the availability of the necessary reactants in sites impacted by high concentrations of sulfate (such as AMD). This makes it an attractive option for large-scale applications in mining regions, where traditional remediation methods may be less feasible (Bijmans, 2008). The adaptability of

SRM to various environmental conditions, including cold climates, further underscores their utility in diverse geographical settings (Zhu et al., 2020). However, the effectiveness of SRM can vary depending on site-specific conditions, such as the availability of organic carbon and the presence of other microbial communities denoting the importance of selecting suitable strains or consortia for effective bioremediation (Ayangbenro et al., 2018). The importance of remediating acidic streams lies in the ability to restore water quality, support ecological recovery, and reduce the environmental footprint of mining activities.

Operating systems at low pH provides several benefits: it allows direct treatment of acidic water in a single bioreactor, reduces methanogenesis for more efficient electron donor usage in sulfate reduction, increases pH during sulfate reduction eliminating the need for alkaline addition, and enables selective metal precipitation when pH is maintained at certain (Bijmans et al., 2008; Bijmans et al., 2010; Koschorreck, 2008).

The pH reached in acid mine drainage can be as low as <2 (extreme acidic conditions), and is consequence of the large amount of protons and sulfuric acid produced during the oxidation of sulfide minerals (Rambabu et al. 2020).

In addition to affecting the acidity of surface water, acidic streams can cause some metals embedded in the mineral structure such as As, Cr, Pb, Co, Au and Zn to leach into the water. Some of these metals can be incorporated into the food chain and reabsorbed in living organisms, including humans, causing serious health problems (Johnson and Hallberg 2005). Mining is not the only industry responsible of generating streams rich in sulfate and metallic ions, other industries such as flue-gas scrubbing, electroplating, and paper and mill produce such effluents as well.

The speciation of sulfur compounds at alkaline, circumneutral, or acidic pH differs greatly and has an impact in the biodiversity developed in each condition. In agricultural soils and plants, elemental sulfur undergoes oxidation state changes, influencing stress tolerance and productivity (Fuentes-Lara et al., 2019). Sulfur is essential for plant growth and development, with sulfate being the major form absorbed from soil (Li et al., 2020a). In the ocean, marine

organisms play a crucial role in the transformation and mineralization of organic sulfur compounds (Tang, 2020).

A pH below 4.5, which is typically regarded as acidic, H_2S is the predominant type of sulfur at this low pH; sulfate is much less common. However, the pH range in AMD can vary based on the source and other factors, and not all AMD streams have a pH below 4.5. But rather than necessarily buffering the complete system, it serves as a store for sulfate ions that are currently available (Figure 1.2) (Egbueri, 2019).

The pH level significantly influences sulfur species' distribution, as demonstrated by the interplay among $H_2S/HS^-/S^{2-}$ showed in Figure 1.3. The occurrence of S^{2-} becomes notable specifically at a pH exceeding 16, whereas slight variations in pH within the range of 6.0 to 8.0 lead to a rapid alteration in the concentration of H_2S (Johnson and Sánchez-Andrea, 2019). At these acidic conditions, hydrogen sulfide prevails as non-dissociated gaseous H_2S, rather than its soluble forms HS^- and S^2, which is advantageous for microorganisms because the potential toxicity of sulfide is reduced (Gouvêa de Godoi et al., 2017).

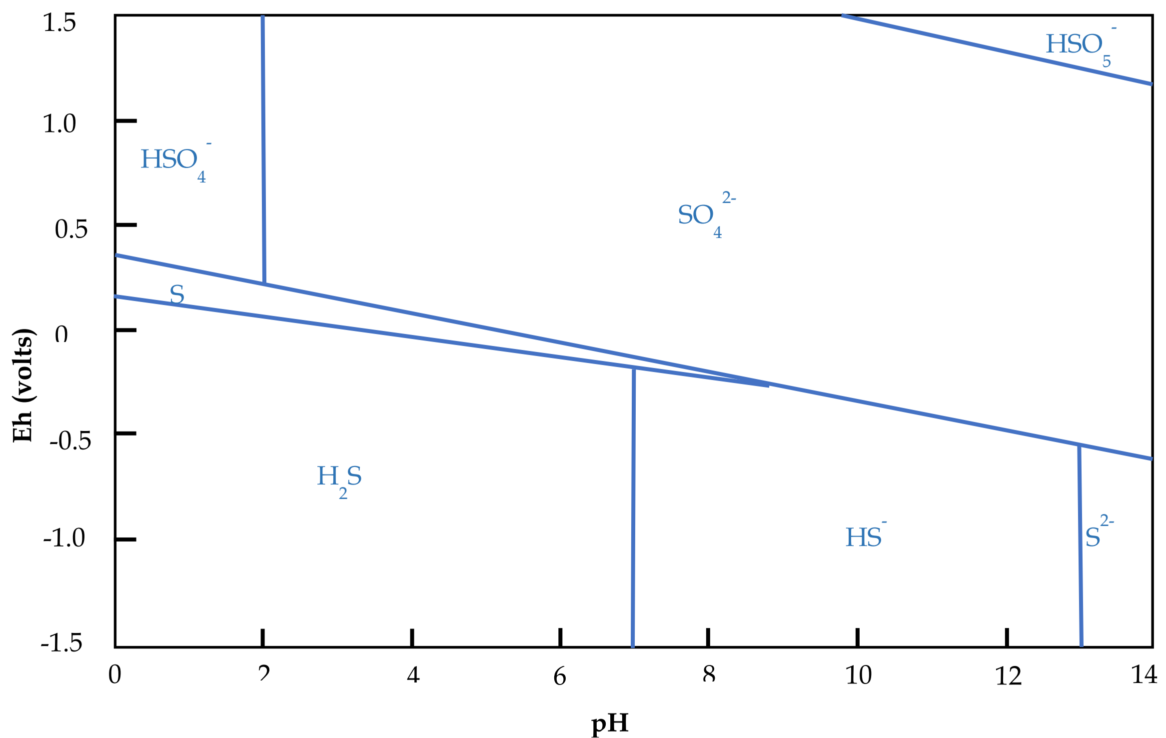


Figure 1.2. Equilibrium species Eh-pH diagram for sulfur (Anderson et al., 2005).

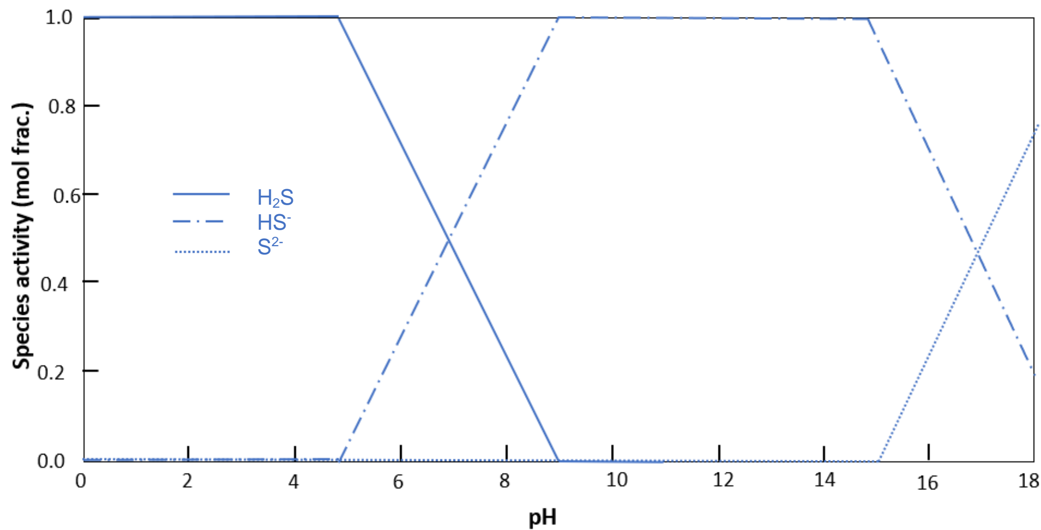


Figure 1.3. Total concentration of sulfide species in function of pH (Costa et al, 2016).

1.3 Sulfate reducing microorganisms (SRM)

Dissimilatory sulfate reduction is an anaerobic respiration process that produces sulfide, and is based on the oxidation of an electron donor, which can be an organic substrate or molecular hydrogen, coupled with the reduction of sulfate as terminal electron acceptor. The reduction of sulfate requires 8 electrons (e-), which come from the electron donor (Dannenberg et al., 1992).

Reaction 1 outlines the stoichiometry of sulfate reduction, where CH_2O represents the electron donor (organic matter).

$2\ CH_2O + SO_4^{2-} \rightarrow H_2S + 2\ HCO_3^-$ Reaction 1

Sulfate-reducing bacteria play a crucial role in neutralizing acidic streams. These bacteria produce hydrogen sulfide, which can precipitate metal sulfides (Reaction 2) and bicarbonate that helps to neutralize acidity. When bicarbonate reacts with a hydrogen ion, it forms carbon dioxide gas and water (Reaction 3).

$H_2S + M^{2+} \rightarrow MS(s) + 2H^+$ Reaction 2

$HCO_3^- + H^+ \rightarrow CO_2(g) + H_2O$ Reaction 3

Metal sulfides precipitates can be recovered and can be used in other industrial processes such as electronics, agriculture, mining, catalysts, pigments, water purification (Habib et al., 2019). In this manner, one can exploit

the metabolic processes of SRM for the remediation of acidic effluents contaminated with heavy metals and sulfate.

Metal ions, the pH of the solution, and the sulfide ion concentration are among the myriad factors influencing the solubility of metal sulfides (Lv et al., 2022; Wang and Zhang, 2019; Xia et al., 2021). For instance, the presence of calcium ions can decrease the amount of free sulfide ions available for metal precipitation (Wang 2019). The release rate of H_2S also plays a crucial role in the particle size and settling performance of metal sulfides in acidic wastewater (Lv et al., 2022). The hydrophilicity/hydrophobicity of metal sulfide particles can further impact their aggregation performance in wastewater (Xia 2021). For example, the pH of the fluid is a primary factor controlling the precipitation of lead and zinc sulfides (Zhang 2019).

The solubility product constant (Ksp) serves as an indicator of the balance between the concentrations of ions in a saturated solution of an ionic compound (Li et al., 2020b). The Ksp values for metal sulfides change based on the metal ion present. The Ksp of zinc sulfide (ZnS), for instance, is about 1.6×10^{-25}, while the Ksp of lead sulfide (PbS) is roughly 4.8×10^{-28}. Given that PbS has a much lower Ksp value, it is less soluble than ZnS. This means PbS will precipitate first from the solution as its ions reach saturation more quickly. Therefore, in an acidic stream (pH <4), lead sulfide (PbS) is more likely to precipitate compared to zinc sulfide (ZnS) (Zárate-Gutiérrez et al., 2015).

SRM are also exceptional in that they can reduce sulfate to sulfide in a variety of environments, including soils, freshwater sediments, and marine sediments. Anaerobic conditions are necessary for the development and metabolism of strictly anaerobes because they cannot grow in the presence of oxygen. While facultative anaerobes usually prefer anaerobic environments, they can grow in either the presence or absence of oxygen. Since dissimilatory sulfate reduction is the metabolic pathway by which sulfate is used as a terminal electron acceptor in the lack of oxygen, both strict anaerobes and facultative anaerobes can be categorized as SRM (Plugge et al., 2011; Rabus et al., 2015).

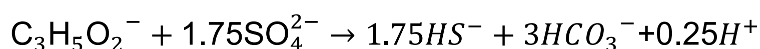
SRM can be classified into two groups based on their capacity to perform either full or incomplete oxidation of substrates. Those SRM able to

oxidize completely the substrate using sulfate as the electron acceptor produce bicarbonate and sulfide as the lactate products. In contrast, those SRM unable to completely oxidize the substrate produce acetate and sulfide as the products because this group of SRM lacks a mechanism to oxidize acetyl-CoA, (Terrett et al., 2014). Lower energy yields are obtained from incomplete oxidation of the substrate than from complete oxidation.

The complete oxidation of propionate through sulfate-reduction yields a Gibbs free energy change (ΔG) -85.4 kJ/reaction (Reaction 4), whereas for the incomplete oxidation is -37.8kJ/reaction, (Reaction 5). This discrepancy underscores the relevance of determining the spontaneity of a reaction, as both reactions are deemed non-spontaneous (Ozuolmez et al., 2015).

$C_3H_5O_2^- + 0.75SO_4^{2-} \rightarrow CH_3COO^- + 0.75HS^- + HCO_3^- + 0.25H^+$

$\Delta G^{0\prime}$=-37.8kJ/reaction Reaction 4



$C_3H_5O_2^- + 1.75SO_4^{2-} \rightarrow 1.75HS^- + 3HCO_3^- + 0.25H^+$

$\Delta G^{0\prime}$=-85.4kJ/reaction Reaction 5

1.4 Taxonomic classification of SRM

SRM play a crucial role in the global sulfur cycle, with diverse taxa identified across various environments (Diao et al., 2023; Florentino et al., 2018; Hausmann et al., 2016; Hemme et al., 2015; Jantharadej et al., 2020; Jördening and Winter, 2005; Li et al., 2018b; Pelikan et al., 2015). These microorganisms are found in peat soil, industrial wastewater treatment plants, high-temperature oil reservoirs, and haloalkaliphilic bioreactors, among other environments. The taxonomic classification of SRM is complex, with a wide range of bacterial and archaeal phyla harboring these microorganisms (Diao et al., 2023). Until 2014, it was known that sulfate reducers in the bacterial domain had been identified in different phyla, which included *Chloroflexi*, *Chlorobi*, Proteobacteria (classes *Alpha*, *Beta*, Gamma, Delta and *Epsilon*-Proteobacteria), *Firmicutes*, Actinobacteria, *Planctomycetes, Spirochaetes, Acidobacteria, Bacteroidetes, Verrucomicrobia, Gemmatimonadetes, Synergistetes*, and *Caldiserica* (Sanchez-Andrea, 2014).

Metagenome-based findings have provided a novel perspective on the undisclosed variety of SRM. It is now possible to acknowledge that in addition

to the four bacterial and two archaeal phyla known to house cultured SRM, the capacity for carrying out dissimilatory sulfate/sulfite reduction is present in a combined total of 23 bacterial and 4 archaeal phyla; a comprehensive analysis of 950 genomes from sulfate reducers, primarily derived from metagenomes, uncovered genetic repertoires that challenge earlier generalizations about their energy metabolism (Diao 2023). It has enlarged our comprehension of sulfate-reducing microorganisms, uncovering a higher diversity of potential SRM, including those from the *Acidobacteriota*, mesophilic *Nitrospirota*, and *Bacteriodata* family UBA2268 (*Kapabacteria*) (Li et al., 2022a). The use of dsrAB gene-based surveys has been highlighted as a valuable tool for exploring this diversity (Santos et al., 2015).

Waite et al., (2020) reclassified the classes Deltaproteobacteria and Oligoflexia, and the phylum Thermodesulfobacteria, into four novel phylum-level lineages, *Desulfobacterota phyl. nov*., *Myxococcota phyl. nov*., SAR324, and *Bdellovibrionota* (formerly the class, *Oligoflexia*). This reclassification is supported by the analysis of 1,000+ type-strain genomes, which substantially improves the taxonomic classification of *Alphaproteobacteria* (Hördt et al., 2020). The genomic characterization of three novel *Desulfobacterota* classes has expanded the metabolic and phylogenetic diversity of the phylum (Murphy et al., 2021). A new approach to delineate genera based on a standard genome relatedness index has been proposed, which excludes the direct use of the 16S rRNA gene (Barco, 2020).

This novel categorization enhances our comprehension of the functions of the microorganisms found in our samples. It ensures that most of our sequences are categorized rather than simply labeled as unclassified. Subsequently, if we aim to isolate or enrich specific community types in the future, we will possess more detailed information to execute the process accurately.

The *Desulfobacterales*, a prominent order within the Deltaproteobacteria class, has been reclassified into the *Desulfobacterota* phylum, reflecting its diverse functional capabilities (Waite 2020). This reclassification has led to the proposal of two new families, *Desulfocapsaceae* and *Desulfurivibrionaceae*, within the order (Waite et al., 2020). The phylum *Desulfobacterot*a has been expanded

with the characterization of three novel classes, each with distinct metabolic and phylogenetic diversity (Murphy et al., 2021).

The *Desulfovibrionia* class. nov. has been reclassified at various taxonomic levels to resolve inconsistencies in the current taxonomy of the order *Desulfovibrionales*. This reclassification includes the subdivision of the genus *Desulfovibrio* into 13 genera (Adeolu et al., 2016). The genus *Desulfotomaculum* has been reclassified into four novel genera (Watanabe et al., 2018).

Syntrophobacterales order has been reclassified into four class-level lineages, with the proposed class *Syntrophia* including the current family *Syntrophaceae* (Waite et al., 2020). This reclassification is supported by the establishment of genome-based criteria for the classification of the family *Desulfovibrionaceae*, which includes sulfate-reducing bacteria (Park 2022). Further, the proposal of two novel genera within the *Desulfovibrionaceae* family, *Alkalidesulfovibrio* and *Salidesulfovibrio*, has contributed to the understanding of sulfate-reducing bacteria (Park et al., 2022). These reclassifications have been further supported by the proposal of a novel genus, *Conexivisphaera*, within the phylum *Thaumarchaeota*, which includes thermophilic sulfur- and iron-reducing archaea (Kato et al., 2021).

1.5 Electron donors used by SRM at low pH

The addition of electron donors is crucial for promoting sulfate-reducing activity in oligotrophic waste streams (Yildiz et al., 2019). These electron donors can range from simple molecules like formate to more complex ones such as molasses (Johnson and Sánchez-Andrea, 2019). In acidic streams with low organic carbon, the inclusion of electron donors like formate, lactate, ethanol, or acetate can support sulfate-reducing activity and aid in the removal of sulfate and heavy metals from wastewater (Costa et al., 2021). Some SRM have the ability to engage in autotrophic sulfate reduction, which is important in oligotrophic settings with low organic carbon concentrations (Shi et al., 2020). Using metal-organic frameworks in sulfate radical-based advanced oxidation processes can enhance the catalytic performance for removing organic pollutants (Du and Zhou, 2021). Various methods, including biological treatment, ion exchange, and adsorption, can be used to tackle stringent sulfate

removal requirements in mine water treatment (Runtti et al., 2018). The use of a sulfur-based sulfidogenic system in the treatment of Cu-laden electroplating wastewater has been shown to be effective, with real domestic sewage serving as the electron donor (Cai et al., 2021). A novel photoelectric microbial electrolysis cell has been developed for simultaneous sulfate reduction and elemental sulfur recovery, with sulfate reducing bacteria and sulfur oxidizing bacteria playing key roles (Luo et al., 2020).

Acetate, a simple molecule, can be a more effective electron source for SRM in oligotrophic environments (Yin et al., 2024). Acetate accumulation in sulfate-reducing processes has been widely observed, with potential explanations including incomplete oxidation of organic matter and inhibition of SRM by high sulfate or heavy metal concentrations (Yu et al., 2022). SRM enriched sludge has been shown to effectively remove sulfate and heavy metals from electroplating effluent (Xia et al., 2021). The choice of electron donor in bioreactors can influence sulfate reduction and metal recovery (Costa et al., 2021).

Bioelectrochemical sulfate reduction in flow mode has been shown to significantly increase sulfate removal rates and Coulombic efficiencies (Dai et al., 2023). Sulfidogenic sludge acclimated to acetate has been found to increase sulfate reduction and COD removal, while also improving the performance of microbial fuel cells (González-Paz et al., 2020). Additionally, the potential of autochthonous sulfate-reducing microbial communities for treating acid mine drainage has been demonstrated (Giordani et al., 2019). The use of acetate as a substrate in biological sulfate reduction has been further explored, with the identification of specific sulfate-reducing microorganisms and the development of kinetic models (Hessler et al., 2022). Lastly, the impact of nitrate on sulfate reduction has been investigated, with a focus on substrate competition and microbial function (Cai et al., 2021)

Also, at low pH acetate may be toxic to microorganisms, at pH values lower than its pKa (4.8), acetic acid is found as a neutral molecule that permeates the cell membrane and inhibits cellular respiration (Kimura et al. 2006; Koschorreck 2008). Due to this limitation, SRM cultures fed with an acidic stream (pH 2.5-3) containing acetate, in a neutral reactor pH 6.5-8, did not

consume more than 40% of the substrate (Kaksonen et al., 2003; Kaksonen et al., 2004a).

The concentration of the protonated form of an organic acid at a certain pH can be calculated using various methods, including the direct method, proton exchange scheme, and hybrid cluster-continuum and implicit-explicit models (Ho and Coote, 2009). The pKa values of typical organic acids, such as lactate and acetate, can be determined using first-principles calculations in nonaqueous solvents (Ding et al., 2009). Lactate and lactic acid are both protonated (RH) and non-protonated (R^-) types of organic acids. Due to the abundance of hydrogen ions (H^+), the protonated form has a high concentration at low pH (< 3.86), whereas the non-protonated form has a greater concentration at higher pH (pH > 5).

Since lactate has a pKa of about 3.86, it primarily appears in the protonated form (i.e., lactic acid) at pH values below this one. At pH 3.86 lactic acid is present in 50%, at pH 5 in 5%, and at pH 7 the predominant species (100%) is lactate anion. Low pH beyond the pKa is an issue because the protonated form can pass through their cell membranes and interfere with their metabolic processes, which can be toxic. Because of this, lactate is not the best electron source for SRM when the environment is acidic (Ramanaiah and Sailaja, 2014).

Acidophilic SRM have been found to efficiently oxidize substrates like ethanol to acetic acid, making them suitable for bioremediating highly acidic waste waters (Kolmert and Johnson, 2001). Glycerol, a non-ionizable substrate, can be selectively oxidized to high-value chemicals such as glyceric acid and dihydroxyacetone (Katryniok et al., 2011). However, its oxidation can also produce acetate, a common by-product in sulfate reduction. For instance, glycerol has been used successfully as the substrate for SRM, especially when cultivated at acidic pH (< 4)(Nancucheo and Johnson, 2012 and 2014).

1.6 Low pH sulfidogenic reactors

By encouraging the activity of SRM, which in turn reduce sulfate into sulfide and raise pH, sulfidogenic reactors are useful to remediating acid streams (Sánchez-Andrea et al., 2014). This is because sulfate reduction causes a net rise in alkalinity due to the consumption of protons (SO_4^{2-} + 8 H^+

+ 8 e- → HS^- + $4H_2O$). These reactors help the formation of metal sulfide precipitates, like zinc, copper and lead, which can be used to treat acidic steams and remove harmful heavy metals (Bratkova, 2021; Nancucheo et al., 2017).

In order to maximize sulfate reduction while minimizing the formation of hydrogen sulfide gas, pH is usually maintained in the range of 6 to 8 in sulfidogenic reactors, because is the optimal pH known for sulfate-reducers. However, the precipitation of metal ions is more advantageous and the solubility of metal sulfides diminishes at lower pH values. Therefore, the metabolism of sulfate-reducing microorganisms can be inhibited if the pH is too low, which lowers the efficiency of the reactor. To balance the precipitation of metal ions with the activity of the sulfate-reducing bacteria, the pH of the reactor must be carefully regulated(Bijmans, 2008).

The use of autochthonous sulfate-reducing microbial communities as inoculum in acid mine drainage treatment has also been shown to be effective (Giordani et al., 2019). Kolmert et al. (2001), evaluated how efficient a sulfate-reducing acidophilic community could be compared to a neutrophilic community under acidic conditions $pH < 5$, both adhered to glass beads, using a mixture of electron donors (glycerol, ethanol, and lactic acid). The sulfate-reducing rates were higher when the acidophilic sulfate-reducing community (0.25g/L·d) was used, although they obtained acetic acid as product of the incomplete oxidation of the substrates used. Lastly, chitinous materials have been found to be highly effective in removing metals from sulfate-reducing bioreactors, with the main mechanism being metal precipitation as sulfides. This has been demonstrated in the treatment of mining-influenced water (Al-Abed et al., 2017), acidic mine drainage (Vasquez et al., 2018; Yildiz et al., 2019), and synthetic wastewater (Gopi Kiran et al., 2017). The use of chitinous substrates has been particularly successful, with higher metal and sulfate removal rates compared to ligneous substrates (Al-Abed et al., 2017).

The use of bioreactors in the remediation of acid streams using sulfate reducing communities offers several advantages. However, there are also some disadvantages, such as the need for large land parcels and prolonged treatment periods in passive systems (Rambabu et al., 2020), and the high

investment costs and lack of expertise in active bioreactors (Rambabu et al., 2020). The use of two bioreactors with different acidophilic microbial consortia has been shown to be effective in treating acidic mine water, and the use of Fischer-Tropsch waste water as a feedstock for dissimilatory sulfate reduction has been successful in removing sulfate and COD (Frederico et al., 2022).

Operational parameters such as temperature and hydraulic retention time play a crucial role in the performance of SRM in acid streams in bioreactors. These parameters can significantly impact the efficiency of SRM in treating AMD and recovering metals from wastewater (Kaksonen and Puhakka, 2007; Vasquez et al., 2018). Various operational parameters influence the competition between SRM and methanogenic bacteria in sulfate-containing wastewater treatment. These include pH, temperature, and sulfide concentration, which can affect the growth rates and affinities of these bacteria (Lens et al., 1998). Salinity can also impact this competition, with higher salinity levels favoring sulfidogenesis over methanogenesis (Zampieri et al., 2021). In the context of high-retention membrane bioreactors, the impact of salinity and the need for steady-state operation are highlighted (Bijmans et al., 2009a; Bijmans et al., 2010).

The establishment of a sulfidogenic environment under thermophilic acidogenic conditions can enhance sulfate removal and acetate production (Gil-Garcia et al., 2018). High-rate sulfate reduction under acidic conditions has been achieved, with a specific activity of 81 SO_4^{2-} mmol per gram of volatile suspended solids per day (Bijmans et al., 2009b). The optimization of process parameters, including pH, has been shown to improve sulfate and metal removal from acid mine drainage (Dev et al., 2017). The effectiveness of sulfate removal and metal precipitation has been demonstrated at lower pH values (Yuan et al., 2022).

Johnson and colleagues have conducted research on continuous upflow biofilm reactors using acidic effluents with glycerol as the principal electron donor for sulfate reduction. For instance, Santos and Johnson, (2018) demonstrated the use of glycerol as the primary electron donor at a pH value of 4 in an upflow biofilm sulfidogenic bioreactor for metal removal. Bertolino et

al., (2014) compared glycerol and lactate as electron donors for sulfate reduction, finding glycerol to be a cost-effective alternative.

The inoculum used by Johnson and coworkers since 2012, is an acidophilic microbial mixed built community containing different strains of SRM (D. *acididurans*, *Peptococcaceae* strain CEB3 and *strain* CL4) and acidophilic microorganisms (strain AR3, *Acidithiobacillus* (At.) *ferrooxidans* and Ac. *aromatica*) that play key roles the in sulfate-reduction activity at low pH. Different operational parameters (temperature, pH inside the reactor, and pH of the inlet-fed media) resulted in changes in the relative abundances of the bacteria within the consortium (Nancucheo and Johnson 2012; Nancucheo and Johnson 2014; Santos and Johnson 2017; Santos and Johnson 2018). For example, *D. acididurans* was more relatively abundant at higher pH (5) and lower temperatures (30 °C), and *Desulfobacillus* CEB3 at lower pH (4) and higher temperatures (35 °C) (Santos and Johnson 2018).

From the previous works studying sulfate reduction at acidic pH in continuous reactors, an important characteristic is that the microbial community is immobilized to increase biomass retention (biofilm) within the reactor and maintain high cell retention times to prevent reactor wash-out (Silva et al. 2006; Zhang et al. 2016). The carrier materials used for the retention of sulfate-reducing communities are granulated activated carbon (GAC) (Sánchez-Andrea et al. 2012), polyurethane foam (Silva et al. 2006), polyethylene particles (Piña-Salazar et al. 2011; Montoya et al. 2013), porous glass beads (Nancucheo and Johnson 2012), and zeolite (Zhang et al. 2016), among others.

The sulfate-reducing bacteria *Desulfobacca acetoxidans*, *Desulforhabdus amnigenus*, and *Desulfovibrio spp*. have been found to be more prevalent in acidic feed media, but their efficiency is hindered by the production of acetate (Kaksonen et al., 2004b; Montoya et al., 2013; Watanabe et al., 2018).

1.7 Acidophilic SRM

The anaerobic acidophilic SRM that have been characterized are categorized within the genera *Thermodesulfobium*, *Desulfosporosinus*, *Desulfothermobacter*, and *Acididesulfobacillus*. The acidophilic SRM are either moderate thermophilic or mesophilic, with variations in their temperature needs

for optimal growth conditions. Nevertheless, it is crucial to emphasize that all known acidophilic SRM present a similar pH range (3.5-5.5) Table 1.

Acidophilic archaea are microorganisms that play a crucial role in the sulfur cycle by breaking down sulfur compounds, which helps in nutrient cycling and maintaining ecosystem balance and thrive in acidic environments, often with a pH level below 3, such as hot springs, hydrothermal vents, and acidic mine waste sites. Examples of acidophilic archeae are: Thermoproteota, Halobacteriota, and Thermoplasmatota (Diao, et al. 2023).

A range of acidophilic sulfate-reducing bacteria have been isolated from various environments. For example, Sánchez-Andrea et al, (2013) and Mardanov et al., (2017) isolated acidophilic sulfate-reducing bacteria from mine sediments (Rio Tinto (Spain) and gold recovery mine (Russia)). Willis et al., (2019) enriched and isolated acid-tolerant sulfate-reducing microorganisms from anoxic, acidic hot spring sediments. Frolov et al., (2018), isolated a thermoacidophilic sulfate-reducing bacterium from a terrestrial hot spring. Johnson and Sanchez-Andrea, (2019), provided a review of the biodiversity and metabolisms of sulfate- and sulfur-reducing prokaryotes in low pH environments. The studies presented by Johnson 2019 collectively demonstrate the diversity and potential applications of acidophilic sulfate-reducing bacteria.

The discovery of these acidophilic species adds to the growing body of knowledge on extremophiles, which are organisms that thrive in extreme environments (Johnson and Hallberg, 2003; Johnson and Schippers, 2017).

Various studies have isolated and characterized acidophilic sulfate-reducing bacteria with unique physiological traits. For instance, *Thermodesulfobium narugense,* a moderate thermophile, uses H_2/CO_2 and grows between pH 4 and 6.5 (Mori et al., 2003)*. Desulfosporosinus acidiphilus and Desulfosporosinus acididurans,* both members of the Peptococcaceae family, are acidophilic sulfate-reducing bacteria, with the latter being spore-forming and having an optimum pH of 5 (Sánchez-Andrea et al., 2015).

Recent research has identified two new acidophilic species within the *Thermodesulfobium genus,* and the strains, *T. acidiphilum* and *T. sp*. strain 3baa, both of which thrive in low pH (4.8-6.0) environments at a temperature

above 60 °C (Egas, 2024; Meier et al., 2012). These findings are significant as they contribute to our understanding of how sulfate-reducing prokaryotes can thrive in natural and engineered systems at low pH (Rüffel et al., 2018).

Acidophilic SRM are widespread in two domains of life with the ability to tolerate high metal concentrations (Baker-Austin and Dopson, 2007). The acid-tolerant *Desulfovibrio sp.* VK and *Desulfovibrio sp.* ED, both of which are Deltaproteobacteria, have been identified as potential candidates for the remediation of acidic streams (Sánchez-Andrea et al., 2013). Additionally, the acid tolerant sulfur-respiring bacterium *Desulfurella amilsii* has been isolated from acidic river sediments (Florentino et al., 2018).

Table 1.1 A summary of documented species belonging to moderately acidophilic sulfate-reducing microorganisms. Describing pH optimum, temperature range, source of inoculum and the consumption of acetate

sulfate-

Acidophilic and moderate SRM						
Name of the microorganism	**Reference**	**Energy Source and Metabolism**	**Range temperature**	**Optimum pH**	**Isolated from**	**Consumption of acetate/acetic acid**
Desulfosporosinus acidiphilus sp.	Alazard D. et al., 2010	Sulfate-reducing bacteria; oxidation of organic matter; SO_4^{2-} as electron acceptor	25–40°C	5.2	Acid mining effluent decantation pond sediment sample	Yes (H_2 was used too)
Desulfosporosinus acididurans sp.	Sánchez-Andrea I., et al., 2015	Sulfate-reducing bacteria ; Fe^{3+}, NO_3^-, SO_4^{2-}, S_8 and $S_2O_3^{2-}$ as electron acceptors; oxidation of organic matter.	15–40°C	5.5.	Acidic sediments (White river and Tinto river)	No
Acididesulfobacillus acetoxydans gen. nov.	Sánchez-Andrea I., et al., 2022	Sulfate-reducing bacteria; SO_4^{2-} as electron acceptor; organic electron donors.	25–42°C	5.0	Tinto River	Yes
Thermodesulfobium narugense sp.	Mori K., et al, 2003	Sulfate-reducing bacteria; sulfate, thiosulfate, nitrate, and nitrite as electron acceptors	50–55°C	5.5-6	Hot spring in Narugo	No
Thermodesulfobium acidiphilum sp.	Frolov E, et al. 2017.	Sulfate-reducing bacteria; SO_4^{2-} as electron acceptor; organic electron donors.	37–65°C	4.8-5	Geothermally heated soil	No
Desulfosporosinus metallidurans sp.	Panova I., et al, 2021	Sulfate-reducing bacteria; sulfate, sulfite, thiosulfate, nitrate and fumarate as electron acceptors	42-37°C	5.5	Microbial mat in a tailing dam at a gold ore mining site	No
Desulfothermobacter acidiphilus	Frolov E., et al, 2018	Sulfate-reducing bacteria; SO_2 and SO_4^{2-} as electron acceptor	42-70°C	4.5	Terrestrial hot spring	No
Archaeoglobus profundus sp.	Burggraf S. et al, 1990	Sulfate-reducing archaeon; utilizes acetate and CO_2 for biosynthesis.	70–80°C	4.5-7	High-temperature oil fields	Yes
Caldivirga maquilingensis gen. nov., sp	Itoh T. et al, 1999	Hyperthermophilic archaeon; sulfur, thiosulfate or sulfate as electron acceptors.	60-92 "C	3.7-4.2	Acidic hot spring “Mud Spring”	No
Vulcanisaeta spp.	Diao M., et al. 2023	Heterotrophic archaeon; organic carbon sources; Sulfur and thiosulfate as electron acceptors.	85–90°C	3.5	Kamchatka Peninsula hot springs	No

reducing bacteria (aSRB) that play a crucial role in the treatment of acid gases and associated impurities (Kuever, 2014). Almstrand et al., (2016) reported the reconstructed genome from metagenome of *Desulfatirhabdium* showing heavy metal and acid resistance. A variety of sulfate-reducing bacteria, including *Desulfobulbus, Desulfobacterium, Desulfobacca, Desulfotomaculum, and Desulfomonile* spp., have been detected in low pH sulfidogenic bioreactors (Miletto et al., 2010; Montoya et al., 2013; Muyzer and Stams, 2008). These bacteria play a crucial role in the sulfur cycle, with some species being dominant community members in hydrothermal vent sites (Muyzer and Stams, 2008). Other related genera, such as *Thiomicrospira, Desulfovibrio, Desulfocapsa, Desulfuromusa, and Desulfosporosinu*s, have also been identified in similar environments (Van Den Brand et al., 2016; Brito et al., 2014; Pereira et al., 2011; Sánchez-Andrea et al., 2022).

The use of SRM that can oxidize acetate at extreme acidic pH is crucial for several reasons, particularly in the context of bioremediation of acidic environments such as AMD. These microorganisms, such as the newly identified *Acididesulfobacillus acetoxydans*, are capable of thriving at low pH levels (as low as 3.8) and can completely oxidize organic acids like acetate to CO_2. This complete oxidation is significant because it reduces the toxicity associated with the protonated form of organic acids prevalent at low pH, thereby enhancing the microorganisms acidic stress resistance and contributing to environmental alkalinization (Sánchez-Andrea et al., 2022).

The role of fermentative acidogenic bacteria and SRM in lactate degradation and sulfate reduction has also been studied. The use of acidogenic sulfate-reducing bacteria in enhancing sulfate reduction has been explored (Zhao et al., 2008).

Vulcanisaeta is a genus of hyperthermophilic, anaerobic archaea that play a crucial role in environmental and industrial processes (Chernyh et al., 2020). Members of the *Vulcanisaeta* genus, such as *Vulcanisaeta thermophila*, exhibit unique metabolic capabilities, utilizing various electron acceptors like fumarate, malate, sulfur, and thiosulfate (Yim et al., 2015). *Vulcanisaeta* unique metabolic capabilities and evolutionary insights make it a significant organism in

understanding extremophilic microbial ecosystems and biogeochemical processes (Gumerov et al., 2011).

1.8 Research aim and thesis outline

This thesis focused on the suitability of sulfate-reducing activity at low pH by enriching an acidophilic sulfate-reducing community and isolating key players as well as evaluating its biotechnological potential in a continuous reactor. The primary source of microorganisms was an abandoned sulfur mine (Guaxacama, Mexico). From this source, we retrieved seven consortia that were able to grow at acidic pH <4. One of these consortia, using glycerol as the substrate, was evaluated to grow on different carrier materials and then used as the inoculum of a continuous sulfidogenic reactor. Finally, *Desulfofarcimen acidiphilus*, a novel acetotrophic sulfate-reducer was isolated and characterized.

Chapter 2 describes the enrichment of bacterial communities with sulfate-reduction activity from sediments by successive transfers. From these enrichments it was possible to obtain seven consortia that completely consumed the substrate (glycerol or lactate), and its byproducts (including acetate) at initial acidic pH (3 or 4). The activity of all the consortia was reproducible after five successive transfers. The microbial analysis highlighted some SRM and fermenters as responsible for the complete oxidation of the substrate at acidic pH (3 or 4).

Chapter 3 describes the evaluation of different inert materials suitable for the attachment and development of a well-established acidophilic consortium at initial low pH (3). The three materials evaluated were: GAC, porous glass beads, and zeolite. Using glycerol as the electron donor, batch assays were conducted to establish the sulfate-reduction rate and formation and consumption of intermediaries (acetate and propionate). Also, the effect of the carrier material was evaluated on zeolite and glass beads in the biofilm and planktonic phase.

In **Chapter 4** the performance of an acidophilic sulfate-reducing consortium capable of degrading acetate in a sulfidogenic continuous reactor is evaluated. The bioreactor operated 150 days in continuous conditions (IX

periods) at controlled acidic pH (from pH 5 to 3.25) by using an extremely acidic stream inlet (pH 2.5 to 1.7), glycerol was used as the electron donor. In this chapter the microbial diversity of the biofilm and planktonic communities were also evaluated, and the main differences assessed by statistical analysis.

Chapter 5 summarizes the outstanding aspects of this thesis, discusses the outcome, and provides perspectives and directions for future research regarding the biology and biotechnological application of acidophilic sulfate-reducing microorganisms.

Chapter 2

In search of sulfate-reducing consortia able to degrade acetate under acidic conditions

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Abstract

SRM can help to remediate acidic effluents containing metals. One drawback of sulfate reduction is that some SRM do not oxidize completely the substrate to CO_2 and acetic acid may remain as a byproduct, affecting the process efficiency. Acidic environments are a potential source of sulfate-reducers able to thrive acidic conditions. This work aimed to develop cultivable consortia of sulfate-reducing microorganisms able to consume acetate at acidic pH and analyze their community composition. Starting from sediment enrichments from a natural acidic source, by successive transfers and combinations of electron donors and pH we obtained seven sulfate-reducing consortia. All the consortia consumed the acetate produced from the incomplete oxidation of the substrate (lactate or glycerol) and used 53-75% of the reducing equivalents for sulfate reduction. The sulfide production rate of the consortia was between 0.22-0.26 mmol/L·day in the range of pH 3 – 6, being slightly higher at acidic conditions (4 – 5). The microbial diversity of the consortia was dominated by 21 OTUs, including taxa of acetotrophic sulfate reducers (i.e., *Desulfotomaculum* and *Desulfatirhabdium*) and fermenting bacteria. The consortia reported here have the potential to serve as inoculum for sulfate-reducing bioreactors and could help to overcome acetate accumulation at low pH.

Keywords

Acetate; Acidic-pH; Acidophilic; Consortia; Community; Sulfate-reduction

2.1 Introduction

The biological sulfate reduction process is based on the oxidation of an electron donor, which can be an organic substrate or molecular hydrogen, coupled to the reduction of sulfate (terminal electron acceptor) to produce sulfide. SRM are responsible for sulfate reduction and are a group of prokaryotes, remarkably adaptable, that can be found in terrestrial and aquatic environments, mainly in sulfate-rich anoxic environments in very diverse natural environments such as saline, alkaline, acidic, or thermal habitats (Muyzer and Stams, 2008).

Recently, sulfate reduction at low pH raised interest for the treatment of metal-containing effluents (Kaksonen et al., 2004a; Nancucheo and Johnson, 2014), such as AMD, the biologically produced sulfide can react with heavy metals such as Fe^{+2}, Zn^{+2}, Cu^{+2}, or Cd^{+2} and precipitate them as insoluble metal sulfides (Gallegos-Garcia et al., 2009; Rezadehbashi and Baldwin, 2018; Utgikar et al., 2003; Zhang et al., 2018). Such effluents are somewhat acidic (pH < 5) due to the acidification of the waste generated from the exploitation of minerals, either by chemical or biological processes and generally contain low amounts of organic carbon (< 10 mg/L), these characteristics diminish the efficiency of the sulfate reduction process (Sánchez-Andrea et al., 2014).

The activity of SRM retrieved from environmental samples (i.e., sediments or streams) has been observed under extremely (pH 1-3) and moderately (pH 4-5) acidic conditions and many efforts have been made to enrich, cultivate, and eventually isolate SRM at those conditions (Sánchez-Andrea et al., 2013). The development of several types of reactors for the treatment of AMD became possible by using communities from this kind of acidic environments. For instance, Nancucheo and Johnson (2012) treated synthetic AMD successfully in a continuous reactor inoculated with an enrichment obtained from the stream of an abandoned copper mine, and bioaugmented with pure cultures of *Desulfosporosinus* M1 and *Desulfobacillus acidavidus.* The community developed on glass beads was the key to the successful operation of the reactor at pH as low as 2.1. In another work, sulfate-reducing consortia and four isolates of SRM were eventually retrieved from the extremely acidic environment of Rio Tinto in Spain (Sánchez-Andrea et al.,

2013). The isolates were cultivated at pH 5.5-4.0 using glycerol, methanol, and lactate as substrates, but glycerol and lactate were incompletely oxidized to acetate. Up to date, only a few isolates of the genera *Desulfovibrio*, *Desulfosporosinus*, *Desulfobacillus,* and *Desulfurella* have been identified as acid-tolerant or acidophilic; none of these isolates can oxidize acetate (Qian et al., 2019).

Lactate and ethanol are the substrates typically used to promote the activity and growth of SRM at neutral pH (Plugge et al., 2011; Smith et al., 1981). However, a challenging area in the field of sulfate reduction at acidic pH is that when incomplete oxidation of these substrates occurs, the efficiency of substrate oxidation *via* sulfate reduction is lower because acetate remains as a by-product (Kaksonen et al., 2003). The acidic pH adds another constraint to the use of acetate by SRM because at pH values lower than 4.76 (i.e*.,* the pKa of acetic acid), undissociated acetic acid is the predominant form, and this non-ionized molecule will cross the cell membrane and inhibit cellular respiration (Qian et al., 2019; Reis et al., 1990). In contrast, glycerol has been used successfully as a substrate for the enrichment, cultivation, and even isolation of SRM, at acidic conditions (pH ≤ 4.0) (Sánchez-Andrea et al., 2013; Santos et al., 2018). Glycerol does not ionize at acidic pH, avoiding the harmful effects that ionizable substrates such as organic acids may cause, but acetate is still a common by-product of glycerol oxidation (Nancucheo and Johnson, 2012). Therefore, to efficiently apply sulfate-reduction for AMD treatment, it is critical to count on acetate consuming sulfate-reducing communities thriving at acidic pH. This work aimed to expand the scope of SRM at acidic pH by developing and characterizing sulfate-reducing consortia. Using the acclimation approach, we were able to obtain seven sulfate-reducing communities cultivated at low pH (3 or 4) that can consume acetic acid.

2.2 Materials and methods

2.2.1 Source of microorganisms

Enrichments previously cultured were used as inoculum to develop the acetotrophic sulfate-reducing consortia reported here; these enrichments

originated from the sediments of the acidic leachates from an abandoned sulfur mine and were cultivated with different carbon sources (acetate, lactate, or glycerol) at different pH (3, 4, or 5) as reported elsewhere (Moreno-Perlin et al. 2019). To start the cultures of the consortia, we screened 45 enrichments and selected a total of 38 to be used as inoculum, based on the sulfide production and acetate consumption capacity of each enrichment (Fig. 1.1). In this work, we aimed to obtain consortia free of sediment.

2.2.2 Culture medium and cultivation conditions

The following minimal anaerobic medium was used to develop the consortia (mM): 3 KH_2PO_4, 3 $Na_2HPO_4 \cdot 2H_2O$, 50 NH_4Cl, 30 NaCl, 40 $MgCl_2 \cdot 6H_2O$, 75 $CaCl_2 \cdot H_2O$, 1 mL/L trace element solution (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 $ZnCl_2$), and 0.1 g/L of yeast extract, modified from Stams et al. (1993). The medium was supplemented with 10 mM Na_2SO_4 as the electron acceptor and the stoichiometric amount of electron donor: 10 mM acetate, 6.6 mM lactate, or 5.71 mM glycerol. All cultures were developed in 120 mL serum bottles, containing 80 mL of minimal anaerobic medium supplemented with the corresponding substrate, sodium sulfate; anaerobic atmosphere (N_2/CO_2; 80:20%) and were incubated at 30°C in the dark without agitation.

2.2.3 Development of the consortia by successive transfers

To develop the consortia by successive transfers, we started from the 38 initial enrichments selected as inoculum. These enrichments were divided into two groups. Group 1: those initial enrichments incubated at initial pH 4.0 and fed with lactate, acetate, or glycerol, six bottles each. The successive transfers of this group were inoculated with 20% of slurry from the enrichment or 20% of the previous transfer (Fig. 1.1).

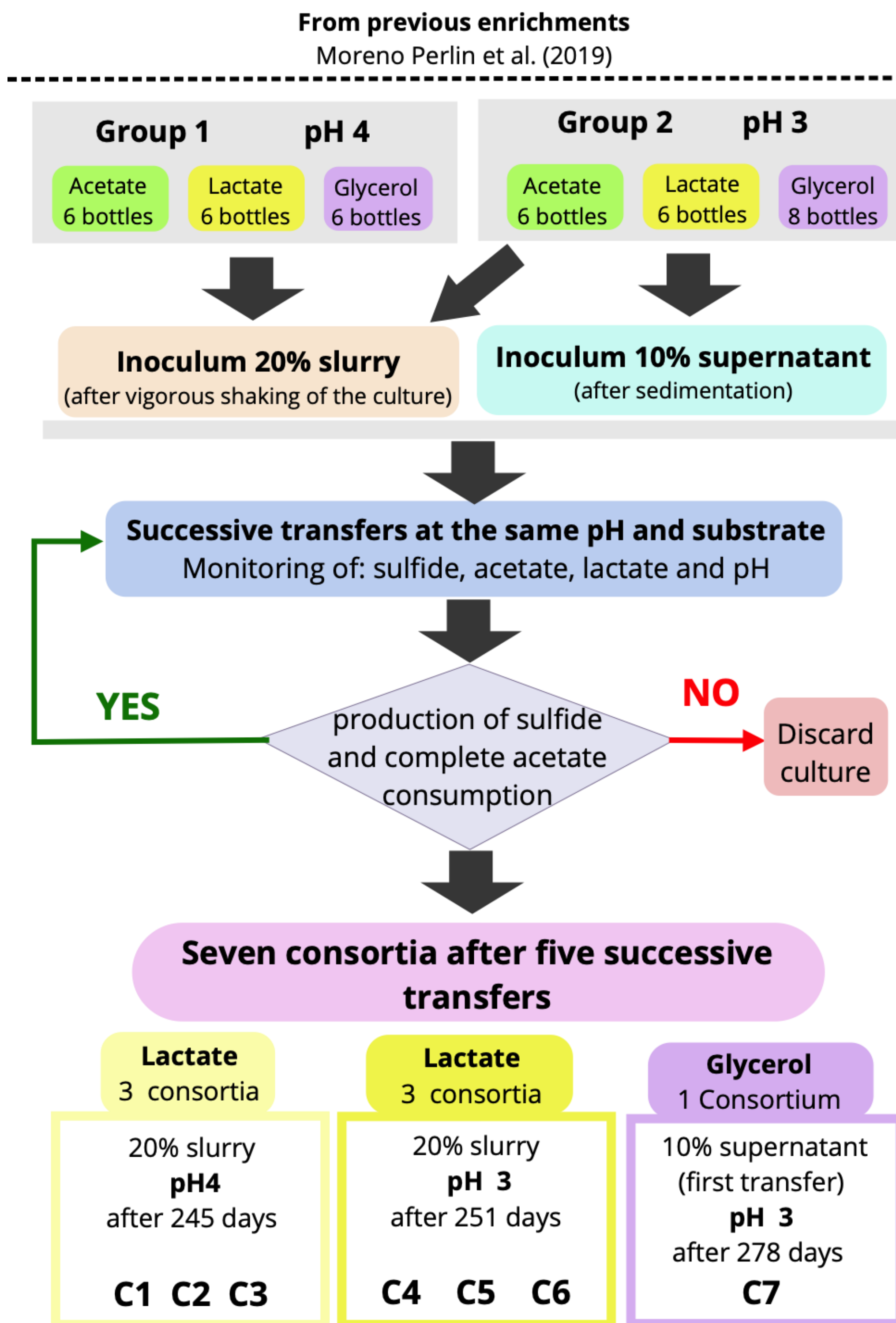

Figure 2.1 Diagram of the experimental strategy followed to obtain the seven cultivable and reproducible consortia at acidic conditions (initial pH 3 or 4).

Group 2 consisted of 20 bottles in total, enriched at initial pH 3 with lactate or acetate (6 bottles each) or glycerol (8 bottles); for starting-up the successive transfers from this group, we assayed two ways of inoculation: 1) inoculation with 10% supernatant (liquid fraction after sedimentation) and 2) inoculation with 20% slurry (the mixture of liquid media and sediment after vigorous agitation). The development of the cultures was monitored periodically through the concentration of substrates (acetate, lactate, sulfate), the concentration of sulfide, and the pH until the sulfide concentration was constant and almost complete consumption of acetate was observed (around 30 days). At this point, the cultures that showed sulfide production and acetate consumption were transferred again to new media with the corresponding substrate and initial pH; in this way, another transfer was obtained. In total, five successive transfers were needed to obtain each one of the seven consortia presented here; all the consortia were devoid of the original sediment. Those cultures that did not produce sulfide and did not consume acetate were discarded (Fig. 2.1). During the successive transfers, the pH of the cultures was not controlled.

2.2.4 Characterization of the final consortia

Each final consortium (fifth transfer) was characterized by sulfide production, sulfate consumption, acetate production, pH, and optical density (600 nm) in triplicate. The time profiles obtained in this assay were used to calculate the maximum rates of lactate and acetate consumption and sulfide production to verify the reproducibility of the activity of the consortia.

2.2.5 Favorable pH interval

The final consortia were cultivated (in duplicate) with their corresponding substrate but varying the initial pH of the culture medium with the addition of 1 N HCl or 1 N NaOH. For the consortia originally cultivated at pH 4.0, we screened the following initial pH values: 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0. For the consortia originally cultivated at pH 3.0, the initial pH was adjusted to 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0. The concentration of sulfide, pH, and optical density were determined every seven days; the sulfate reduction rate was indirectly obtained from the slope of the sulfide production curve (in mM)

vs. time. Subsequently, the rates obtained were plotted at each pH value to obtain the interval of favorable pH of each consortium.

2.2.6 Chemical analyses

Dissolved sulfide was quantified by the Cord-Ruwisch (1985) method with the corresponding calibration curve (0-20 mM, in triplicate; maximum error 5%) using $Na_2S \cdot 9H_2O$ as standard. Volatile fatty acids (lactate and acetate) and sulfate were determined by capillary electrophoresis with a diode array detector according to the method of Soga and Ros (1999) from calibration curves (50-1000 mg/L), using high purity standards, after centrifugation (10000 *g*) and filtration (0.22 μm) of the samples. The pH was measured with a Thermo Scientific TM Orion TM VersaStar potentiometer. To quantify the increase of biomass, the optical density (600 nm) was determined from fresh samples of the cultures.

2.2.7 Molecular characterization

To characterize the diversity of each final consortium (fifth transfer), the DNA was extracted from each bottle of the triplicate assay (Characterization of the final consortia) using the SPIN FastDNA-T DNA Extraction Kit for Soil (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instructions. Then, the DNA was pooled into one composite sample, amplified, and cloned. Amplification of the 16S rRNA gene was performed with primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) to obtain a 1465 bp fragment. The PCR mix (50 μL) contained: 5X PCR Green GoTaq® reaction buffer, 0.2 mM dNTPs, 0.1 μM of each forward and reverse primer, GoTaq® DNA Polymerase (1.25 u), and 1 μL of template DNA. The PCR program was: 97ºC for 5 min, followed by 30 cycles at 95 °C for 2 min, 52 °C for 40 sec, 72 °C for 1.3 min, and a final extension at 72 °C for 10 min. The PCR products with the expected size (1465 bp) were cleaned by DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, United States), and ligated (overnight) using the pGEM-T Easy vector (Promega) following the manufacturer's instructions. Ligation was plated on Luria-Bertani (LB) agar with ampicillin (100 mg/L), IPTG (0.00238 mg/L) and X-gal (0.0040 mg/L) as selection media. Positive white colonies were selected

(48 per sample) and grown in LB medium for 18 h at 37 °C, the grown cultures were plated into GATC plates and sent for Sanger sequencing with SP6 primer (Eurofins *GATC Biotech,* Konstanz, Germany). The DNA sequences were checked using Chromas (version 2.32, Technelysium Pty. Ltd.), and contigs were constructed from the partial sequences using DNAbaser (version 2.71.0, Heracle Software, Lilienthal, Germany) resulting in sequences of 800-1200 bp of the 16S rRNA gene. To find the phylogenetic affiliation of the clones, the bacterial 16S rRNA sequences were checked for anomalies using Pintail online software(Ashelford et al., 2005), and compared to the blastn GenBank (NCBI). Sequences were also aligned with SINA (v1.2.11), of the SILVA ribosomal database project, to find the phylogenetic affiliation of the clones using SILVAngs (*version*: 1.9.4 / 1.3.9) for Sanger sequencing analysis and to construct rarefaction curves. The sequences are deposited in the NCBI nucleotide sequence database GenBank under accession numbers MT022112-409. We used the R program (2005) to calculate the Euclidean distance matrix and construct a dendrogram using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) building the tree by the upside-down approach. The richness, Shannon-Wiener index, Simpson index of dominance, evenness, and principal component analysis (PCA) were calculated with R Studio program using the vegan community ecology R package (version 2.5.6).

2.3 Results

2.3.1 Development and performance of the consortia-successive transfers

Acetate-consuming sulfate-reducing consortia were enriched from previous incubations of sediments at acidic conditions (pH < 4.0) by successive transfers. To be transferred again, the cultures should produce sulfide and consume acetate completely. Only the first transfers inoculated with 20% of slurry and lactate showed sulfide production and acetate consumption; the cultures inoculated with 10 or 20% of the supernatant produced less than 2 mM of sulfide and consumed less than 80% of the substrate. Therefore, the following successive transfers with lactate as substrate were inoculated with 20% (v/v) of slurry. Interestingly, Consortium 7, fed with glycerol, was the only

one that was obtained using 10% (v/v) of the supernatant as inoculum in the first transfer; nonetheless, due to the long-time needed (68 days) to consume the acetate completely and produce sulfide, the successive transfers were also inoculated using 20% (v/v) of supernatant.

Table 2.1 Initial pH, electron donor, and type of inoculum used to obtain the consortia.

Consortium	Initial pH	Electron donor	Inoculum
1	4	Lactate[a]	20% of slurry
2	4		
3	4		
4	3		
5	3		
6	3		
7	3	Glycerol	10% of supernatant[b]

[a]Lactate was used as substrate in consortia 1-6.
[b] just in transfer 1; transfers 2 to 5 were inoculated with 20% of supernatant.

Following this methodology, from a total of 365 incubations, only seven consortia were obtained after five successive transfers (Fig. 2.2 – 2.4). These consortia were free of sediment, able to produce sulfide at acidic conditions (pH 3 or 4) and consume acetate using lactate (Consortium 1-6) or glycerol (Consortium 7) as the substrates. It is worth noting that, at this stage of the experiment, each consortium was unique because there was only one culture of each consortium. Table 1 shows the combinations of pH, substrate, and type of inoculum that yielded the seven consortia coupling sulfate-reducing activity with complete oxidation of the substrates.

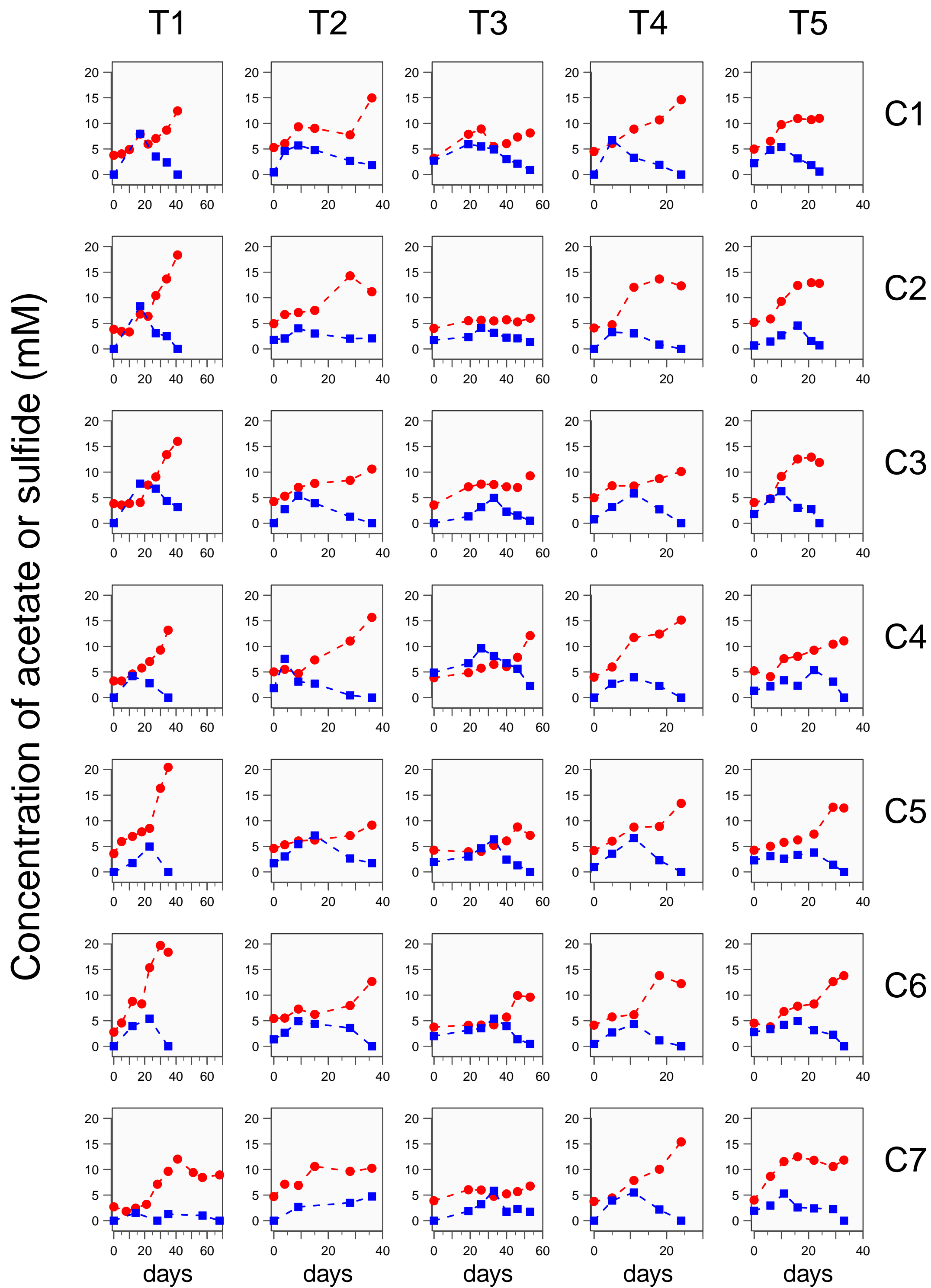

Figure 2.2 Kinetic profiles of sulfide (●) and acetate (■) of the seven consortia, Consortium 1 (C1) to Consortium 7 (C7), from successive transfer one (T1) to successive transfer five (T5). C1 - C3 substrate lactate, initial pH 4; C4 - C6 substrate lactate, initial pH 3; C7 substrate glycerol, initial pH 3.

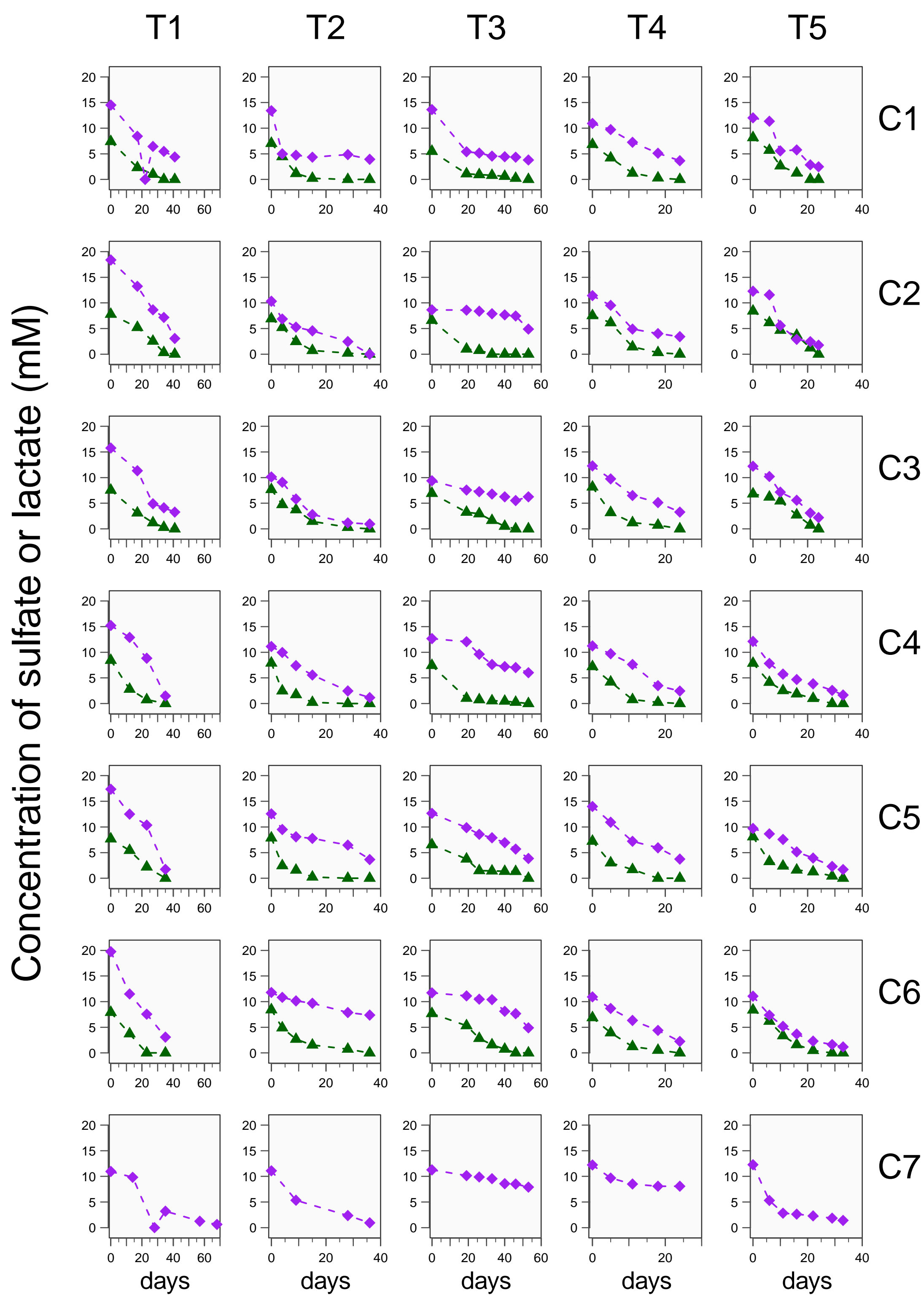

Figure 2.3 Kinetic profiles of sulfate (◆) and lactate (▲) of the seven consortia. Consortium 1 (C1) to Consortium 7 (C7), during successive transfer one (T1) to successive transfer five (T5). C1-C3: lactate (substrate), initial pH 4; C4-C6 lactate (substrate), initial pH 3; C7 glycerol (substrate), initial pH 3.

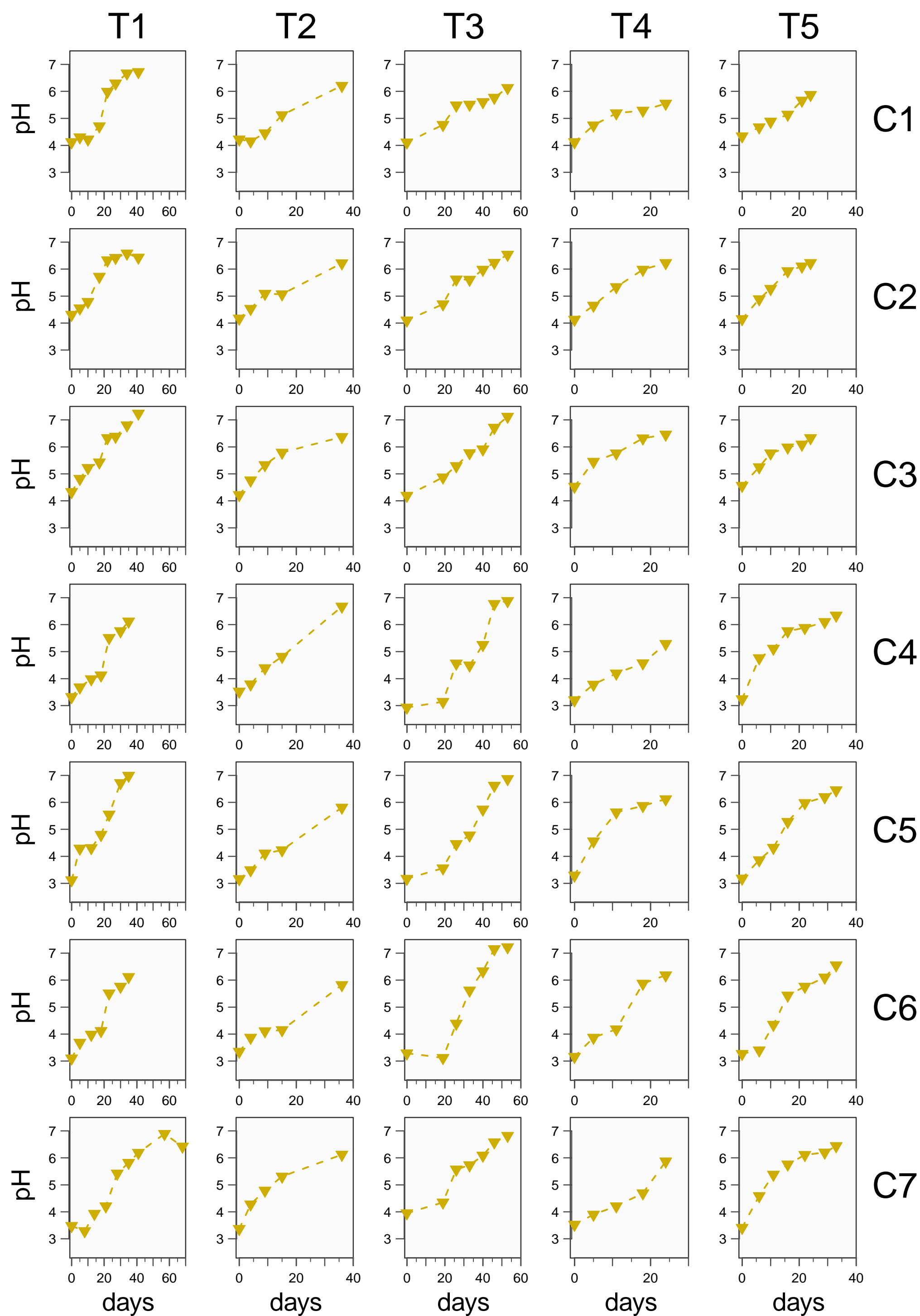

Figure 2.4. Kinetic profiles of pH (▼) of the seven consortia. Consortium 1 to Consortium 7 (C1-C7), during the successive transfer one (T1) to successive transfer five (T5). C1- C3: lactate (substrate), initial pH 4; C4 - C6 lactate (substrate), initial pH 3; C7 glycerol (substrate), initial pH 3.

Figure 2.2 shows the time profiles of acetate production/consumption and sulfide production during the five successive transfers of the seven

consortia. In most of the transfers, acetate accumulated between days 5 and 30; later, the communities consumed acetate and continued producing sulfide.

Most probably, acetate accumulated due to the incomplete oxidation of lactate or glycerol; according to the stoichiometry 1 mM of lactate can produce 0.5 mM of H_2S, 1 mM of acetate and 1mM of CO_2, and 1 mM of glycerol can produce 0.75 mM of H_2S, 1 mM of acetate and 1 mM of CO_2 (Rabus et al., 2015).

The first two transfers of Consortia 1 to 6 still had remains of the sediment due to the strategy of using 20% of slurry as inoculum. Nevertheless, from the third transfer onward, all the cultures were planktonic and free of sediment, producing sulfide and consuming acetate in a more reproducible way. In the third transfer, sulfate-reduction and acetate consumption were slower compared with the previous transfers, possibly as a consequence of getting rid of the remaining sediment, all the seven consortia behave the same (Figs. 2.2 and 2.3). The pH profiles showed that no matter at which pH value each of the consortia started, the pH increased to values between 6.1 and 7.3 (Fig. 2.4). Interestingly, the consortia started to consume acetate when the pH reached a value close to 5.5, this trend occurred in all the transfers (Figs. 2.2 and 2.4). Attempts of developing consortia using acetate as the sole electron donor for sulfate-reduction, at initial pH 3 or 4, were unsuccessful due to the high concentration of undissociated acetic acid (9.8 mM and 8.4 mM at pH 3 and 4, respectively). The sulfate-reducing rates of the successive transfers varied widely (Table 2.2) and did not show any clear tendency to increase; on the contrary, the sulfate-reducing rates decreased from transfer 1 to 3. Eventually, in the last two transfers (4 and 5), the sulfate-reducing activity increased in some cases.

2.3.2 Reproducibility of the acetate-dependent sulfate-reducing activity

In the fifth successive transfer, the cultures were devoid of sediment, and the sulfate-reducing activity remained. At this point, we considered that the consortia were cultivable and reproducible, as shown by the assays performed in triplicate (Fig. 2.5). From these results, it was possible to calculate the percentage of substrate used for sulfate reduction of each consortium based on the stoichiometry of sulfide production (Table 3). Consortia 2 and 7 used

around 75% of the electron donor (lactate or glycerol) to perform sulfate reduction, the rest of the consortia used close to 50% of the substrate for sulfate reduction that was the target activity of the culturing approach. These results indicated that the consortia were not only composed of sulfate-reducers and the successive transfer technique was accurate and appropriate for the cultivation of sulfate-reducers.

Table 2.2 Rates of acetate consumption (A) and sulfide production (B) of the seven cultivable consortia obtained in each one of the five successive transfers (T1 to T5).

A. Acetate consumption rate (mmol/L·day)

Consortium	T1	T2	T3	T4	T5
1	0.321	0.146	0.156	0.335	0.333
2	0.331	0.072	0.098	0.189	0.494
3	0.200	0.199	0.129	0.449	0.396
4	0.183	0.202	0.256	0.302	0.470
5	0.413	0.179	0.213	0.513	0.344
6	0.449	0.163	0.148	0.339	0.265
7	0.016	-0.069	0.182	0.424	0.185

B. Sulfide production rate (mmol/L·day)

Consortium	T1	T2	T3	T4	T5
1	0.209	0.159	0.225	0.427	0.259
2	0.361	0.215	0.029	0.413	0.368
3	0.495	0.158	0.080	0.348	0.397
4	0.308	0.324	0.209	0.258	0.199
5	0.440	0.108	0.126	0.347	0.140
6	0.504	0.170	0.121	0.404	0.332
7	0.160	0.135	0.035	0.476	0.173

We also calculated the acetate consumption, once acetate concentration reached a maximum and started to decrease, and sulfide production rates (Table 2.2). The rates of acetate consumption varied between 0.20 to 0.44 mmol/L·d.

Consortium 1, fed with lactate, showed the highest acetate consumption rate; the rest of the consortia were also able to use acetate as substrate at lower acetate consumption rates. Regarding sulfide production rates, these were between 0.22 and 0.28 (mmol/L·d), and Consortium 7 showed the highest sulfide production rate.

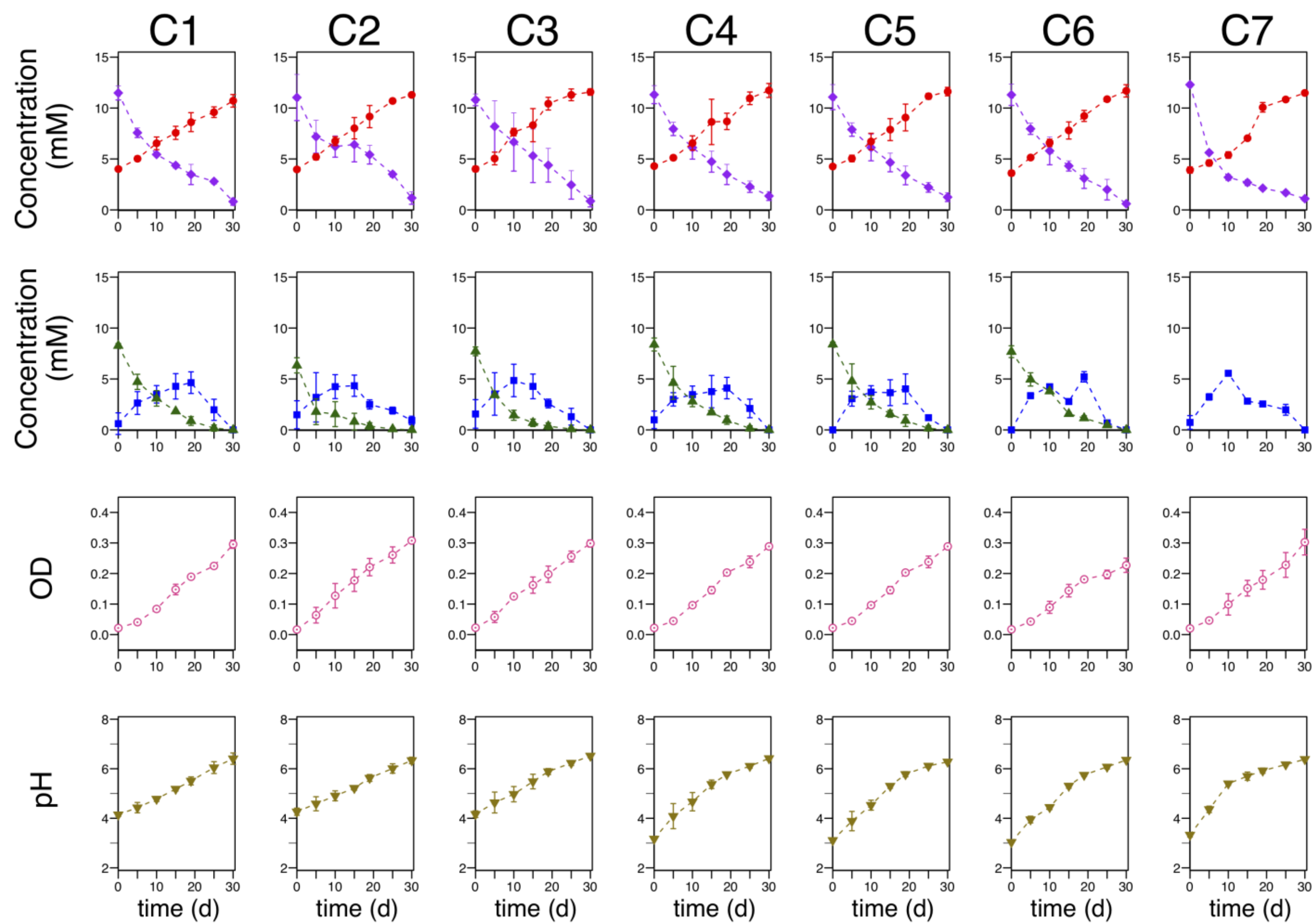

Figure 2.5 Profiles of sulfide (●); sulfate (◆); lactate (▲) and acetate (■); optical density at 600 nm (○); and pH (▼) in the triplicate assays of the seven consortia (C1 - C7) after successive transfer 5.

2.3.3 Range of favorable pH

We attempted to determine the most favorable pH at which the sulfate-reducing activity occurred comparing the rates of sulfide-production at each pH (Fig. 2.6); the selection criterion was that the difference of the sulfide production rate obtained at the different initial pH values, was lower than 0.2.

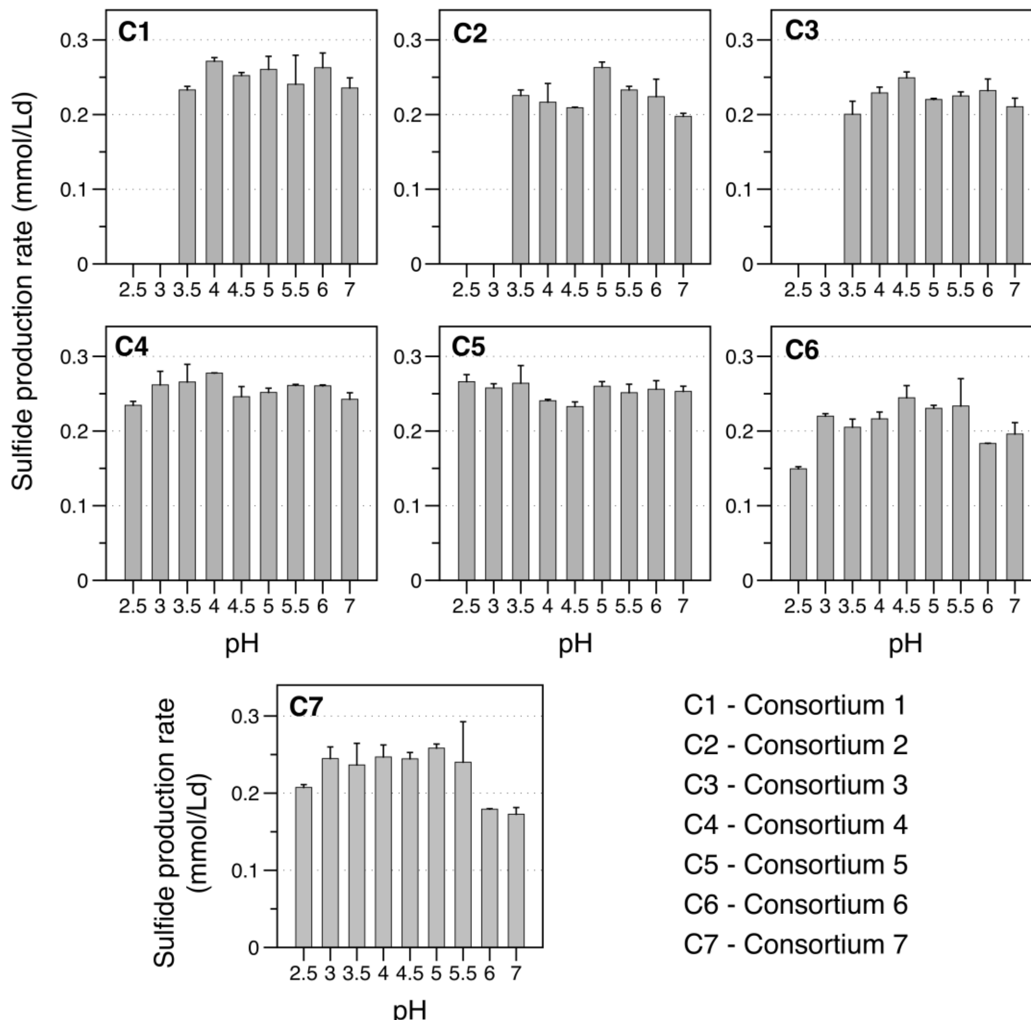

Figure 2.6 Sulfide production rates obtained at different initial pH values for each cultivable consortium.

The results showed that there was not one favorable pH value but a range at which each consortium carried out sulfate reduction optimally (Fig. 2.6 and Table 2.3). The consortia developed at initial pH 4 and fed with lactate (Consortium 1-3), performed better in the range of pH 4-6 than at pH 3.5 or pH 7. On the other hand, the consortia initially cultivated at pH 3.0 and fed with lactate performed better in a pH interval from 2.5 to 6.0 than at pH 7.0 (Consortia 4-6).

Consortium 7, fed with glycerol, showed a clear preference for acidic pH (3.0 to 5.5) to perform sulfate reduction. The initial optical density increased from a value around 0.019 ± 0.001 to values between 0.23 and 0.34 in all consortia, which is in agreement with the optical density values obtained in the sulfate-reducing activity assays (Fig. 2.5), confirming that the microorganisms of the consortia are cultivable, showing growth and not just activity.

Table 2.3 Rates of sulfide production and acetate consumption, percentage of substrate used to perform sulfate-reducing activity, and interval of favorable pH of the seven cultivable consortia.

Consortium	Sulfide production rate (mmol/L day)	Acetate consumption rate (mmol/L day)	Percentage of substrate used to perform sulfate-reducing activity	Interval of favorable pH
1	0.22 ± 0.017	0.44 ± 0.076	53.9 ± 2.17	4.0-6.0
2	0.25 ± 0.008	0.20 ± 0.070	77.8 ± 8.75	5.0-6.0
3	0.25 ± 0.018	0.28 ± 0.053	60.6 ± 4.93	4.0-6.0
4	0.26 ± 0.019	0.39 ± 0.073	59.1 ± 4.54	3.0-6.0
5	0.26 ± 0.002	0.39 ± 0.123	58.3 ± 0.625	2.5-6.0
6	0.25 ± 0.011	0.34 ± 0.059	54.1 ± 2.09	3.0-5.5
7	0.28 ± 0.007	0.25 ± 0.018	[a]75.1 ± 3.74	3.0-5.5

[a]Theoretical value

2.3.4 Microbial composition of the consortia

A total of 21 OTUs (genus level) were obtained per sample at 80-99% similarity (from 336 sequences) (Fig. 2.7). At the phylum level, all the consortia were composed of members belonging to *Bacteroidetes* (20-70%), *Firmicutes* (6-58%), and *Proteobacteria* (2-17%). Other taxa were found exclusively in some consortia.

Figure 2.7 Dendrogram based on relative abundances of the 21 OTUs, at the genus level, obtained from the seven consortia (C1-C7).

For instance, only Consortium 2 and 7 contained sequences resembling *Caldiserica* (2-7%); and sequences related to *Sphaerochaeta* (2-71%) were only present in Consortium 1, 4, and 7. Interestingly, sequences related to the unclassified *Synergistetes JGI-0000079-D21* (2-11%) were present in all the consortia except in Consortium 7. Uncultured bacteria were retrieved from almost

all the consortia (2-9%) except from Consortium 3, 4, and 6, while unclassified bacteria (non-relative) amounted to 2-15%. According to the diversity indices (Table 4), consortia 3 and 7 showed the highest richness value (S=12) in comparison with the rest of the consortia, but the Shannon-Wiener index indicated that Consortium 7 was the most diverse (H=2.106) and the less diverse was Consortium 4 (H=1.145).

Table 2.4 Diversity indices of the seven consortia. S) Richness; H) Shannon-Wiener index; D) Simpson index of dominance; and E) Evenness.

Consortium	S	H	D	E
1	11	1.675	0.708	0.698
2	10	1.745	0.768	0.758
3	12	1.958	0.797	0.788
4	9	1.145	0.472	0.521
5	9	1.694	0.753	0.771
6	9	1.181	0.497	0.537
7	12	2.106	0.839	0.847

Consortium 6 (dominated by *Lentimicrobium*) and Consortium 4 (dominated by *Sphaerochaeta*) showed the lowest Simpson' index values, whereas the rest of the consortia were equally dominated. The consortia grouped in two different clusters (Fig. 2.7), Consortium 1 and 3 (lactate, initial pH 4) showed the most similar microbial structure, as well as Consortium 2 (lactate, initial pH 4) and 5 (lactate, initial pH 3), because they grouped in the same branch. Consortium 4 and 6 (lactate, initial pH 3) clustered together in another branch having a different microbial structure from the rest of the consortia. Consortium 7 (glycerol, initial pH 3) showed a more similar structure to the cluster formed by consortia 2 and 5. The rarefaction curves of all the consortia are shown in Figure 2.8.

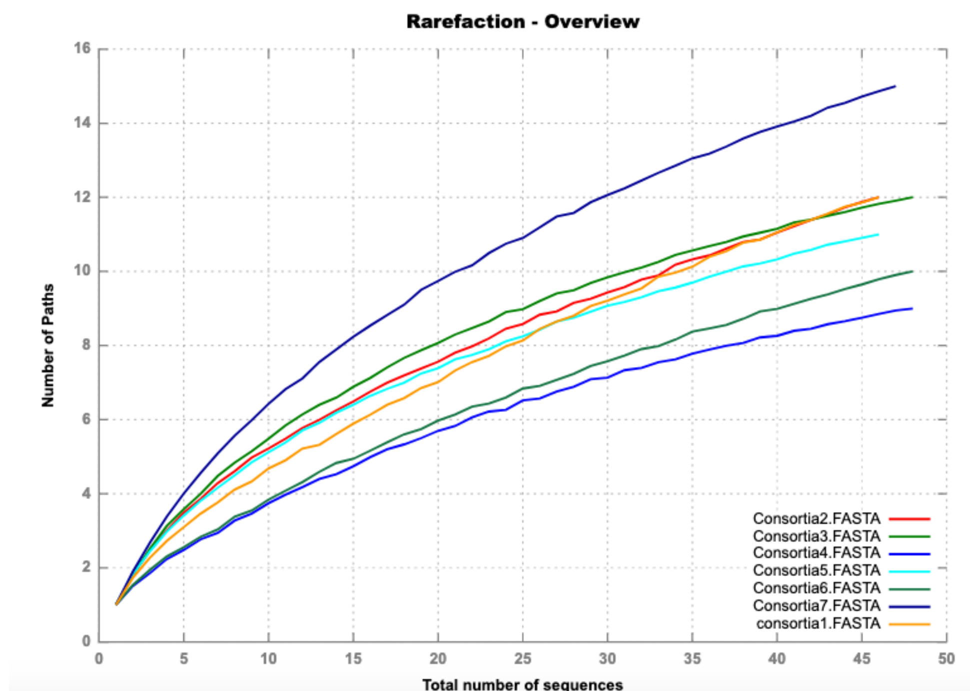

Figure 2.8 Rarefaction curves of the seven consortia.

The most dominant members of the communities at the genus level (21 OTUs) were mainly fermentative bacteria and SRM. The PCA showed no clear relationship between the initial pH value (3 or 4) and the substrates (glycerol or lactate) with the composition of the microbial community in each of the seven consortia (Fig. 2.9).

Using glycerol or lactate as electron donors, we retrieved sequences similar to *Desulfovibrio* (delta-Proteobacteria) representing 2-11% of the sequences in consortia 2, 4, 5, 6, and 7. Sequences similar (92-96%) to the genus *Desulfotomaculum* (Firmicutes) were obtained from consortia 1 and 3 representing 2% of the sequences; while *Desulfatirhabdium* (delta-Proteobacteria) was present in six consortia (91-93% similarity), with relative abundances between 2 and 13%. Sequences 94-96% similar to *Desulfurella* (delta-Proteobacteria) were found in all the consortia except in Consortium 1, the relative abundance of sequences was between 2-22%.

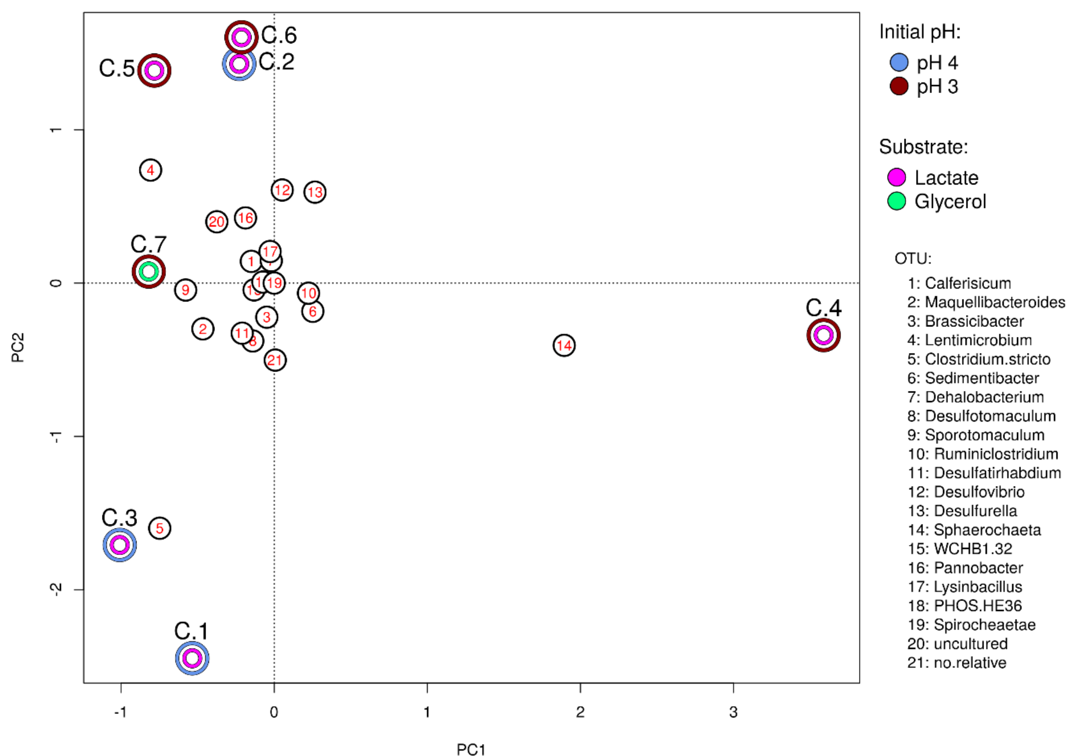

Figure 2.9. Principal component analysis (PCA) of the microbial communities of the seven consortia. C.1=Consortium 1, C.2=Consortium 2, C.3=Consortium 3, C.4=Consortium 4, C.5=Consortium 5, C.6=Consortium 6, and C.7= Consortium 7.

2.4 Discussion

Here, we report the enrichment and cultivation of seven sulfate-reducing microbial consortia able to consume acetate coupled to sulfate reduction at acidic pH. The microbial communities thriving in these enrichments carried out sulfate reduction, for over a year, in successive transfers using lactate or glycerol as the substrates. We pursued sulfate-reducing consortia free of sediment to avoid the "endogenous noise" that the sediment may cause in their characterization and further studies with them.

The percentage of substrate used to perform sulfate reduction confirmed the main function of the consortia (Table 3). Although the consortia came from the same source of inoculum (sediment) and despite using the same substrate in six of them (lactate, consortia 1-6), each consortium showed different consumption rates, denoting the presence of distinct active members in each

community, in agreement with their composition (Fig. 2.7), diversity indexes (Table 4), and PCA (Fig. 2.9). This result may be due to the unpredictable processes shaping the communities, such as random dispersal and stochastic drift, as these forces have been identified to cause some systems to exhibit divergent communities when culturing microorganisms from a heterogeneous source, such as sediments or soils (Justice et al., 2017; Wawrik et al., 2005).

During the course of each transfer, all the consortia presented the same tendency to increase the pH gradually, from the corresponding initial pH 3 or 4 to values close to neutrality (Fig. 2.4). This behavior is related to the conversion of a strong acid such as sulfuric acid to a weak acid like hydrogen sulfide and the CO_2 produced from microbial metabolism that in turn contribute to the alkalinity of the system and increment of pH (Ňancucheo et al., 2016; Rowe et al., 2007). Therefore, if sulfate reduction occurs, the drift of the pH is unavoidable in batch assays and the initial conditions (pH and substrate) have a strong influence on the functional traits (consumption/production rates) of the communities developed under such conditions (Moreno-Perlin et al., 2019).

We also observed that acetate accumulated and then consumed when the pH reached a value close to 5.0; at this pH, only 35% of acetic acid will remain undissociated, contributing to decreasing the potential toxicity of this organic acid (Figs. 2, 4, and 2.5). Possibly, when reaching pH 5, acetotrophic SRM could have coupled the oxidation of acetate with sulfate reduction (Fig. 2.5). In this study, the consortia were cultivated at initial pH 4 or 3 (Table 2.1), which in principle constrained the cultures fed with lactate; it is well known that organic acids (lactic and acetic, among others) are inhibitory at low pH because the undissociated form predominates and can cross the cell membrane lowering the intracellular pH (Bayraktarov et al., 2013). The amount of the undissociated species depends on the dissociation constants; the pKa of lactic acid is 3.08 and for acetic acid is 4.76 (Kleikemper et al., 2002). Therefore, in the experiments initiated at pH 4 or 3, the undissociated species of lactic acid amounted to 42% or 87%, respectively. In the case of undissociated acetic acid, the percentages were higher (84% at pH 4 and 98% at pH 3). Most probably, these high percentages of acetic acid prevented the cultures to succeed when we used acetate as the sole substrate. Sánchez-Andrea et al. (2013) reported the inhibition of the acidophilic sulfate

reducer *Desulfosporosinus acididurans* strain D with 5 mM lactic acid at pH 5, while nonionic substrates (glycerol, H_2, and methanol) allowed sulfate-reduction at pH values of 4.0. Given that glycerol is not toxic at acidic pH, because it does not ionize, this substrate has been used successfully to obtain sulfate-reducing consortia from natural environments (Johnson and Schippers, 2017; Qatibi et al., 1991; Sánchez-Andrea et al., 2013), nevertheless, the cultures obtained do not consume acetate.

We identified a range of pH at which each consortium performed sulfate reduction (Table 2.3 and Fig. 2.6). All the consortia showed the highest rates of sulfide production in a range of pH predominantly acidic (i.e., between 3 and 6), indicating that the enrichment technique was appropriate to obtain cultures with reproducible activity in a wide range of pH values. According to the previous classification of acidophilic microorganisms (Johnson and Schippers, 2017), all of the consortia obtained in the present work could be considered as moderately acidophilic because the communities exhibited sulfate-reducing activity at pH lower than 4. Overall, the performance of the seven consortia was very reproducible at acidic pH, which shows the robustness of the microbial communities; the consortia also consumed acetate, making them an asset for further application in the treatment of acidic effluents that contain metals. As expected, the structure of the consortia was not only composed of SRM and also included fermenters and chemoheterotrophs, in agreement with previous reports when enriching SRM from marine sediments or wastewater treatment reactors (Dar et al., 2008; Xu et al., 2014).

The majority of the consortia contained approximately 2-9% of the sequences related to thus far non-cultivable microorganisms. The sequences related to known species were between 80 to 99% similar to their closest relative, denoting the relevance and potential novelty of some of the microorganisms in the consortia. Most of the fermenters had the lowest percentage of similarity 80%, highlighting their novelty.

In all the consortia, at least one SRM was present in the community, and their global relative abundance was low (< 17%), concurrently with previous observations in sulfate-reducing communities enriched from peatlands where

SRM were present in low abundances (Hausmann et al., 2016). Regarding the SRM found in the consortia, members of *Desulfovibrio* can incompletely oxidize a wide variety of substrates including lactate, ethanol, and a few of them use glycerol (Rabus et al., 2015). They also can use hydrogen as electron donor, which was possibly produced by the fermenters present in the consortia. Microorganisms resembling *Desulfovibrio* could be responsible for the initial consumption of lactate or glycerol in the consortia and left the residual acetate for other microorganisms able to consume it, such as *Desulfotomaculum* or *Desulfatirhabdium*. Some members of the genus *Desulfotomaculum* (Firmicutes) can degrade a great variety of simple organic compounds, including acetate, formate, ethanol, lactate, and glycerol (Widdel, 2006).

The genus *Desulfotomaculum* includes spore-forming microorganisms that enable them to survive and grow in habitats that exhibit desiccation periods and low pH (O'Sullivan et al., 2015). This characteristic may explain their presence in the consortia since the primary inoculum (sediment) was retrieved from a semi-arid zone. Microorganisms resembling *Desulfatirhabdium* could be the main contributors to the sulfate-reducing activity in most of the consortia because they are classified as complete oxidizers that can use a wide variety of long- and short-chain fatty acids, including acetate (Kuever, 2014). The draft genome of *Desulfatirhabdium,* reconstructed from a metagenome, includes heavy metal and acid resistance traits that could be important for AMD remediation (Almstrand et al., 2016).

Fermentative bacteria are ubiquitous in sulfate-reducing communities, and bacteria of the genera *Lentimicrobium*, *Clostridium*, *Sphaerochaeta*, *Sedimentibacter*, *Ruminiclostridium, Sporotomaculum* and *Macellibacteroides*, may compose anaerobic microbial communities. All of them gain energy from the fermentation of complex organic matter and most probably played a key role in providing hydrogen and acetate to sulfate reducers (Grigoryan et al., 2018; Purkamo et al., 2017; Zhang et al., 2018). For instance, *Clostridium* and *Desulfovibrio* coexisted in mixed sulfidogenic cultures and cooperated in the resistance of heavy metals like Cu, Zn, and Fe (Alexandrino et al., 2014).

Overall, the performance of the seven consortia showed that the successive transfer approach was appropriate to develop stable cultures of sulfate reducers from environmental samples (i.e., sediments) with lactate or glycerol as substrates at low pH (3 or 4). Despite that obtaining the consortia was time-consuming (245 days), after five successive transfers, the cultures were devoid of the original sediment and allowed to corroborate the cultivability of the consortia and confirm that the sulfate-reducing activity remained. Our results showed that although the enrichments were cultivated at the same initial conditions, each one of the consortia turned out to be unique, as confirmed by the molecular analysis. These consortia, retrieved from the same source, represent an opportunity to use them as model communities that could help to understand the complexity of the natural community. Also, the value of the consortia is in their potential biotechnological application, given the reproducibility of the sulfate-reducing activity at acidic pH.

Chapter 3

Acetotrophic sulfate-reducing consortia develop active biofilms on zeolite and glass beads in batch cultures at initial pH 3

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Abstract

Sulfate-reducing microbial communities remain a suitable option for the remediation of acid mine drainage using several types of carrier materials and appropriate reactor configurations. However, acetate prevails as a product derived from the incomplete oxidation of most organic substrates by sulfate-reducers, limiting the efficiency of the whole process. An established sulfate-reducing consortium, able to degrade acetate at initial acidic pH (3.0), was used to develop biofilms over GAC, glass beads, and zeolite as carrier materials. In batch assays using glycerol, biofilms successfully formed on zeolite, glass beads, and GAC with sulfide production rates of 0.32, 0.26, and 0.14 mmol H_2S/L·d, respectively, but only with glass beads and zeolite, acetate was degraded completely. The planktonic and biofilm communities were determined by the16S rRNA gene analysis to evaluate the microbial selectivity of the carrier materials. In total, 46 OTUs (family level) composed the microbial communities. *Ruminococcaceae* and *Clostidiaceae* families were present in zeolite and glass beads, whereas *Peptococcaceae* was mostly enriched on zeolite, and *Desulfovibrionaceae* on glass beads. The most abundant sulfate-reducer in the biofilm of zeolite was *Desulfotomaculum* sp., while *Desulfatirhabdium* sp. abounded in the planktonic community. With glass beads, *Desulfovibrio* sp. dominated the biofilm and the planktonic communities. Our results indicate that both materials (glass beads and zeolite) selected different key sulfate-reducing microorganisms able to oxidize glycerol completely at initial acidic pH, which is relevant for a future application of the consortium in continuous bioreactors to treat acidic streams.

Keywords

Acidic pH; Acidophilic consortium; Acetate biodegradation; Glass beads; Sulfate-reduction; Zeolite

3.1 Introduction

In the last 20 years, biological sulfate reduction has attracted attention for the remediation of effluents that contain metals. Sulfate reduction offers several advantages when compared to other remediation options such as chemical precipitation, making possible the recovery of metals as metal sulfides. SRM couple the reduction of sulfate (SO_4^{2-}) to sulfide (H_2S/HS^-) with the oxidation of hydrogen and low molecular weight organic substrates such as lactate, propionate, or butyrate, among others (Kaksonen and Puhakka, 2007). Sulfide reacts with metal divalent anions forming metal sulfides that precipitate due to their low solubility (Sánchez-Andrea et al., 2014). A wide variety of reactor types can be used to treat metal-containing acidic streams by sulfate reduction (Sahinkaya et al. 2011; Habe et al. 2020). High-rate reactors with biomass retention offer the advantage of forming the metal sulfides in one step, such as the fluidized bed reactors (Papirio et al., 2013), fixed bed reactors (El Bayoumy et al., 1999), and continuous flow reactor with biomass retention (Nancucheo and Johnson, 2012).

A drawback for implementing sulfate reduction for the treatment of acidic metal-containing streams at full scale is the typical acidic nature of such effluents (Ayangbenro et al., 2018). Most known SRM are neutrophiles that are negatively affected by low pH (<5) (Kaksonen and Puhakka, 2007). Also, the efficiency to produce sulfide is limited, as not all SRM degrade the substrate completely to CO_2, usually leaving acetate as a product (Kleikemper et al., 2002). For the efficient treatment by sulfate reduction of acidic effluents that contain metals, it is desirable to use SRM communities that can oxidize the substrate completely at acidic pH (<5) (Sánchez-Andrea et al., 2014), otherwise the produced acetate may cause toxicity (Koschorreck et al., 2002). Also, since several reactor configurations for the treatment of acid streams rely on biomass retention, the sulfate-reducing community should be able to attach to the carrier material and form a biofilm (Sánchez-Andrea et al., 2014). Several types of carrier materials have been used to form sulfate-reducing biofilms; for instance, glass beads have been used in percolating columns to evaluate the tolerance to acid stress (pH 4.0-2.5), and to determine the sulfate reduction efficiency with different

combinations of carbon sources (glycerol, lactate, and ethanol) using enrichments of acidophilic and neutrophilic SRM (Kolmert and Johnson, 2001). In continuous biofilm reactors, glass beads also served as carrier material of SRM to remove sulfate from extremely acidic synthetic groundwater (pH 1.6-3.0) using glycerol as the substrate (Nancucheo and Johnson, 2014).

Nevertheless, acetate remained as the end product, but it was reported that after lowering the pH of the reactor from 4.5 to 3.0, the acetate concentration in the effluent decreased. GAC is another carrier material used to form biofilms of SRM at acidic pH (pH 5) that helped to increase the accumulation and retention of biomass, achieving sulfate removal efficiencies up to 82% and sulfate removal rates of 340 mg SO_4^{2-}/L·d in UASB reactors (Sánchez-Andrea et al., 2012). Sahinkaya et al. (2011) used activated carbon in fluidized reactors fed with real acid mine drainage and ethanol as the substrate, at initial pH of 2.7 but the substrate was incompletely degraded to acetate. Similarly, silicate minerals helped to develop sulfate-reducing biofilms and treat acidic synthetic water (pH 2.5-5.2) with lactate and ethanol as the substrates (Kaksonen et al., 2006). In this case, acetate oxidation was the rate-limiting step even when the pH of the reactor, which was not controlled, reached values from 6.8 to 7.9. Other carrier materials used to develop sulfate-reducing biofilms include polyurethane foam (Rodriguez-Freire et al., 2016; Silva et al., 2006), porous scouring pads and sand particles (Baskaran and Nemati, 2006), alumina (Silva et al., 2006), zeolite (Kim et al., 2015), and pozzolana (Battaglia-Brunet et al., 2012).

Despite the clear advances in the treatment of acidic and metal containing effluents by sulfate reduction in biofilm reactors, there is a lack of information on the composition of the communities prevailing in the biofilms and the liquid phase (planktonic community), particularly in the early stages of biofilm formation. In addition, just a few studies analyzed the microbial communities developed in the carrier material at stress conditions, such as acidic pH, reporting sulfate-reducers and fermenting bacteria as the main guilds composing the communities (Baskaran and Nemati, 2006; Montoya et al., 2013). In this work, the performance of sulfate-reducing biofilms at acidic conditions (initial pH 3) using three different carrier materials (i.e., porous glass beads, zeolite, and granular activated carbon) were evaluated with the aim of obtaining biofilms able to oxidize completely the

substrate (acetate oxidation) and characterize them. The microbial composition during biofilm development of the attached and planktonic communities was studied by Ilumina Hi-seq analysis of PCR-amplified 16S rRNA gene products. The results showed complete acetate oxidation at initial pH 3 only with zeolite and glass beads, probably by *Desulfotomaculum* and *Desulfatirhabdium*, and microbial community selectivity depending on the carrier used.

3.2 Materials and methods

3.2.1 Mineral basal medium

The mineral basal medium used in all the experiments contained (mM): 3 KH_2PO_4, 3 $Na_2HPO_4 \cdot 2H_2O$, 50 NH_4Cl, 30 NaCl, 40 $MgCl_2 \cdot 6H_2O$, 75 $CaCl_2 \cdot H_2O$, 1 mL/L trace element solution (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 $ZnCl_2$), and 0.1 g/L of yeast extract (modified from Stams et al. 1993). The medium was supplemented with 10 mM Na_2SO_4 as the electron acceptor and the stoichiometric concentration of glycerol (electron donor) 5.71 mM. The pH was adjusted to 3 using 1 N HCl before autoclaving.

3.2.2 Carrier materials

Three different carrier materials: glass beads, GAC , and zeolite were used to develop the sulfate-reducing biofilms, Table 3.1 provides details about the carriers. Before use, each material was washed several times with deionized water until the rinse liquid was clear; then, the materials were dried at 105°C for 4 hours. Each carrier was left overnight in a serum bottle containing mineral basal medium (pH 2.5, without glycerol, sulfate and yeast extract) under continuous stirring (100 rpm).

Table 3.1 Main characteristics of the support materials.

Material	Supplier	Shape	Size (mm)	Density (g/mL)	External surface area[a] (m^2/g)	Total specific surface area (m^2/g)	pH[d]
Porous glass beads (Poraver)	Dennert GmbH, Germany	Regular spheres	0.5-2.0	0.4	0.008-0.03	Not available	10.7
Granular activated carbon 830W (GAC)	Cabot Norit, Nederland B.V.	Irregular	0.7-2.0	0.7	0.003-0.067	950-1150[b]	Alk
Zeolite	Zeolite Products, Varsseveld, Netherlands	Irregular	0.1-2.5	0.9	0.004-0.012	300 – 700[c]	9.0

[a]Potentially colonizable, corresponds to the outer surface area. Zeolite and GAC were approximated to spherical particles.
[b]Combarros RG et al. 2014. Influence of biofilm on activated carbon on the absorption and biodegradation of salicylic acid in wastewater. Water Air Soil Pollt. 225: 1858. DOI 10.1007/s11270-013-1858-9 and Data Sheet Cabot Norit Activated Carbon – Norit® GAC 830W.
[c]Reeve and Fallowfield (2018)
[d]According to the manufacturer's specifications. Alk: alkaline

Subsequently, the medium was discarded, and fresh mineral basal medium was replenished to the bottle with the carrier material, this time the medium contained sulfate, glycerol, yeast extract and adjusted to pH 3. The bottle was autoclaved (20 min, 15 psi), and finally inoculated under anaerobic conditions (N2/CO2, 80:20%).

3.2.3 Source of microorganisms

The inoculum was an acid-tolerant sulfate-reducing consortium that degraded glycerol and acetate to CO_2 at initial pH 3. The consortium used 75% of the substrate (glycerol) for sulfate reduction and formed microbial aggregates (Campos-Quevedo et al., 2021a). The sample to develop this consortium was originally retrieved from a contaminated sulfur mine in Mexico that was enriched for over one year by gradually reducing the pH from 6 to 4 (Moreno-Perlin et al. 2019).

3.2.4 Experimental design

For each carrier material, a total of 15 experimental units (serum bottles) were set-up (Fig.3.1). Three of the experimental units served to conduct the sulfate-reducing activity (triplicate assay); the other 12 experimental units were opened (sacrificed) at regular intervals to obtain DNA samples. Each bottle (120 mL) contained 40 mL of anaerobic medium, 15 mL of carrier material, and 20% (v/v) of inoculum. Additionally, abiotic controls were set-up, which only contained medium (40 mL) and the carrier material (15 mL); while the biologic control for all the experiments only contained medium and inoculum. The headspace of all the bottles was flushed with 1.5 atm (N_2/CO_2; 80:20%). Before adding the inoculum, all the bottles containing zeolite, glass beads, granular activated carbon, and the bottle of the biological control, were sterilized (120 °C, 20 minutes). After inoculation, the bottles were incubated at 30°C in the dark and agitated (75 rpm). Samples were taken from the triplicate and control assays to determine sulfate, sulfide, pH, and volatile fatty acids at days 0, 5, 7, 10, 12, 15, 20, 25 and 30. On

the same days, one bottle (from the remaining 12 sacrifice bottles) was opened to obtain DNA from the carrier material and the liquid phase, all the DNA samples were stored at -20 ºC. Before opening the bottle, samples were taken to determine sulfate, sulfide, pH, and volatile fatty acids and make sure of the reproducibility compared to the triplicate assays. After finishing and analyzing the kinetic profiles of the triplicate assays, only those experiments that performed similarly to the biological control, this is, the experiments that achieved complete oxidation of the substrate, were selected for subsequent DNA analysis and SEM imaging; therefore, we discarded GAC for further processing.

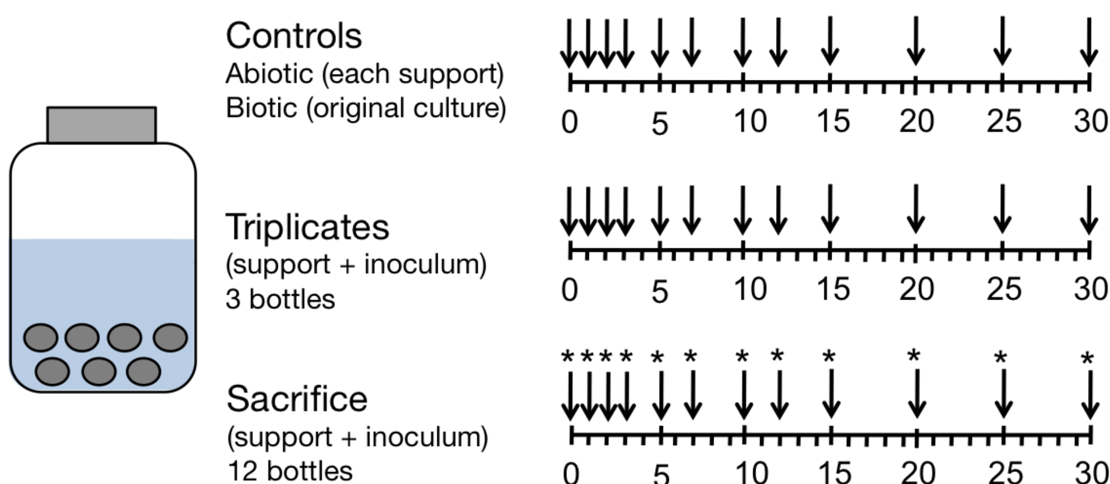


Figure 3.1 Experimental set-up showing the sampling days. Arrows refer to samples for chemical analysis, and asterisks indicate that DNA was extracted from the biofilm and liquid, and stored at -20ºC.

To analyze the communities, from the stored DNA samples, we selected those corresponding to the consumption of glycerol and acetate. With zeolite, glycerol was consumed on day 7 and acetate on day 25, whereas with glass beads, the consumption was on days 10 and 30, respectively. Only the DNA samples from zeolite and glass beads were analyzed by Ilumina Hi-seq sequencing because only in these two experiments the acetate was consumed completely.

3.2.5 Physicochemical analyses

Glycerol and its products (acetate, propionate, butyrate, ethanol, and 1,3 propanediol) were quantified using LKB High-Performance Liquid Chromatography (Thermo Scientific SpectraSystem HPLC, Waltham, MA) fitted with a Varian Metacarb 67H 300 mm column (Varian, Walnut Creek, CA), using H_2SO_4 (0.01 N) as eluent at a flow rate of 0.8 mL/min. The methylene blue spectrophotometric method determined sulfide in the liquid phase (Broenkow and Cline, 1969). Sulfate concentrations were quantified using a Dionex ICS-1000 ion chromatograph (Thermo Scientific, Waltham, MA). The pH was measured with a Thermo Scientific TM Orion TM VersaStar potentiometer.

3.2.6 Scanning electron microscopy analysis (SEM)

Samples of zeolite and glass beads were taken for SEM images when glycerol and acetate were depleted. Since the consumption occurred at different times, the samples for zeolite were taken at days 7 (glycerol consumption) and 25 (acetate consumption) and for glass beads at days 10 (glycerol consumption) and 30 (acetate consumption). Samples of the raw materials were also observed. The samples of zeolite and glass beads were fixed to poly L-lysisn 12 mm coated coverslips (Corning, BioCoat), and incubated for 5 hours at room temperature. The biofilms attached to the carrier materials were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4) for 2 hours and then rinsed with 0.1 M of PBS buffer and post-fixed with 1% osmium tetroxide for 1 hour. The samples were then dehydrated with ethanol series (10, 30, 50, 70, 80, 96, and 100%), dried to the critical point in 100% ethanol in a Leica EM CPD300 system (Leica Microsystems); finally, the samples were mounted onto aluminum stubs and coated with tungsten.

3.2.7 Community composition

DNA was extracted from the zeolite and glass beads experiments, from both the liquid and solid phases, using the FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA), following the manufacturer's instructions. The samples were taken from the sacrifice bottles of zeolite at days 7 and 25, and from glass beads

at days 10 and 30, these days corresponded to depletion of glycerol (day 7 or 10) and acetate (day 25 or 30). The DNA of the inoculum was also extracted. The DNA concentration and purity was checked with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and adjusted to a concentration between 10−20 ng/μL, and used as the template for PCR amplification. PCR was performed in a final volume of 100 l containing 1X HF PCR buffer, 0.2 mM dNTPs, 2 U/μL of Phusion Hot Start II DNA polymerase (Promega, Madison, WI), 10 μM of forward and reverse primer mixture, 200 μM of barcoded forward primer with titanium sequence adaptor, 338R-I+II (Biolegio BV, Nijmegen, The Netherlands), 0.1−0.6 ng/μL of the template DNA and nuclease-free water (to final volume). The PCR program was as follows: initial denaturation (98 °C, 30 s), 30 cycles of denaturation (98 °C, 10 s), annealing (54 °C, 30 s), and extension (72 °C, 30 s), and a final extension step (72 °C for 10 m). The amplicons were visualized after gel electrophoresis in agarose (1% w/v) with 1x SYBR Safe (Invitrogen, Carlsbad, CA). The negative control (water) was amplified in parallel with no product.

The PCR products were purified (High Pure Cleanup Micro Kit, Roche, Basel, Switzerland) and pooled in equimolar amounts at a final DNA concentration of 200 ng/μL. High-throughput sequencing of the pooled amplicons was performed in an FLX Genome Sequencer combined with titanium chemistry (GATC-Biotech, Konstanz, Germany). 16S rRNA gene sequencing data was analyzed using NG-Tax (Ramiro-Garcia et al., 2016). This pipeline was used to demultiplex the reads by sample using the barcodes. Operational taxonomic units (OTUs) were defined using SILVA 16S rRNA gene reference database (Quast et al., 2013). For subsequent analysis QIIME 2 (v.2.2019.1) was used.

Two mock communities Mock3 and Mock4, developed in the MolEco Laboratory of Wageningen University, were used as controls; the correlation coefficient between the reads of the standard and the analyzed mock communities was 0.18 for both (Mock 3 and Mock 4). The negative control (reactants with water) yielded only 173 reads representing 0.09% of the average of the total reads of all the samples (~190,000). We used the ecology package 'vegan' (Oksanen et al., 2017) and R version 3.4.2 (R Core Team, 2005) to calculate the distance among the samples, and to obtain the richness, Shannon-

Wiener index and Simpson index of dominance and evenness. The sequences are deposited in the NCBI nucleotide sequence database GenBank under the BioProject accession number: PRJNA646005.

3.2.8 Statistical analysis

Non Metric Multidimensional Scaling (NMDS) and Redundancy Analysis (RDA) were done with Software R (version 3.4.2) (R Core Team, 2005) and package 'vegan' (Oksanen et al., 2017) using RStudio software (version 1.1.383; RStudio Inc., Boston, MA).

3.3 Results

3.3.1 Sulfate-reducing activity profiles

The assays performed in triplicate helped to monitor the kinetics of glycerol and sulfate consumption, and the production of sulfide, acetate, propionate, and other by-products. In the assays with GAC and zeolite, it was not possible to detect sulfide because these materials have functional groups that adsorbed sulfide. Therefore, the production of sulfide was calculated from the reduction of sulfate, taking into account the abiotic controls of each material (Figs. 3.2, and 3.3). In this way, the kinetic profiles of the sulfide production with zeolite and GAC were constructed. Zeolite and glass beads allowed the sulfate-reducing consortium to perform similarly to the biological control, in terms of sulfide production (Fig. 3.2) and glycerol and acetate consumption (Fig. 3.4). The results also show that the sulfate-reducing activity improved when using zeolite as carrier material. In the case of GAC, it was not possible to reproduce the performance of the consortium without support, moreover, acetate was not consumed within 30 days as in the original inoculum (biological control) (Figs. 3A and 3.E).

In the majority of the assays sulfate was gradually consumed, reaching a final concentration close to 3 mM at day 30 and concentrations of sulfide between 6-7 mM (Figs. 3.2 and 3.3), including the biological control without carrier (8.6 mM) (Fig. 3.2E). Only in the assays with zeolite there was no sulfate at day 30, and the sulfide produced (9 mM) was the highest (Fig. 3.2A). The assays with

glass beads showed a sulfide concentration of 7.5 mM, whereas with GAC the lowest sulfate-reduction activity was attained (< 6 mM H_2S) (Figs. 3.2B and 3.3B). In every experiment the pH increased gradually to neutrality (*ca.* 7, Fig. 3.2 and 3.3B) because the pH was not controlled.

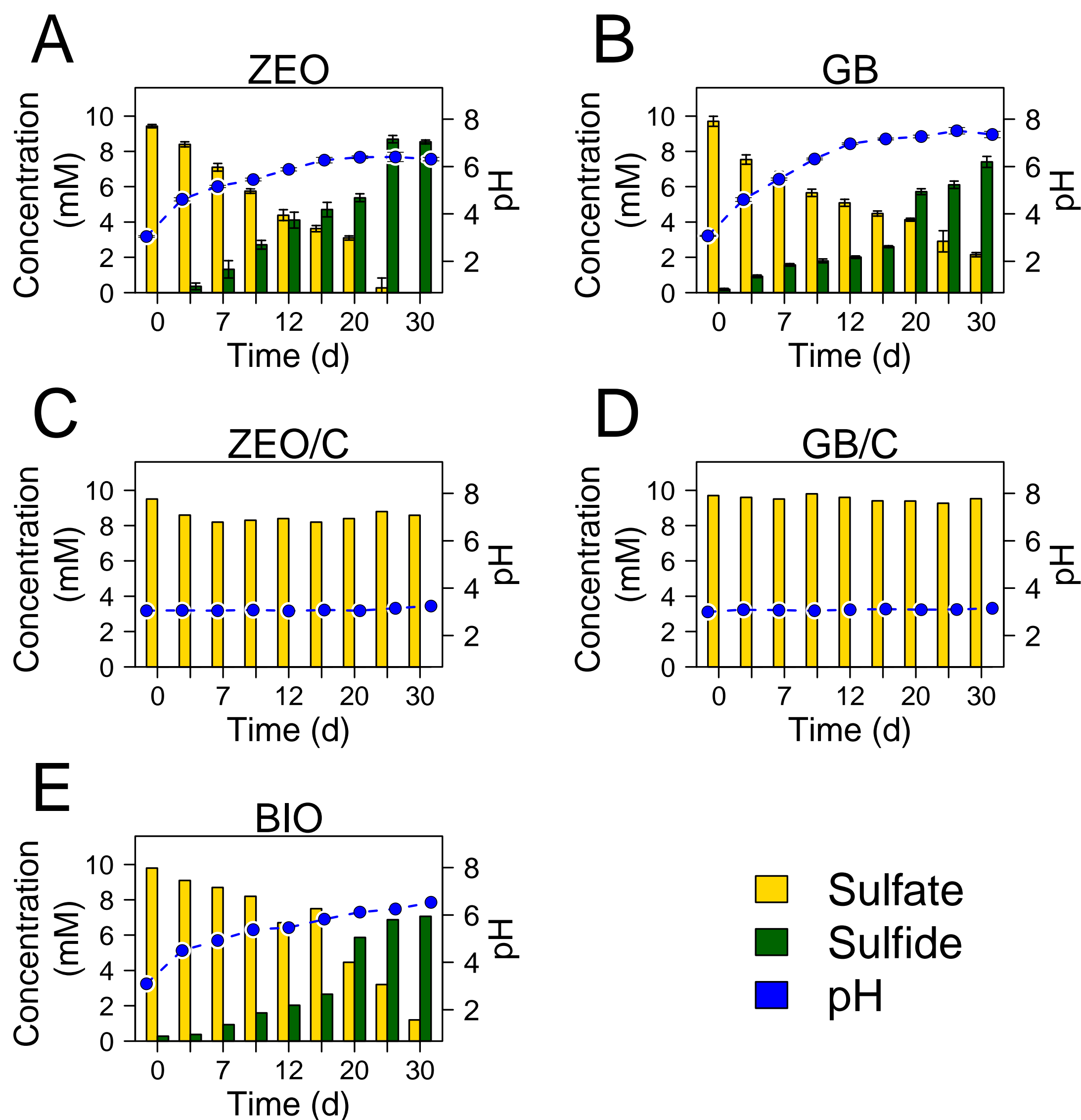


Figure 3.2 Kinetic profiles of sulfate consumption, sulfide production, and pH in the experiments with A) Zeolite (ZEO); B) Glass beads (GB); and E) Biological control (BIO) without carrier material. Profiles of sulfate and pH in the abiotic experiments with C) Zeolite (ZEO/C) and D) Glass beads (GB/C).

The main differences among the experiments are appreciated from the analysis of the substrate fate (Figs. 3.3 and 3.4). Glycerol was completely consumed within 7 to 10 days depending on the carrier material, being faster with

zeolite (7 days) similar to the biological control (Fig. 3.4E), than with glass beads or GAC. Using zeolite or glass beads as carrier material, glycerol was first degraded to acetate and propionate.

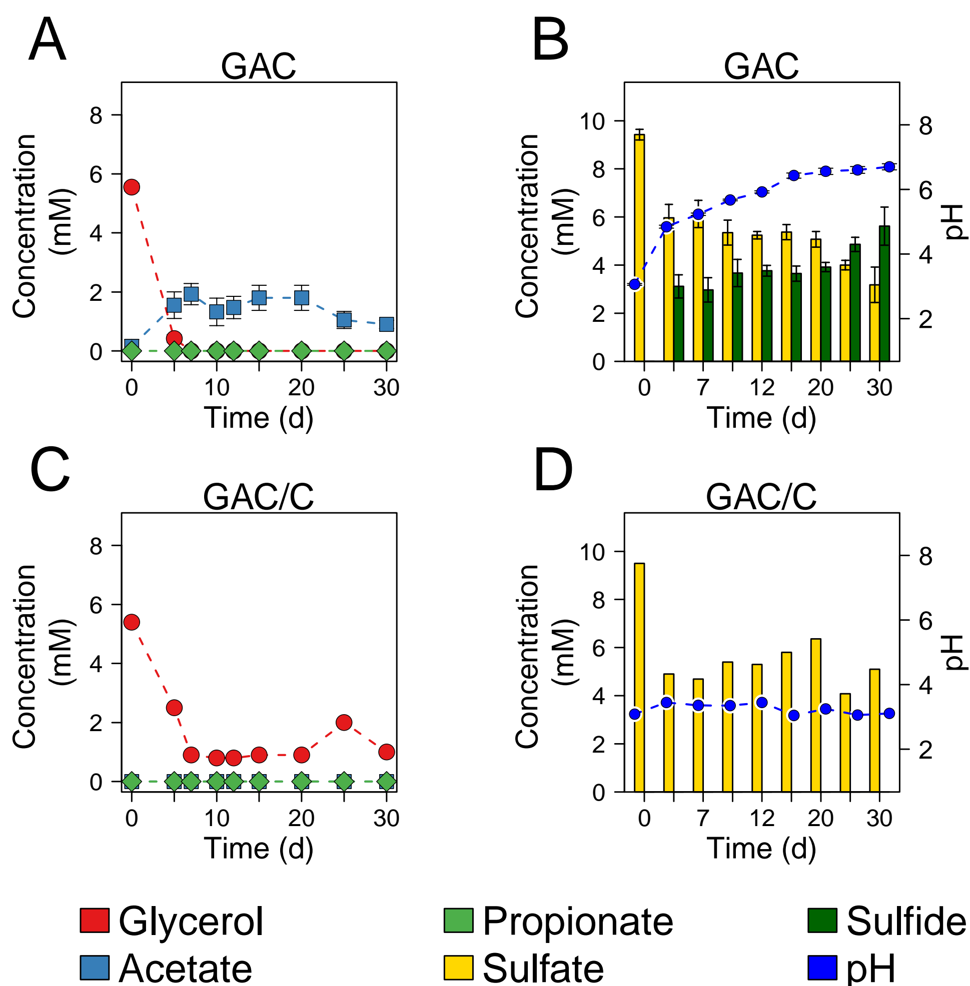


Figure 3.3 Kinetic profiles of the experiments with GAC. A) Glycerol consumption and intermediates production. B) Consumption of sulfate, production of sulfide and pH. C) Concentration of glycerol and intermediates in the abiotic control. D) Concentration of sulfate and pH in the abiotic control.

In the biological control and with glass beads, acetate was completely consumed by day 30 (Figs. 3.4E and B), while acetate was consumed earlier (day 25) in the zeolite experiments (Fig. 3.4A). The consumption of glycerol also yielded some propionate (1.5 mM, day 7), with zeolite as carrier, that was consumed by day 10. In the assay with glass beads the produced propionate was

consumed by day 20 (Fig. 3.4B). In contrast, the acetate produced in the GAC assays was not completely consumed and 1 mM remained on day 30 (Fig. 3A). The analyses of the abiotic control experiments gave a better understanding of the possible effects of the carrier materials on the development of the sulfate-reducing process (Figs. 3.2 to 3.4). The adsorption processes in glass beads are negligible because no substantial change occurred in sulfate (~9.3 mM), pH (~3.08), and glycerol (~5.5 mM) concentrations in the abiotic control (Figs. 3.2D and 3.4D).

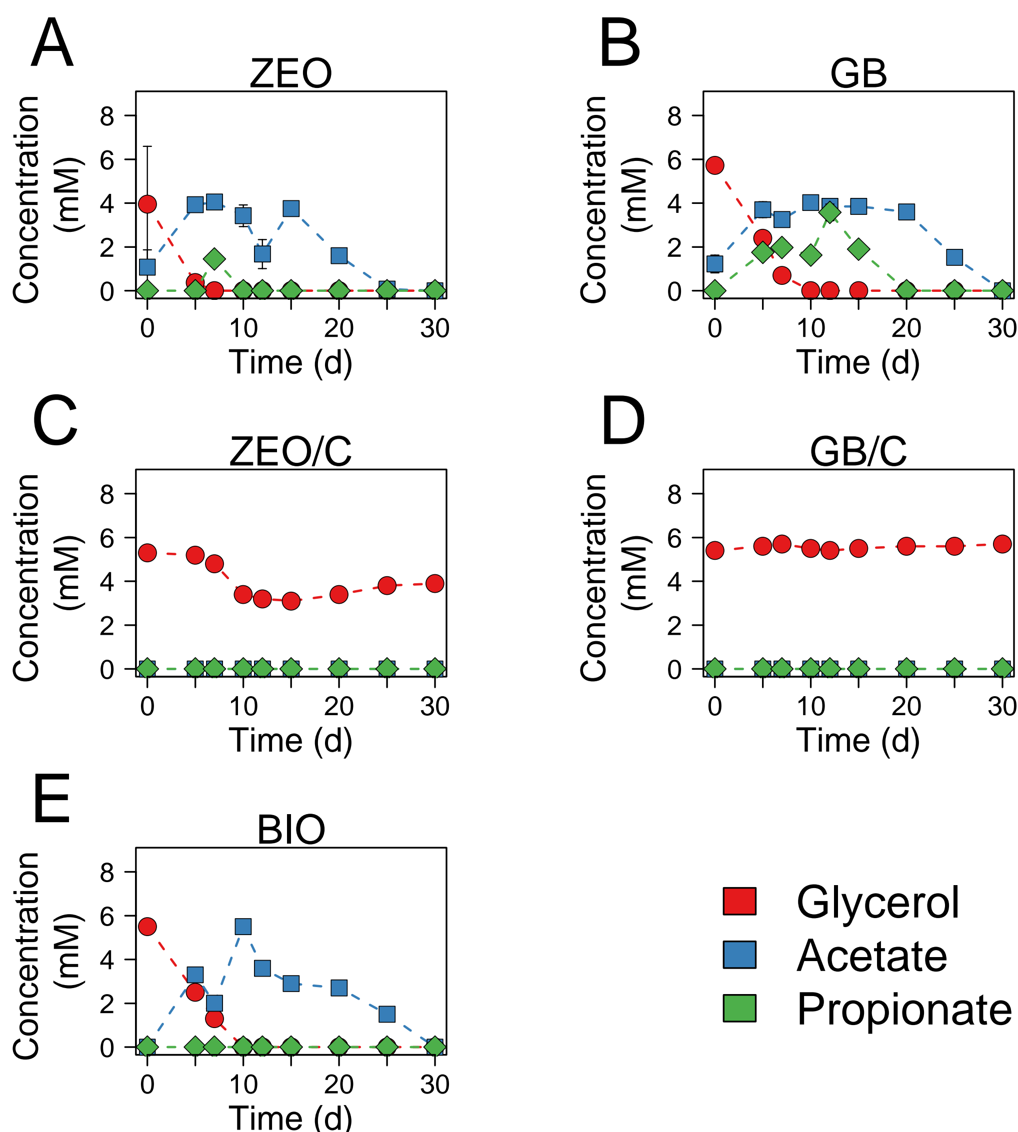


Figure 3.4. Consumption of glycerol and formation of intermediaries with the different carrier materials and abiotic controls: A) Zeolite (ZEO); B) Glass beads (GB); C) Abiotic control of zeolite (ZEO/C), D) Abiotic control of glass beads (GB/C), and E) Biological control (BIO) without carrier material.

Conversely, when using zeolite or GAC, some adsorption and desorption effects were observed. With zeolite, there was a slight absorption of sulfate and glycerol, whereas the pH did not change (~3.09) (Figs. 3.2C and 3.4C), but when comparing with the biotic experiments, the biological consumption of glycerol (Fig. 3.4A) predominated being faster than the absorption process (Fig. 3.4C). The abiotic assays with GAC presented a more notorious effect of the adsorption/desorption of sulfate and glycerol showing maximum adsorption values, ~5.68 mM of sulfate and ~4.6 mM mM of glycerol (Figs. 3.3D and C). The adsorption/desorption in the abiotic controls with zeolite and GAC were considered to calculate the sulfide concentration with these carrier materials.

Even though we did not determine the adsorption of acetate and propionate on zeolite or GAC in abiotic experiments, comparing with glass beads and with the biological control we can infer that the absorption of acetate was not relevant.

3.3.2 Rates of sulfate reduction in the batch assays

The kinetic profiles obtained with zeolite, glass beads, GAC, and in the absence of carrier material (biological control) served to calculate the rates of acetate consumption and sulfide production, sulfide yield, and the percentage of substrate used for sulfate reduction (Table 2).

The values obtained with zeolite were the highest, even higher than in the biological control, the electron donor was almost completely used for sulfate reduction (~ 90%), followed by the glass beads and the biological control (*ca.* 70%). The assays with GAC used close to 60% of the substrate for sulfate reduction, acetate remained as by-product and was not further metabolized despite that glycerol was completely consumed (Fig. 3.3). Regarding the yield of sulfide, the assays with zeolite had the highest calculated yield of sulfide produced per glycerol consumed, followed by the glass beads, biological control, and GAC. Zeolite increased the yield of sulfide by 21% compared to the biological control with no carrier material. Accordingly, zeolite also showed the highest rates of sulfide production and acetate consumption, followed by the biological control,

glass beads, and GAC. Considering these results, we discarded GAC as carrier material for further analysis.

Table 3.2 Sulfide yield, percentage of substrate used for sulfate-reduction, sulfide production rate, and acetate consumption rate with the different carrier materials and in the biological control without carrier material.

Experiment	Yield (mmol H_2S/mmol glycerol)	Substrate used for sulfate reduction (%)	Sulfide production rate (mmol/L d)	Acetate consumption rate (mmol/L d)
Glass beads	1.3 ± 0.05	72.7 ± 0.43	0.26 ± 0.004	0.20 ± 0.012
Zeolite	1.5 ± 0.18	92.6 ± 1.2	0.32 ± 0.011	0.40 ± 0.032
Granular activated carbon	0.98 ± 0.13	58.0 ± 8.2	0.14 ± 0.024	0.15 ± 0.093
Biological control	1.23	71.4	0.27	0.23

SEM images helped to corroborate the attachment of microorganisms on zeolite and glass beads (Fig. 3.5). Initially, after inoculation (day 0), we observed the surface of both materials with practically no attached microorganisms. On days 7 and 10, when glycerol was depleted in the experiments with zeolite and glass beads, respectively, several microorganisms were attached to the surface of the materials indicating the initial formation of a biofilm. At the end of each experiment, on days 25 or 30, more microorganisms were attached to the surface of both materials (Fig. 3.5). The change in the aspect of the carrier materials (zeolite and glass beads) due to the formation of biofilms was also visible to the naked eye (Fig. 3.6).

3.3.3 Microbial composition

The community analysis of the DNA samples, withdrawn when glycerol and acetate were depleted, yielded information about the microorganisms attached to zeolite and glass beads and those that remained in the liquid (planktonic community). The OTUs richness (S) was different between the carrier materials, glass beads had the lowest richness and the values were similar between the phases (planktonic and biofilm) (S = 30-38) (Table 3.3).

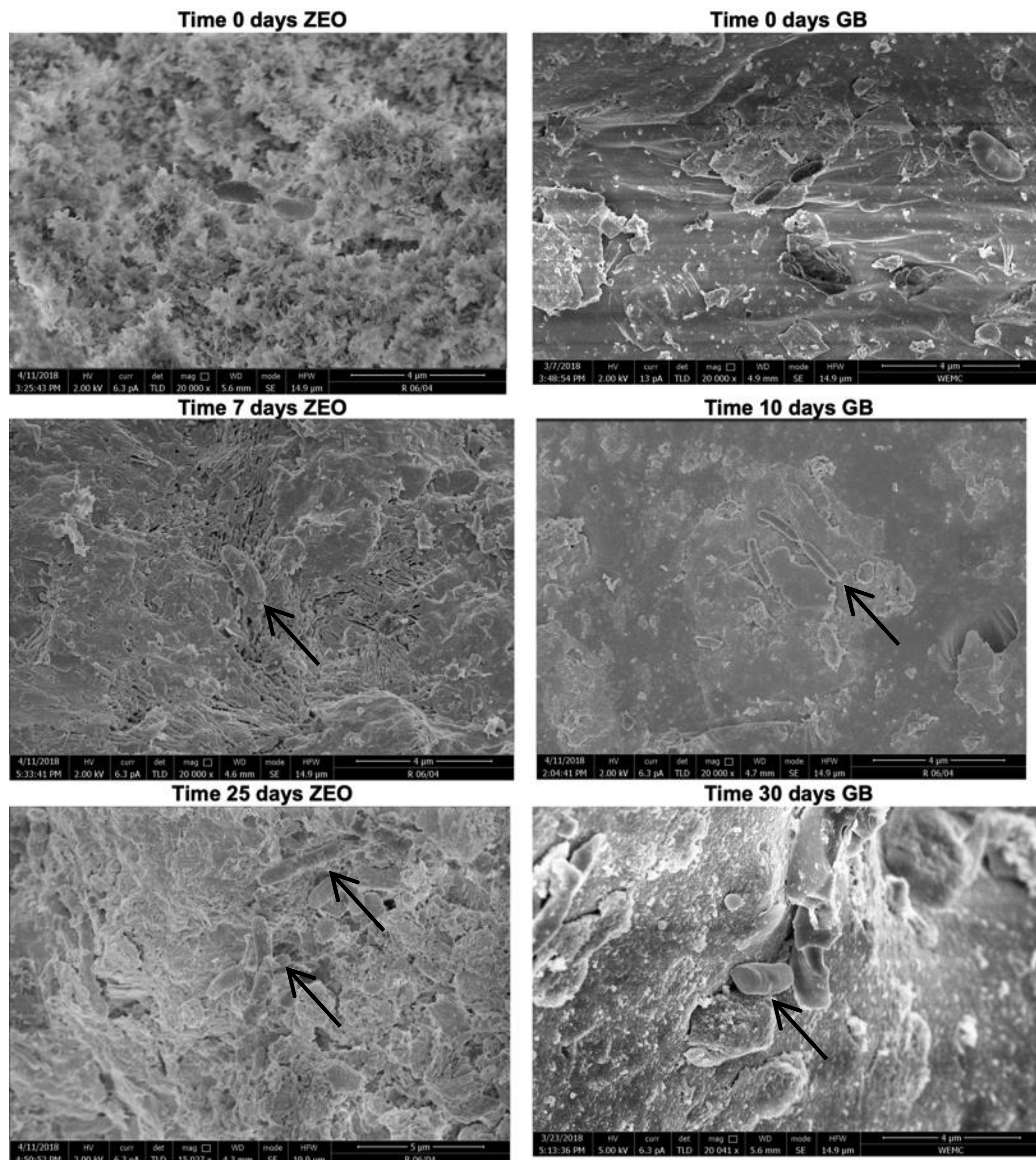


Figure 3.5 Scanning electron microscopy (SEM) images of zeolite (ZEO) and glass beads (GB). The arrows show the microorganisms attached to the surface of glass beads and zeolite.

The samples from the zeolite experiments had the highest values of richness (S = 48-61) in both phases. Accordingly, the Shannon-Wiener index (H) indicated more diversity in the zeolite samples than in the glass beads samples (Table 3.3).

A total of 5,134,558 reads with an average of ~190,168 reads per sample were obtained. The family level's taxonomic affiliation revealed that 46 OTUs composed the communities (Figs. 3.7 and 3.8). All the samples, including the inoculum, shared a basic core of four families (relative abundance >1%) Clostridiaceae, Desulfovibrionaceae, Porphyromonadaceae, and Ruminococcaceae. The inoculum was dominated by Ruminococcaceae (55 ± 4.5%), followed by Desulfobacteraceae (11.2 ± 2.0%), Lentimicrobiaceae (9.0 ± 0.8%) and Clostridiaceae (5.5 ± 0.8%).

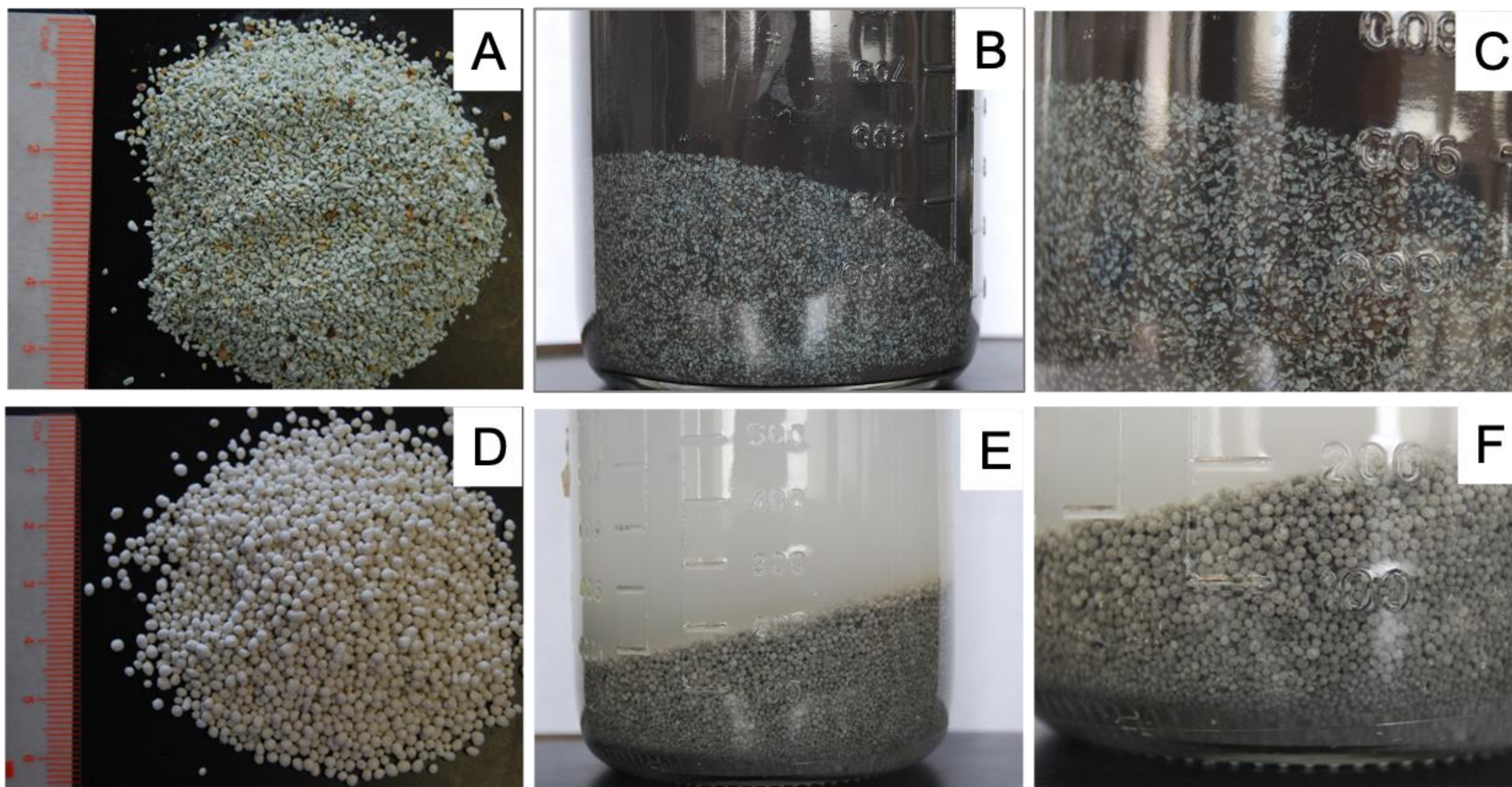


Figure 3.6. A) Zeolite before the experiment. B) Zeolite after 25 days of experiment. C) Detail of zeolite at day 25. D)Glass beads before the experiment. E) Glass beads after 30 days of experiment. F) Detail of glass beads at day 30. Nikon D40X.

Table 3.3 Diversity indexes of the consortia developed on zeolite and glass beads. Richness (S), Shannon-Wiener index (H), Simpson index of dominance (D), and Evenness (E).

Sample[a]	S	H	D	E
GB solid 10	37.00 ± 1.00	2.58 ± 0.05	0.87 ± 0.01	0.71 ± 0.01
GB solid 30	38.67 ± 5.03	2.48 ± 0.08	0.84 ± 0.01	0.68 ± 0.01
GB liquid 10	37.33 ± 1.00	2.91 ± 0.05	0.93 ± 0.00	0.80 ± 0.00
GB liquid 30	30.00 ± 3.61	2.74 ± 0.04	0.91 ± 0.00	0.81 ± 0.02
ZEO solid 7	61.67 ± 1.53	3.11 ± 0.02	0.90 ± 0.00	0.76 ± 0.01
ZEO solid 25	51.67 ± 4.04	2.85 ± 0.05	0.89 ± 0.01	0.72 ± 0.01
ZEO liquid 7	48.00 ± 2.00	2.68 ± 0.02	0.86 ± 0.00	0.69 ± 0.01
ZEO liquid 25	61.33 ± 6.11	3.20 ± 0.05	0.92 ± 0.00	0.78 ± 0.01

[a]GB: Glass beads; ZEO: Zeolite; the number indicates the day when the sample was taken.

The relative abundances of Desulfovibrionaceae, Porphyromonadaceae, Family XI, Caldisericaceae, Mesoaciditogaceae, Pseudomonadaceae, Spirochaetaceae and Thermodesulfobiaceae were between 4 and 1.1%, the rest of the families were represented in less than 1%; including families related to the sulfur cycle (Desulfurellaceae and Peptococcaceae, Figs. 3.7 and 3.8).

The structure of the community was different depending on the carrier material, the type of sample (planktonic/biofilm), and the incubation stage (early/late). For instance, in the incubations with zeolite (Fig. 3.8), *Clostridiaceae* (37–51%) was consistently the most abundant family in all the samples (planktonic/biofilm). Members of the other families were mainly present in the carrier material in the early (7 days) and late stages (25 days), such as *Ruminococcaceae* (15.6 and 12.8%, respectively). Conversely, *Peptococcaceae,* initially abundant in the planktonic community (32.3%), was found mostly attached to the carrier material (22.7%) than in the liquid (4.5%) after 25 days. It is worth to note that the relative abundance of *Peptococcaceae* in the inoculum was low (0.07%). Overall, in the late-stage (25 days) of the assays with zeolite, the biofilm held seven dominating families with relative abundances higher than 1%: *Clostridiaceae* (40.2%), *Peptococcaceae*

(22.7%), *Ruminococcaceae* (12.8%), *Desulfurellaceae* (6.8%), *Porphyromonadaceae* (4.3 %), *Desulfobacteraceae* (2.2%), and *Desulfovibrionaceae* (1.9%).

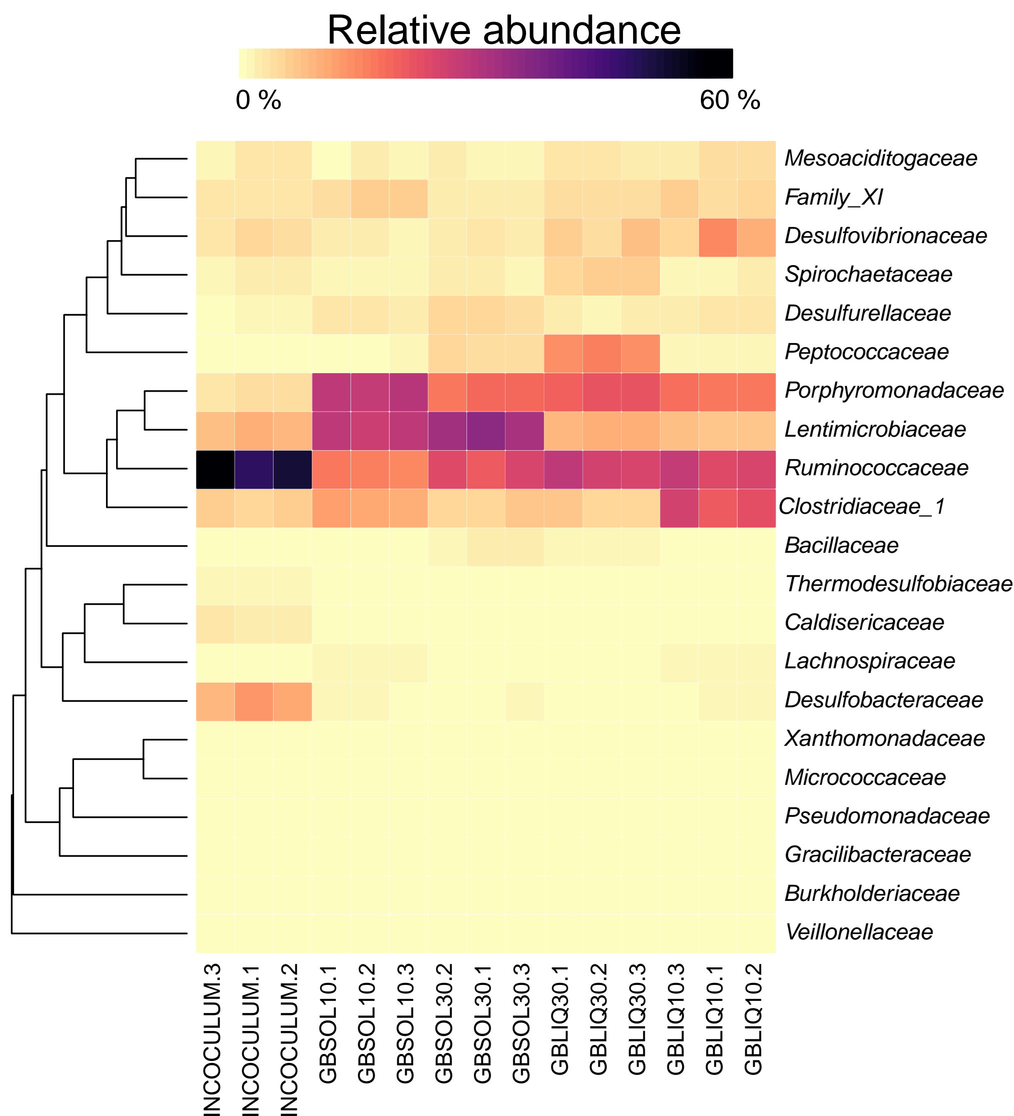


Figure 3.7 Heatmap of the microbial community at the family level in the experiment with glass beads. SOL denotes the biofilm community developed over the carrier material and LIQ the planktonic community, in the early (day 10), and late (day 30) stages. The community of the inoculum is also shown. The number after the period indicates the number of the replica.

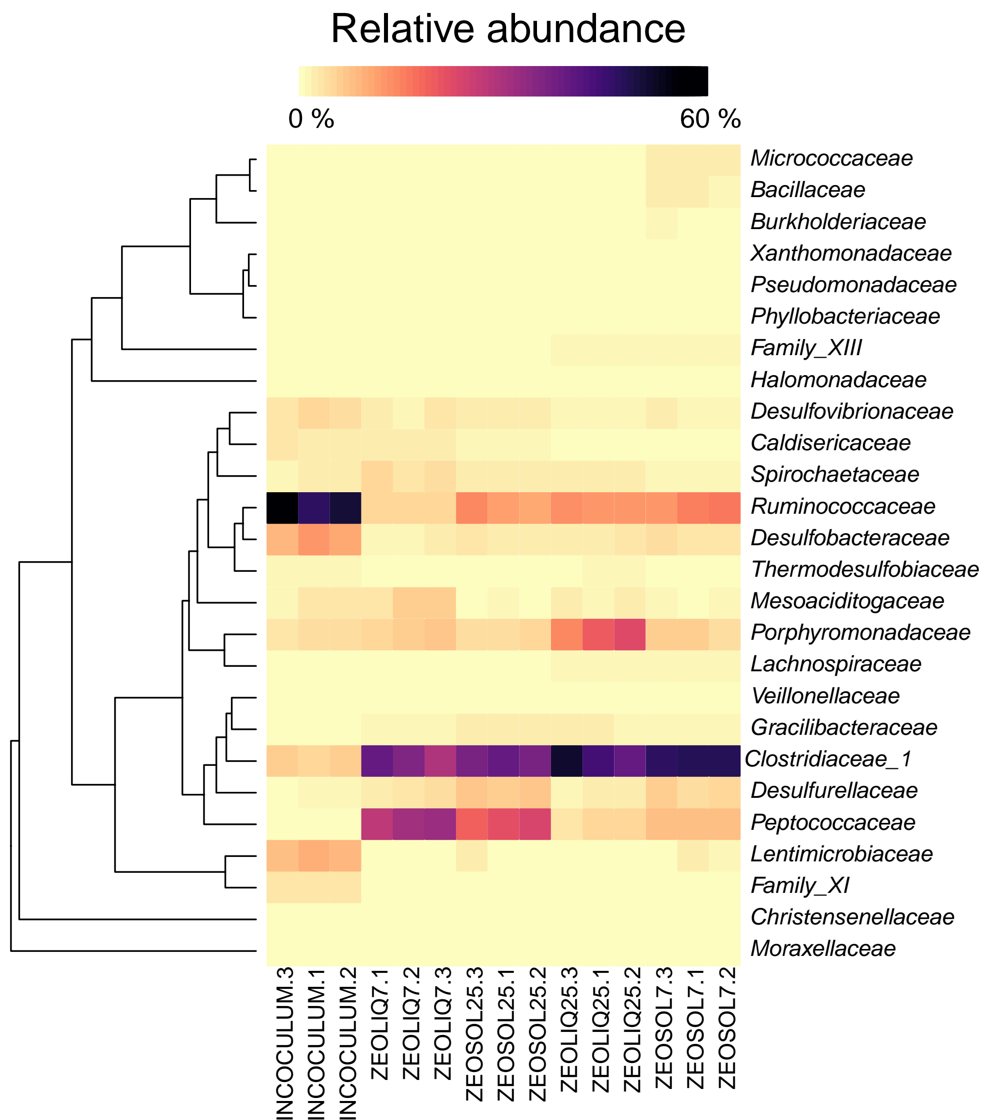


Figure 3.8 Heatmap of the microbial community at the family level in the zeolite experiments. Samples from the biofilm are identified as SOL and those from the liquid phase as LIQ, at the early, (day 7), and late (day 25) stages of the experiments, in comparison with the inoculum. The number after the period indicates the number of the replica.

With glass beads (Fig. 3.7), the structure of the communities of the liquid phase changed with time, the most abundant families (relative abundance > 5%) in the early stage (10 days) were *Ruminococcaceae* (24.6%), *Clostridiaceae* (23.3%), *Desulfovibrionaceae* (10%), *Lentimicrobiaceae* (7.30%), and *Family XI* (5.2%). The

relative abundances of *Porphyromonadaceae*, *Desulfurellaceae*, and *Peptococcaceae* were between 4.3 and 1%.

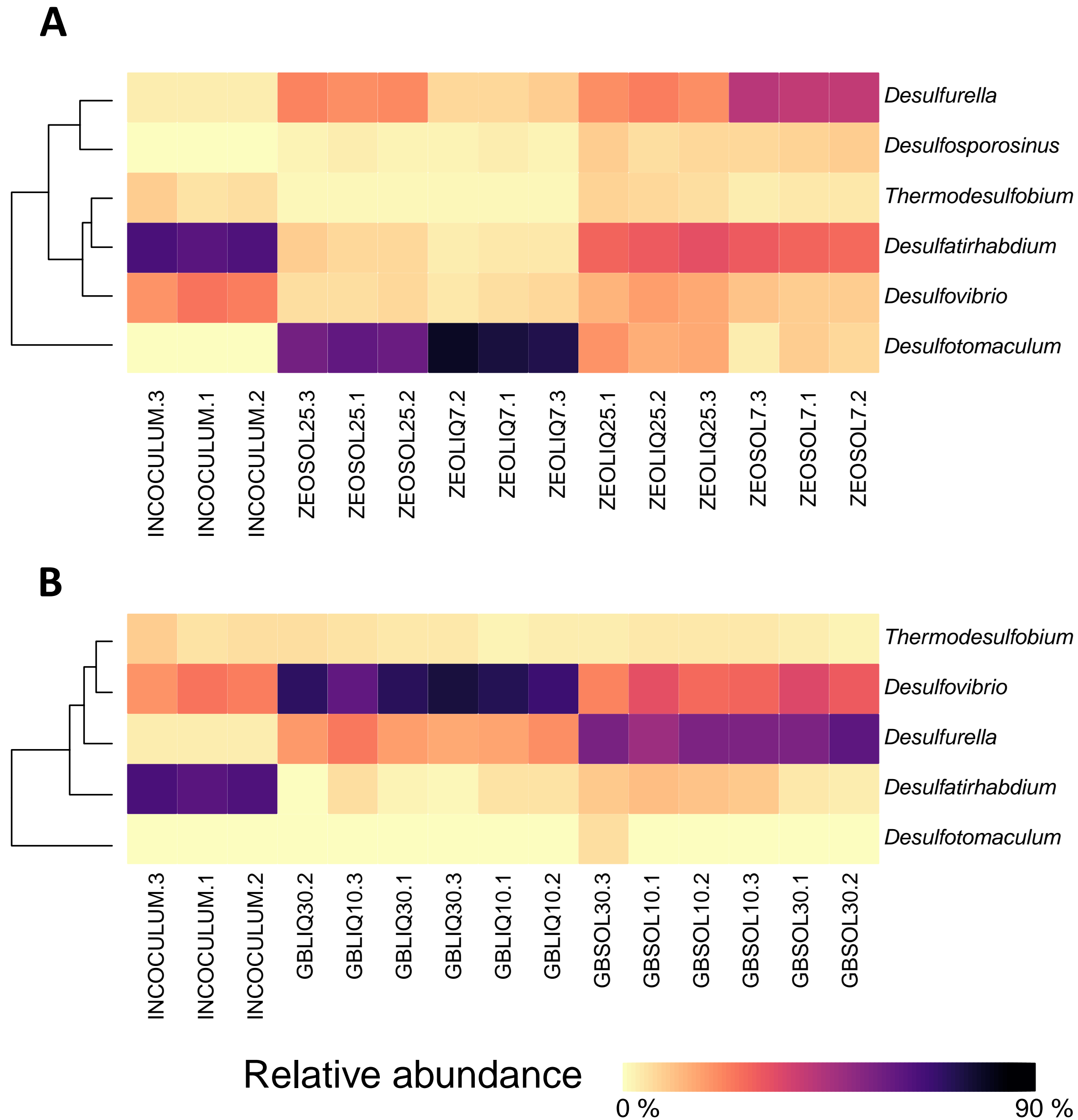


Figure 3.9 Heatmap of the sulfur cycle microbial community at genus level with zeolite (ZEO, top panel A) and glass beads (GB, bottom panel B) in the different matrices liquid (LIQ) or solid (SOL) in the early stage, day 7 or 10, and in the late-stage, day 25 or 30. The community of the inoculum is also shown. The number after the period indicates the number of the replica.

Glass beads did not favor the attachment of *Clostridiaceae* because the relative abundance of this family in the biofilm at day 30 was only 5.7%. Also, the relative abundance of *Desulfovibrionaceae* decreased to 2.3%, whereas in the biofilm community (30 days), the relative abundance of *Lentimicrobiaceae* and *Porphyromonadaceae* sequences increased to 33.4 and 18.5%, respectively.

The diversity of the sulfur cycle microorganisms (Fig. 3.9, A and B) revealed the affiliation to two classes (*Delta-proteobacteria* and *Clostridia*), five families (*Desulfovibrionaceae*, *Desulfobacteraceae*, *Desulfurellaceae*, *Peptococcaceae*, and *Thermodesulfobiaceae*), and six genera (*Desulfovibrio*, *Desulfatirhabdium*, *Desulfurella*, *Desulfotomaculum*, *Desulfosporosinus*, and *Thermodesulfobium*). Except for *Desulfurella,* which has only a sulfur-reducing metabolism, the other genera are sulfate reducers.

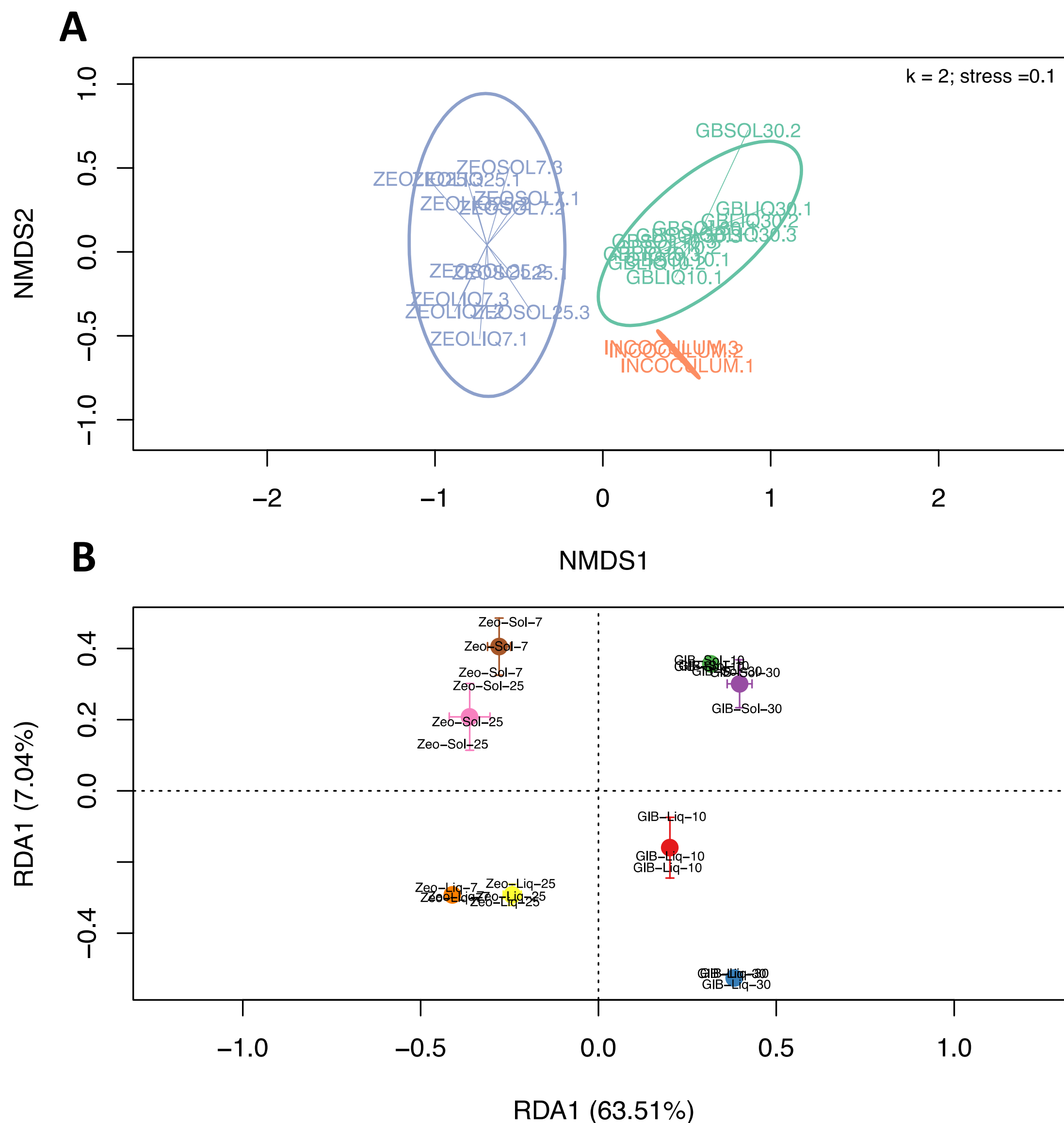


Figure 3.10 A.- Non-metric multidimensional scaling (NMDS) plot and B.- Redundancy analysis (RDA) of the microbial communities obtained with zeolite (ZEO) or glass beads (GB) in the different matrices liquid (LIQ) or biofilm (SOL), sampled at 7, 10, 25, or 30 days.

In the zeolite assays, depending on the stage and the type of sample (planktonic or biofilm), the microorganisms of the sulfur cycle represented between 7.3 and 35.7% of

the community of all the samples; with glass beads, the percentages were lower between 5.5 and 14.1% (Fig. 3.9 A).

Some of the identified SRM preferred to be attached to the carrier material. For example, in the experiments with zeolite after 25 days, the relative abundance of *Desulfotomaculum* reached 18.9% compared to the abundance found in the liquid (1.2%). *Desulfurella* was also detected in the biofilm in higher abundances (5.0 and 6.8%) than in the liquid (2.9 and 1.6%), irrespectively of the stage. In contrast, the relative abundances of *Desulfovibrio* (1.2–1.9%), *Desulfosporosinus* (0.5–1.0%), and *Thermodesulfobium* (0.3–0.5%) were approximately the same in the liquid and the biofilm. Interestingly, after 25 days, the relative abundance of the sulfur cycle microorganisms decreased in the planktonic community of zeolite from 36% to 7.3%; with the consequent increase in the biofilm to 31% (Fig. 3.9 A).

In the experiments with glass beads, the relative abundances of *Desulfurella* were higher in the biofilm (4.6%) than in the liquid (1.4%) at the end of the experiment (30 days, Fig. 3.9 B). *Desulfovibrio* showed the highest relative abundances (10%) in the liquid phase and in the early stage. It appeared that *Desulfovibrio* could not attach to the glass beads. The other genera (*Desulfatirhabdium, Thermodesulfobium, Desulfosporosinus,* and *Desulfotomaculum*) had relative abundances lower than 1%, and it was difficult to identify a preference for the carrier material (Fig. 3.9 B).

The Non-metric multidimensional scaling (NMDS) analysis showed that the communities developed with the carrier materials are different from each other and the inoculum (Fig. 3.10A). The redundancy analysis (RDA) indicated that the constrained variance represented 73% of the total variance, suggesting that most of the variation in the composition of the communities may be accounted for the combination of time, phase (planktonic/biofilm), and the type of carrier (Fig. 3.10A). Zeolite and glass beads grouped in different quadrants, indicating that the communities of the two carrier materials were different. Further, the communities from the liquid and biofilm of both materials were also different. In the case of glass beads, the planktonic communities grouped depending on the sampling time (10 or 30 days), unlike the biofilm communities. In the case of zeolite,

the communities from the liquid were different from those of the biofilm, regardless of the sampling time (Fig. 3.10B).

3.4 Discussion

At acidic conditions sulfate-reducing biofilms formed with zeolite and glass beads but the extent of the sulfate reducing process and the community structure depended on the carrier material. Some SRM were more present in the biofilm, others in the planktonic phase; even though SRM did not dominate the microbial communities, the sulfide production yield and percentage of the substrate used to perform sulfate-reduction improved.

The complex communities developed on zeolite and glass beads in the biofilm and planktonic phase differed from the original planktonic culture (inoculum); nevertheless, the most important feature that interested us, which was the complete oxidation of acetate, was achieved at acidic pH. Here, we show that the communities developed with zeolite and glass beads degraded acetate at acidic pH while preserving the sulfate-reducing activity of the inoculum. In contrast, the community formed with GAC did not degrade acetate at the same rate (Table 3.2), despite the concentration of acetate was lower than the observed with glass beads or zeolite (Figs. 3.3 and 3.4). Acetate degradation in sulfate reducing systems at acidic pH is relevant because acetate may be toxic for microorganisms (Sánchez-Andrea et al., 2014), the protonated volatile fatty acids diffuse through the cellular membrane and decouple the electron transport chain. Concentrations as low as 1 mM, may be toxic for acidophilic microorganisms (Kaksonen et al. 2007). Although in experiments with zeolite and glass beads the maximum concentration of protonated acids were 2.1 mM (pH 5.15) and 3.9 mM (pH 4.6), respectively, the communities were able to degrade acetate once the pH surpassed the pKa of acetic acid (4.76). This result confirmed the importance of acetate oxidation to increase the pH when remediating acidic currents (Kaksonen et al., 2003). Overall, acetate degradation in sulfate reducing systems increases the efficiency of the process. The degradation of acetate in our experiments allowed reaching substrate consumption efficiencies via sulfate reduction as high as 58-92.6%, which contrast with other works

reporting close to 50% of substrate consumption via sulfate reduction (Nancucheo et al 2012). A relevant aspect of our study is that we screened for SRM in the biofilms and in the liquid phase of the experiments. Two acetate degraders were present in the communities of the assays with zeolite: *Desulfotomaculum* in the biofilm and *Desulfatirhabdium* in the planktonic phase (Balk et al., 2008; Kleikemper et al., 2002). *Desulfotomaculum*, member of the *Peptococcaceae* family, is a spore-forming sulfate-reducing bacteria (Castro et al., 2002). This genus is very heterogeneous and has recently been reclassified including some complete oxidizing species, and members of this genus have been found in sediments, deep surface samples, lakes, pits, and has also been used to remediate AMD (Aüllo et al., 2013; Watanabe et al., 2018). In the inoculum, *Peptococcacea* family was underrepresented (< 1%), some members of this family (*e.gr. Desulfotomaculum and Desulfosporosinus*) are important sulfate reducers in acidic environments (Nancucheo and Johnson, 2014; Sánchez-Andrea et al., 2013; Sánchez-Andrea et al., 2014). *Desulfatirhabdium* is a genus classified as a complete oxidizer that can use a wide variety of substrates and has genes that confer resistance to acid and metals (Almstrand et al., 2016; Kuever, 2014) that might explain its presence in our systems. Members of these two genera may be responsible for the ~21% improvement of the sulfate-reducing performance using zeolite. In contrast, the overall performance with glass beads was very similar to the performance in the absence of carrier material. Previously, glass beads were used as an ideal support material for the adhesion of sulfate-reducing communities at acid pH in continuous reactors (Nancucheo and Johnson, 2012; Santos and Johnson, 2018). In this work, glass beads did not promote the attachment of members of the Peptococcacea family (such as *Desulfotomaculum* or *Desulfosporosinus*) as compared with zeolite. Glass beads were not an appropriate carrier material for the acetate-degrading SRM that were present in the inoculum (*i.e. Desulfotomaculum* and *Desulfatirhabdium*).

The well-known sulfate-reducing genus *Desulfovibrio* grew mostly in the liquid phase, which was more evident in the experiment with glass beads. This genus belongs to the family *Desulfovibrionaceae* that comprises incomplete oxidizers. Some members of *Desulfovibrio* can oxidize glycerol (Kremer and Hansen, 1987; Qatibi, et al., 1998), and

have been found in acidic streams such as acidic lakes, wetlands, acidic sulfate soils, and bioreactors (Sánchez-Andrea et al., 2014).

Using glycerol as substrate, the communities with zeolite and glass beads achieved complete oxidation of the substrate, including acetate, reaching 58-92% of sulfate-reducing activity (Table 3.2). Typically, glycerol has been used to develop acidic sulfate-reduction at low pH (<5), both in batch cultures (~ 30-85% sulfate-reduction activity) (Dinkel et al., 2010; Moreno-Perlin et al., 2019) and in continuous reactors (~15-75%) (Nancucheo and Johnson, 2012; Santos and Johnson, 2017). However, the complete oxidation of acetate is not always achieved, which is the main drawback of sulfate-reduction at acidic pH specially in continuous systems, most probably due to the lack of complete oxidizers and acetate cannot be degraded (Santos and Johnson, 2018). In the present work, the success of the starting community in degrading acetate may be related to the fact that it has been cultured at acidic pH for more than three years, and its performance is very reproducible (Campos-Quevedo et al., 2021a). Therefore, there was no need to do any bioaugmentation of the microbial community with an acetoclastic acidophile (e.gr., *Acidocella aromatica*) to improve the degradation of acetate, as previously reported (Nancucheo et al. 2017).

Despite performing sulfate-reduction with high efficiencies, the communities developed over zeolite and glass beads were not dominated exclusively by SRM (Figs. 3.7 and 3.8), and were statistically different from the inoculum (Fig.3.10A). Instead, complex communities developed on the biofilm and planktonic phase of both carrier materials, which allowed SRM to resist stress conditions such as the presence of acetate at acid pH.

Low-abundant microorganisms still may play a crucial role in the global process by developing different mechanisms to thrive over adverse conditions and proliferate when favorable conditions prevail, as pointed out before (Hausmann et al., 2016); this could be the case of *Desulfosporosinus,* and *Thermodesulfobium* (Fig. 3.9, A and B). Some species of *Desulfosporosinus* genus have been found in acid mine drainage, and some of them can resist moderate acidic conditions (Alazard et al., 2010). *Thermodesulfobium*

genus has been described in enrichments of an acidic pit lake (pH 3 and 4) suggesting that sulfate-reducing communities were better adapted to extreme conditions (Meier et al., 2012). On the other hand, the fermentative microorganisms (i.e., *Ruminococcacea* and *Clostridacea*) present in the biofilm and in the liquid phase could be responsible for the formation of exopolymeric substances and may helped SRM to survive and handle stressful conditions through synergetic associations (Sánchez-Andrea et al. 2012).

From the four families that dominated all the analyzed communities, members of *Clostridiaceae* could be responsible for glycerol fermentation, and the production of propionic acid in zeolite and glass beads, GAC being the exception in which propionic was not observed as in the biological control (Figs. 3.3 and 3.4). Representatives of this family are very conspicuous in anaerobic communities and have been found in samples of acid mine drainage, sulfate-reducing consortia, and sediments (Lu et al., 2011; Reyes et al., 2017). Members of the family *Ruminococcaceae* have been previously described in microbial communities of a sulfate-reducing bioreactor operated at pH 5 to 6.5 (Shan et al., 2017), and along with sulfate-reducing bacteria in ferruginous sediments (Vuillemin et al., 2018). On glass beads (Fig. 3.7), the majority of the sequences resembling *Peptococcaceae* family were similar to *Sporotomaculum* genus, mostly found in the liquid phase at day 30. It is plausible that their role in the consortium was as fermenters because the cultured representatives cannot use sulfate as an electron acceptor (Brauman et al., 1998).

The results also show important differences between the communities in the early/late stages of the biofilm or liquid phase. In other studies, this issue has remained unexplored highlighting that there is a lack of information about the communities developed in the early stages of sulfate-reducing biofilms at acidic pH, specially of those that degrade acetate. Therefore, the information presented here becomes useful when implementing sulfidogenic cultures in continuous biofilm reactors under acidic conditions. The study of the biofilm communities in the early and late stages also allowed knowing the time needed for the SRM to adhere to the support material (biofilm formation).

Overall, starting with the same inoculum, the carrier materials (glass beads or zeolite) shaped the attachment and development of different microbial communities. Our results confirmed previous observations with sulfate-reducing bacteria (Basu and Baldwin, 2000) or soil communities (Aminiyan et al. 2018). The effect of the carrier material was also confirmed by diversity indexes and statistical analysis, showing that such difference was significant (Fig. 3.10B and Table 3.3). These observations underline that the carrier material is a decisive factor in the formation of biofilms and, consequently, in the performance of biofilm reactors as noted before (Basu and Baldwin, 2000; Silva et al., 2006). The development of biofilms over carrier materials is multifactorial and depends on the surface properties of both the carrier material and bacteria. Surface roughness, hydrophobicity, the composition of the carrier material and species of bacteria are among the most relevant characteristics that determine bacterial attachment (Hadjiev et al., 2007; Pereira et al., 2000). Roughness has been highlighted as more important than internal surface area for bacterial colonization because the surface irregularities (crevices, cracks, grooves, etc.) promote initial colonization (Pereira et al., 2000), and protect microorganisms from abrasion/detachment. Internal pore size, which relates to the specific surface area, can be an important characteristic as long as 70% of the pores have diameters in the micrometric scale. For bacterial colonization, the pores should be between one time the smallest dimension of the bacteria and five times the largest one (Oliveira et al., 2003). In our study, none of the three carrier materials meet this condition, because the internal pore size of zeolite, glass beads, and activated carbon is in the nanometric scale (Huysman et al. 1983). Regarding hydrophobicity, this feature has an impact on the interaction forces between bacteria and the carrier material, these forces become stronger when water is “squeezed out” allowing the contact between bacteria and the carrier material (Habouzit et al., 2011; Pereira et al., 2000). Given this complexity, the selection of the carrier material based on kinetic assays may represent an advantage *versus* using a carrier material based only on its physicochemical characteristics (i.e., superficial area, hydrophobicity, and charge, among others).

The use of carrier materials under acidic conditions has been widely studied due to the advantages that the carrier provides to the community, such as preventing washout

by biofilm formation, allowing high flows, and increasing the cellular retention time (Basu and Baldwin, 2000; Silva et al., 2006), these advantages allow to operate high-rate continuous reactors. With zeolite, the change of the community composition had a positive effect on the sulfate reducing process; this material promoted faster kinetics and improved the efficiencies and yield compared with the original acid-tolerant consortium used as the inoculum. In contrast, the change of the community with glass beads was not reflected in the global performance of the assays, which performed similar to the assay without carrier. Most probably, the differences between zeolite and glass beads communities are due to the surface roughness of the carriers, being zeolite of irregular shape with a rough surface, it presented more crevices for initial colonization than the regular shape and smooth surface provided by glass beads. The cation exchange feature of zeolites could also contribute to this difference as previously reported (Kubota et al., 2008; Wang and Peng, 2010). Exploring different carrier materials in kinetic experiments to reproduce the activity observed in liquid culture is worth to reveal the performance beforehand running a reactor.

Previously, biofilms of acidophilic sulfate reducers formed on GAC were reported to achieve high removal efficiencies of sulfate (75 - 90%) (Sánchez-Andrea et al. 2012). Despite the suitability of GAC to sustain the growth of SRM, in the present study, GAC showed the lowest sulfate reduction rate of all the evaluated carrier materials and acetate remained as by-product. One drawback of using GAC or zeolite as carrier materials is that it was not possible to quantify the concentration of sulfide in the liquid phase, due to the nature of the materials with high porosity and surface area, and with a suite of functional groups that can adsorb sulfide (Liu and Adanur, 2014; Tran et al., 2016). The main functional groups that compose GAC of basic character, as Norit 830W, include chromene structures, diketone or quinone groups, and pyrone like groups (Montes-Morán et al., 2004). Due to its adsorption capacity and high surface area, GAC has been used as carrier material to form biofilms in several biotechnological applications, and also for the adsorption of sulfide (Coppola and Papurello, 2018). The results of the abiotic controls highlight the importance of accounting for the adsorption contribution of each material.

Although adsorption and desorption of glycerol, sulfate, and acetate occurred with zeolite and GAC, in the case of zeolite, these substrates remained bioavailable despite their absorption, as confirmed by the sulfate-reducing activity. Conversely, the bioavailability of glycerol, acetate, and sulfate was compromised when GAC was used as the carrier material. Both materials contain functional groups that promote adsorption of different compounds that have no adverse effect on the microorganisms and can be considered as "inert" carrier materials. Nonetheless, their use in sulfate-reducing batch assays should be analyzed carefully, moreover if metals are involved. Eventually, the absorption of sulfide will reach a saturation point, explaining the successful application of these materials for metal precipitation in continuous reactors (Bertin et al., 2004; Sánchez-Andrea et al., 2012). From this perspective, glass beads could be an ideal inert carrier material as did not interfere with the concentrations of sulfate and glycerol in the abiotic controls. Earlier, glass beads were used for the attachment of sulfate-reducing communities at acidic pH in continuous reactors (Santos and Johnson 2018; Santos and Johnson 2017; Nancucheo and Johnson 2014; Sahinkaya et al. 2011). Overall, the results allowed us to select glass beads and zeolite as appropriate carrier materials for the acidophilic sulfate-reducing consortium because both carrier materials preserved the main characteristic of interest: acetate consumption at acidic pH. Any of these carrier materials could be used in future applications of the consortium to maintain the community within a continuous reactor. Despite the noticeable change of the microbial community with zeolite and glass beads, compared with the initial sulfate reducing community, the complete consumption of acetate at low pH prevailed. With glass beads the performance was almost the same as the inoculum, however with zeolite the change of the community enhanced the yield and the sulfate-reducing activity. These sulfate-reducing communities on zeolite and glass beads could be applied to remediate metal containing effluents, which are typically acidic.

The present study contributes to understand that sulfate-reducing communities thriving at acidic conditions are complex and do not need to be dominated by SRM and that the communities initially attached to the biofilm may change as the biofilm matures.

The carrier material determined the community development of the biofilm and a proper choice is crucial for the whole sulfate-reducing process.

This study also demonstrated that the preference of the SRM to attach or remain planktonic depends on the carrier material, *Desulfotomaculum* preferably attached to zeolite, and *Desulfovibrio* to glass beads in the late stage of the batch incubations (25-30 days). In the planktonic phase, *Desulfatirhabdium* remained present in the zeolite experiments, and *Desulfovibrio* in glass beads. This work underlines the critical role of abiotic controls and having an inoculum already acclimated to specific conditions, in this case, sulfate reduction at acid pH and complete consumption of acetate. Overall, here we highlight the importance of a proper start-up strategy when developing acidophilic sulfate-reducing biofilms in a possible application such as continuous high-rate reactors to treat acidic metal containing effluents. Glass beads preserved the sulfate reducing activity of the inoculum, whereas zeolite enhanced the activity, these two materials could be applied successfully to treat acidic streams with metals in high-rate continuous reactors.

Chapter 4

Continuous bioreactor performing acetotrophic sulfate reduction at pH 3.25 of extremely acidic stream (pH 1.7)

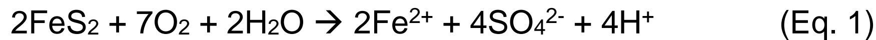
Abstract

Acidic streams, such as acid mine drainage, low pH (pH < 4), high metal and sulfate concentrations, cause significant damage to aquatic life, including fish and stream biota, and can render water unusable for drinking and industrial purposes. SRM offer a sustainable, environmentally friendly, and efficient solution for treating AMD due to their ability to generate alkalinity and neutralize acidity. The activity of SRM in sulfidogenic reactors is limited due to the sensitivity of some SRM to acidic conditions and acetic acid, a byproduct from the sulfate-reducing metabolism. We aimed to develop a sulfidogenic process overcoming these limitations. We developed a continuous biofilm consortium reactor with immobilized biomass using zeolite as the carrier material, for treating extremely acidic synthetic media (pH 2.5 to 1.7), supplemented with glycerol (~478.9 – 994.6 mg/L) as the electron donor and sulfate as the acceptor (~998.1 – 1689.7 mg/L), added stoichiometrically. The sulfate-reduction efficiency, byproducts and its tolerance to continuous acidic media were evaluated varying and controlling the pH from ~5.0 – 3.1, the reactor operated during 159 days. In the most acidic periods (pH ~3.75 - 3.25) the sulfate consumption rate was ~2881.8 – 3103.5 mg/L· d, at the shortest hydraulic retention time (~0.47 d). The microbial diversity of the biofilm and planktonic communities, analyzed by 16S rRNA gene amplicon sequencing, showed a total of 11 genera sulfate-reducing microorganisms. From these, three genera included members known to perform acetate oxidization (*Desulfofarcimen*, *Desulfatirhabdium*, and *Desulfobacter).* Notably, relative abundance of SRM in the planktonic biomass reached ~13.2 - 53% and ~22 - 43% in the biofilm. Such relative abundances are the highest reported so far in continuous reactors under acidic conditions. This research showcases one of the most remarkable high sulfate reduction rates under highly acidic conditions. The consortium not only survived but outperformed in accomplishing sulfate reduction under extremely acidic conditions.

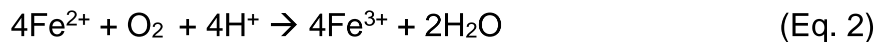
Keywords
Acetate; acid pH; biofilm; continuous reactor; sulfate-reduction; zeolite

4.1 Introduction

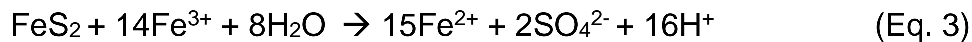
AMD is one of the most severe environmental problems of our time associated with mining activities; it can lead to the degradation of ecosystems and poses a threat to human health (Bratkova, 2021). AMD originates from the interaction of ores, rich in reduced sulfide minerals, with water and oxygen, which results in their oxidation, causing acidity (pH < 4), elevated concentrations of soluble metals, and high concentrations of sulfate. The primer reaction of AMD generation is the oxidation of pyrite that yields Fe^{2+}, sulfate, and protons (Eq 1). In subsequent chemical reactions, Fe^{2+} oxidizes to Fe^{3+} (Eq. 2), and soluble Fe^{3+} catalyzes the oxidation of more pyrite (Eq. 3)(Sánchez-Andrea et al., 2014).



$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+ \qquad \text{(Eq. 1)}$$



$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \qquad \text{(Eq. 2)}$$



$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \qquad \text{(Eq. 3)}$$

The physicochemical characteristics of AMD largely depend on the ore, but the constant is the high content of iron, sulfate, and acidic pH. AMD has a detrimental impact on the biota of water bodies affected by this effluent (Steyn et al., 2019). Among the different technologies to remediate AMD, the addition of acid-neutralizing agents, for example hydroxides or carbonates which also co-precipitate metals has several disadvantages, namely the production and disposal of toxic chemical wastes with the impossibility of metal recovery (Kolmert and Johnson 2001a). Conversely, the biological treatment of AMD by SRM is an environmentally friendly and feasible option to the traditional physicochemical processes (Zhang et al. 2016; Zhao et al. 2017). Two features of the SRM are key for the benefits of the sulfidogenic process for AMD treatment: i) the ability of SRM to reduce sulfate to sulfide and ii) the precipitation of metal cations that react with anionic sulfide, which makes possible the recovery of metals as sulfide precipitates (Celis et al., 2009; Celis et al., 2013; Gallegos-Garcia et al., 2009).

The efficiency to treat AMD using SRM depends on reactor configuration, carbon source, hydraulic retention time (HRT), chemical oxygen demand (COD) to sulfate ratio, pH, temperature, and inoculum source, among others (Sánchez-Andrea et al., 2014; Visser et al., 1993; Yildiz et al., 2019). Nevertheless, a drawback of sulfidogenic reactors is that SRM species characterized so far are highly sensitive to even mild acidity (pH 5 - 6) (Celis et al., 2013).

Continuous sulfate-reducing reactors uphold a consistent state of operation, enabling a reliable and foreseeable outcome, which proves advantageous for processes such as sulfate elimination and acetate consumption. They provide a superior level of regulation over operational parameters like hydraulic retention time (HRT) and sulfate loading rate (SLR), which can be fine-tuned to enhance the effectiveness and productivity of the reactor. Furthermore, in acidic conditions, part of the hydrogen sulfide (H_2S) in the liquid is always in equilibrium with the gas phase, and that equilibrium in the gas phase is highly dependent on the equilibrium of H_2S/HS^- species, which depends directly on the pH value. Continuous reactors reduce the negative impact of dissolved sulfide on the biomass, thereby improving the overall sulfidogenic activity within the system (Gil-Garcia et al., 2018).

At present, full-scale implementation of sulfate reduction AMD remediation requires a carrier material at acidic pH to provide a surface for biofilm formation, which enhances microbial attachment and growth, improving the efficiency of the sulfate reduction processes (Bijmans, 2008; Montoya et al., 2013). Another issue, when carrying sulfate reduction at low pH, is the predominance of acetic acid species, as the result of the production of acetate from the incomplete oxidation of the organic substrate. The protonated form of acetate, i.e. acetic acid (CH_3COOH), becomes predominant at pH values lower than its pk_a (< 4.76). This species is more toxic to cells than the unprotonated acetate (CH_3COO^-) because it can easily penetrate cell membranes and disrupt internal processes (Sánchez-Andrea et al., 2022).

Therefore, it is relevant to enhance the microbial community tolerance to the harmful AMD conditions (Yildiz et al., 2019). Microbial communities enriched from sites exposed to extreme conditions (e.g. acidic pH, high sulfate or metal concentrations) have

been used in sulfidogenic systems to cope with the effects of such conditions (Sánchez-Andrea et al. 2012; Santos and Johnson 2018). Unfortunately, not all the communities investigated so far are able to degrade completely the acetate, limiting the efficiency of the whole process (Celis et al., 2009; Nancucheo and Johnson, 2014; Patel et al., 2020). It is crucial to develop reactors with sulfate-reducing communities capable of consuming acetate at acidic pH to promote an efficient treatment process (Campos-Quevedo et al., 2021b). From the previous works studying sulfate reduction at acidic pH in continuous reactors, one common characteristic is that the microbial community is immobilized to increase biomass retention (biofilm) within the reactor and promote high cell retention times therefore preventing reactor wash-out (Silva et al., 2006; Zhang and Wang, 2016). The carrier materials used for the retention of sulfate-reducing communities were GAC (Sánchez-Andrea et al. 2012), polyurethane foam (Silva et al. 2006), polyethylene particles (Piña-Salazar et al. 2011; Montoya et al. 2013), porous glass beads (Nancucheo and Johnson 2012), and zeolite (Zhang et al. 2016), among others.

It should be emphasized that there is a lack of research documenting a biofilm continuous reactor operating under controlled acidic conditions while completely consuming acetate. For instance, Nancucheo and Johnson (2011) studied a reactor operated between pH 2.8 - 4.5, using glass beads as carrier material and glycerol as the electron donor (460 to 64 mg/L d) with acetate as byproduct (180 to 60 mg/L·d). So far, an accurate estimate about the effect of the long-term change of pH on the reactor efficiency and the microbial community has been barely studied.

Previous research demonstrated that zeolite and glass beads are appropriate carrier materials for cultivating biofilms from an acidophilic sulfate-reducing consortium. Zeolite enhanced the rate of sulfate reduction and the overall efficiency of the sulfate-reducing process (in batch experiments) when compared to activated carbon (Campos-Quevedo et al., 2021a). Both zeolite and glass beads were shown to be advantageous carrier materials for sulfate-reducing processes under acidic conditions. Moreover, zeolite facilitates the development of intricate microbial communities capable of withstanding adverse circumstances and sustaining sulfate-reducing activity. On the other hand, glass beads do not interfere the levels of sulfate and glycerol, thus establishing glass beads as

an optimal inert carrier material for sulfate-reducing biofilms (Campos-Quevedo et al., 2021b).

This study aimed to develop a sulfidogenic process for producing sulfide at low pH (~5 – 3.1) with the complete oxidation of the substrates (i.e., acetate consuming process), thus diminishing the concentration of acetic acid within the reactor. For that, we developed a continuous biofilm reactor with sulfate-reducing biomass immobilized in zeolite. The reactor treated extremely acidic synthetic media containing sulfate (pH 2.5 to 1.7) supplemented with glycerol as the electron donor in pH controlled (pH ~5 to 3.16). We also investigated the microbial diversity dynamics of the biofilm and planktonic communities at different pH values by using 16S rRNA gene amplicon sequencing.

4.2 Materials and methods

4.2.1 Inoculum and pre-colonized zeolite

The source of microorganisms was a highly specialized consortium, identified as Consortium 7, retrieved from a contaminated sulfur mine in Guaxacama San Luis Potosi, S.L.P., Mexico (Moreno-Perlin et al., 2019, Campos-Quevedo et al., 2020, and **Chapter 2 of this thesis)**. This microbial consortium completely oxidizes glycerol to CO_2. The consortium used around 75% of the substrate (glycerol) to perform sulfate-reduction and developed biofilms on zeolite, as observed for another carrier such as GAC or glass beads (**Chapter 3**).

To startup the continuous reactor, pre-colonized zeolite particles were used as the inoculum. To develop the precolonized zeolite, 191.7 g (213 mL) of zeolite were placed in a 1 L anaerobic bottle with 710 mL of acidic synthetic media, thus zeolite corresponded to 23% of the liquid volume. The media used for precolonizing the zeolite was the same used to feed the continuous reactor. The acidic synthetic media composition was based on previous reports (Nancucheo and Johnson, 2012; Stams et al., 1993b). feed and contained (mM): 0.6 KH_2PO_4; 0.6 $Na_2HPO_4 \cdot 2H_2O$; 5.6 NH_4Cl; 5.1 NaCl; 0.5 $MgCl_2 \cdot 6H_2O$; 5 $CaCl_2 \cdot H_2O$, 0.25 mL/L trace element solution (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 $ZnCl_2$), and 0.01 g/L of yeast extract per

liter; the media did not contained any vitamins solution. To precolonize the zeolite, the acidic synthetic was supplemented with 5.71 mM glycerol (525.85 mg/L) as the electron donor and 10 mM sulfate (960.6 mg/L) as the acceptor.; the pH was adjusted to 3. The bottle was incubated (30ºC, no agitation) until complete glycerol, acetate, and sulfate depletion (approximately 1 month). The pre-colonized zeolite was then transferred to the reactor.

4.2.2 Reactor startup and long-term operation

An autoclavable 1-liter tank reactor equipped with an automated controller and a scalable software platform for process control (Applikon Biotechnology B.V.) was used for the continuous experiments.

To feed the reactor, the acidic synthetic media was supplemented stoichiometrically with glycerol and sulfate. Glycerol and sulfate concentrations changed depending on the period, HRT and loading rate (Period I – IX), further specifications are shown in the development of this chapter for your understanding also shown in Table 4.1. Glycerol concentrations ranged from ~478.88 - 994.60 mg/L (5.2 - 10.8 mM) and sulfate ~998.06 - 1689.69 mg/L (10.2 - 17.86 mM). The pH of the media was adjusted between ~2.15 – 1.7. The reactor operated continuously at a controlled temperature (30ºC). The pH was reduced gradually from 5.0 to 3.0 and controlled within the reactor depending on the period. The acidic feed media (pH ~2.5 – 1.7) served to control the pH to the desired value inside the reactor. Therefore, the flow rate fluctuated as needed to maintain the pH inside the bioreactor at the desired set point pH value (~5 – 3.0). With this approach, the feed media with a lower pH value than the pH inside the reactor, compensated the alkalinity generated by the sulfate reducing activity.

Mixing inside the reactor was achieved through a constant N_2 current fed at the bottom of the reactor, below the carrier material. Additionally, from periods VII to IX, mechanical agitation (maximum 100 rpm) was included to help with the medium homogenization.

Figure 4.1. Schematic diagram of the anaerobic bioreactor. The arrows represent the direction of the gas flow. Image created in BioRender.com

The reactor was inoculated with the aforementioned pre-colonized zeolite corresponding to 23% (213 mL) of the total working volume (923 mL). After inoculation, the reactor operated in batch mode for 9 days without pH control (initial pH 3) until glycerol and acetate were completely oxidized. Afterwards, the operation mode changed to continuous.

Samples of the influent and effluent were collected every day (except on the last Period IX when samples were taken each ~3 days) to quantify the pH and the concentrations of sulfate, glycerol, and byproducts (acetate, propionate, and butyrate).

4.2.3 Physicochemical analysis

Glycerol and volatile fatty acids (acetate, propionate, and butyrate) were quantified using high-performance liquid chromatography (LKB) with a Varian Metacarb 67H 300 mm column, using H_2SO_4 (0.01 N) as eluent at a flow rate of 0.8 mL/min. The determination of sulfate levels was conducted by means of a Dionex ICS-1000 ion chromatograph that was configured with an IonPac AS22 column and utilized a 4.5 mM carbonate/1.4 mM bicarbonate eluent at a flow rate of 1.2 ml min-1 (Dionex) (Florentino et al., 2018).

4.2.4 DNA extraction and sequences analysis

For DNA analysis, 15 mL from the planktonic community (liquid phase) and 5ml of the biofilm developed on zeolite (solid) were taken at the end of each period before changing the next phase at lower pH. DNA was extracted using FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, USA), following the manufacturer's protocol. DNA was quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA) and adjusted to a concentration of 20 ng/μL for further use as the template for polymerase chain reaction (PCR) amplification. PCR was performed in a final volume of 50 mL containing 5X HF PCR green buffer, 0.2 mM dNTPs, 5 U μL−1 of Phusion Hot Start II DNA polymerase (Promega, Madison, USA), 10 μM of forward and reverse primer mixture (515F: GAGTGCCAGCMGCCGCGGTAA and 806R: CGGGACTACHVGGGTWTCTAAT) V4 region (Walters et al., 2011), 200 μM of barcoded forward primer with titanium sequence adaptor, 338R-I+II (Biolegio BV, Nijmegen, The Netherlands), 0.5-1 ng/μL of the template DNA and nuclease-free water (to final volume). The PCR program was as follows: initial denaturation (98 °C, 30 s), 25 cycles of denaturation (98 °C, 10 s), annealing (56 °C, 10 s), and extension (72 °C, 10 s), and a final extension step (72 °C for 7 m). The amplicons were visualized after gel electrophoresis in agarose (2.2% w/v) with 1x SYBR Safe (Invitrogen, Carlsbad, USA). Water was used as the negative control, it was amplified with no product. The PCR products were purified (MagBio Beads, High Pure Cleanup Micro Kit, Roche, Basel, Switzerland) and pooled in equimolar amounts at a final DNA concentration of 200 ng

μL^{-1}. Ilumina Mysec of the pooled amplicons were determined by Novogene Netherlands B.V. (Novogene (NL) International Holding B.V., The Nederlands). The sequences were processed using Qiime 2 (v.2.2019.1) (Bolyen et al., 2018; Quast et al., 2013). Silva 132 taxonomy was used to assign the taxonomy (function assignTaxonomy Dada2). Eukaryotic sequences were removed (Callahan et al. 2016). The ecology package 'vegan' (Oksanen et al. 2017) with the R version 3.4. (R Core Team, 2005)was used to calculate the distance among the samples, obtain the richness, Shannon-Wiener' index, Simpson' index of dominance and evenness and to perform the Multivariate Homogeneity of Groups Dispersions (Variances). For the attribute analysis and beta-dispersion (Figs 4.6 and 4.7), only the sequences identified at the family level (92.40% of the sequences) were considered. These sequences represent more than 92.02% of total reads in all the samples and on average covered 97.29% of reads.

4.3 Results and discussion

4.3.1 Reactor performance

The objective was to operate a sulfidogenic reactor addressing challenges such as acetic acid buildup, effective utilization of electron donors, and highly acidic conditions. Through a series of nine controlled reactor pH phases (ranging from pH 5 to 3.25), an acidophilic sulfate-reducing consortium could degrade acetate/acetic acid. Table 4.1 shows the operational data of the reactor, showing the averages of each of the periods and their standard deviation. Figure 4.2 shows the reactor profiles in detail during the 159 days of operation.

Period I was the batch startup period, during which the pH was not controlled. It ended after 9 days, when the glycerol and acetate were completely oxidized.

On Period II, the continuous operation began, at pH 5. The glycerol concentration in the inlet was maintained at approximately ~504.1 – 765.69 mg/ L until Period IV (pH 4.25) (Table 4.1 and Fig. 4.2A). Because at this period, the HRT was ~8.72d, to increase the sulfate-reducing activity and therefore decrease the HRT, on day 70, the concentration of glycerol and sulfate was doubled until day 80. After day 80 the

concentration of glycerol and sulfate decreased stoichiometrically again until Period IX, where glycerol concentration decreased because the microbial activity at this pH of 3 was minimal.

On Period II, glycerol consumption efficiency reached 100% (Fig. 4.2C). However, not all the electrons were directed to sulfate-reduction, being the sulfate reduction efficiency around 80% (Fig. 4.2C). The efficient consumption of glycerol continued during periods III, IV, and V. The slight reduction in the consumption efficiency at the end of Period V was due to the increase in glycerol concentration in the feed. It is worth noting that during Period V, the pH was controlled at 4.07.

During periods VI to VIII, when the reactor pH was less than 4.0, the glycerol and sulfate-reducing efficiencies were ~88% and 81%, respectively (Fig. 4.2C). These efficiencies are slightly higher than those reported previously (~30 – 70 %) in continuous controlled acidic conditions inside the reactor (25 -45 %) and inlet pH (1.3-3) (Nancucheo and Barrie Johnson, 2014; Nancucheo and Johnson, 2012; Santos and Johnson, 2017).

In period VIII, due to high sulfate-reduction rates (~86.22 % efficiency), we decided to decrease the pH of the inlet from 1.8 to 1.7 and inside the reactor from 3.25 to 3.0 in period IX (Table 4.1 and Fig. 4.2C and E).

In Period IX, when reducing the pH of the inlet media to 1.7, and pH inside the reactor decreased to ~3.0 the glycerol consumption dropped to ~ 60% efficiency. These pH values at the inlet and inside the reactor were a breakpoint, and the reactor collapsed. The efficiencies, byproducts, and HRT changed drastically, and the reactor could not recover to the performance of previous periods (Table 4.1 and Fig. 4.2C). In this period, the acetic acid and butyric acid concentrations peaked (Fig. 4.2B). Therefore, the product formation differed from periods VI-VIII, were propionic acid was formed, most probably due to glycerol fermentation (Himmi et al. 2000). Propionic acid can enhance sulfate reduction in anaerobic environments, as reported in paddy soils where some members of *Syntrophobacteraceae* are major propionate-degrading sulfate reducers (Gu et al., 2017).

In the reactor, the acetic acid concentration was quite low ~1.17 – 22.59 mg/L·d (< 0.382 mM), less than 1 mM during all the periods, except in Period IX (Fig. 4.2B). In

periods VI-VIII, acetic acid was not even detected (Table 4.1. and Fig. 4.2B), showing a complete substrate oxidation coupled to the sulfate reduction process. This is especially remarkable at the acidic pH < 4.0 that prevailed inside the reactor, since acetotrophic sulfate reduction is hardly achieved at low pH (Wang et al., 2008).

The HRT varied in response to the metabolic processes as the requirement for protons in the solution was essential to achieve the desired acidic pH level (Table 4.1 and Fig. 4.2D). In the most acidic operational conditions (Periods VI-VIII), the HRT was as short as ~0.5 days or even less, marking a record low under such acidic conditions (pH 3.77 - 3.27), in comparison with 50 hours (Santos and Johnson, 2018), and ~0.41 to 5.83 days (Nancucheo and Johnson, 2014).

Several studies have investigated the utilization of sulfate-reducing bioreactors under acidic conditions. Lopes et al. (2007), illustrated the sustained reduction of sulfate at pH 4.0 in a thermophilic (55°C) upflow anaerobic sludge bed (UASB) reactor that was supplied with sucrose. This accomplishment was achieved over a span of 78 days with acetate as a byproduct, which was not oxidized.

The continuous sulfate reduction reactor presented in this chapter (Periods II – IX) operated at a pH range of 5-3 with a HRT of 6.08 – 0.37 days and achieving a sulfate reduction rate of approximately 62 - 92%, highlighting efficient biological sulfate reduction under acidic conditions. The reactor performance is notable for achieving such a high sulfate reduction rate under these specific conditions, showcasing the effectiveness of the biological sulfate reduction process even at low HRT values. The diverse microbial community, including sulfate-reducing bacteria, in such reactors is crucial for maintaining high-rate sulfate reduction and efficiency (Campos-Quevedo et al., 2021b).

Since the acidic influent was used to maintain the pH inside the reactor, the HRT exhibited significant fluctuations between periods II to IV in comparison to periods V to VIII. This result was governed by the sulfate-reducing activity of the consortium, where the produced sulfide and carbon dioxide tended to increase the pH, therefore demanding more protons to keep the reactor at acidic levels. Sulfide in sulfate-reducing reactors can

lead to an increase in pH, as observed in various studies. Villa-Gómez et al. (2014) found that a rapid increase in sulfide was associated with a rise in pH.

The experimental design involved sequentially lowering the pH value in each period, as illustrated in Figure 4.2 (Panel E), to investigate the consortium minimum limit (Table 4.1; Fig. 4.2E). In period III, the pH was decreased in 0.5 points, afterwards the pH was decreased by 0.25 points each period, because IV (pH 4.25) the pH was below the pK_a value of acetic acid (4.76) and therefore could cause a dramatic change in the microbial community. The pH was decreased once acetate was depleted, and HRT values were maintained the same at least three consecutive times. From these results, we can conclude that the threshold pH for the consortia was at ~3.16. When operating a sulfidogenic reactor, it is desirable to achieve complete mineralization of the substrate because the subproducts (volatile fatty acids) affect the efficiency of the reactor (Kimura et al. 2006; Kaksonen et al. 2006; Montoya et al. 2013).

At pH values lower than its pKa = 4.76, the acetic acid species becomes predominant (50%) resulting in increased cell toxicity. Thus, for sulfate-reducing processes conducted at acidic conditions, acetic acid production should be avoided (Utgikar et al. 2001).

Johnson and colleagues conducted extensive research on continuous acidic sulfidogenic reactors; nevertheless, they identified that the primary limitation of operating under such extreme conditions is the accumulation of acetic acid. In 2014, they operated a sulfidogenic reactor under highly acidic conditions, with an inlet pH ranging from 1.3 to 3 (Nancucheo and Johnson, 2014). Glycerol (2-3 mM) was used as the electron donor, resulting in a pH inside the reactor ~~of~~ between 2.8 to 4.5. However, they discovered that these extreme conditions were not conducive for the sulfate-reducing community, leading to an acetic acid concentration of 2.5 mM. In 2017, they controlled the temperature and pH to support the sulfate-reducing community, operating the reactor at pH levels of 4-5, with an inlet pH of 2.1 (Santos and Johnson, 2017). Glycerol was used as the substrate, yielding 20-40% acetate as a byproduct. To achieve these efficiencies, they had to introduce an acetoclastic microorganism (*Acidocella aromatica*) to prevent acetate

accumulation. Subsequently, in 2018, a reactor was operated at pH 4.5 with an inlet pH range of 2.2 to 2.5, utilizing glycerol (~19 mM) as a the substrate, resulting in residual acetate levels of 1 mM (Santos and Johnson, 2018).

At pH 4, there are 1000 times more protons than at pH 7, causing a diffusion pressure on the cell membrane in which much more protons diffuse through it at low pH compared to neutral pH. To avoid toxicity byproducts like acetic acid, the cells avoid the entrance of protons and then pump out protons, so at low pH, bacteria need to invest energy to maintain a neutral internal pH, and less energy is available for growth (Sánchez-Andrea et al. 2014). This is the reason why the proton rate observed at the lowest pH values inside the reactor (periods VI – VIII) is 10 times higher than at higher pH values (periods II - V; Fig. 4.2F). And the proton effluent rate was lower than the rate of protons entering the reactor. This extremely high proton rate was in the reactor however, the reactor showed efficiencies of sulfate-reduction above 70%, which may be explained by the type of sulfate-reducers that composed the microbial community.

In sulfate-reduction at very low pH, one-stage reactors offer advantages such as cost-effectiveness, efficient metal precipitation, and pH adjustment. Research has shown that biological treatment using sulfate-reducing microorganisms in one-stage reactors can effectively reduce sulfate concentrations and precipitate metals and increase pH levels, providing a comprehensive treatment approach (Waite et al., 2020; Zhao et al., 2017). Also, some one-stage sulfate-reducing reactors utilize a carrier material to enhance the performance and stability of the system. The addition of carriers like diatomite or netted carriers promotes the formation of SRM granules or immobilizes bacteria, improving their adhesion ability and biofilm formation(McMahon and Daugulis, 2008). Zeolite has demonstrated the potential as a support material for sulfate reducers, particularly under acidic conditions (Campos-Quevedo et al., 2021a; Pizarro et al., 2021).

In our experiments, the microbial community was able not only to resist high extreme acidic conditions, but the community was also able to reach high rates of sulfate-reduction when the reactor operated at acidic pH (3.77-3.27).

Table 4.1. Performance of the continuous sulfate-reducing reactor under acidic conditions (controlled pH 5 to 3.25)

Parameter									
Periods	**I[a]**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Days	**0-9**	**9.02-34**	**34.25-52**	**52.33-78**	**78.40-96**	**96.29-105**	**105.33-112**	**112.33-119**	**120.15-159**
pH reactor controller	3.21 - 5.1	5.18 ± 0.23	4.54 ± 0.03	4.28 ± 0.04	4.07 ± 0.04	3.77 ± 0.16	3.52 ± 0.18	3.27 ± 0.07	3.16 ± 0.10
Set point pH	-	5.0	4.5	4.25	4.00	3.75	3.5	3.25	3.0
pH inlet media	-	2.51 ± 0.01	2.26 ± 0.06	2.24 ± 0.28	2.32 ± 0.03	2.14 ± 0.07	1.84 ± 0.08	1.82 ± 0.00	1.70 ± 0.00
HRT (d)	-	6.08 ± 6.60	2.31 ± 1.02	7.57 ± 5.01	1.67 ± 0.86	0.53 ± 0.33	0.47 ± 0.32	0.37 ± 0.05	5.73 ± 3.55
Glycerol inlet (mg / L · d)	-	504.21 ± 44.6	624.92 ± 55.81	765.69 ± 140.84	648.33 ±152.88	732.4 ± 71.67	666.73 ± 66.01	656.16 ± 32.18	1177.82 ± 64.80
Sulfate inlet (mg / L · d)		1228.85 ±44.67	1162.46 ± 351.14	1700.69 ± 214.12	1167.18 ± 231.84	1350.05 ± 133.09	1233.22 ± 129.20	1177.82 ± 64.80	1333.79 ± 49.03
Acetate production rate (mg / L · d)	-	22.59 ± 24.41	1.17 ±2.53	7.25 ± 14.06	4.42 ± 12.85	7.59 ± 17.12	0.0 ± 0.0	0.0 ± 0.0	15.35 ± 10.05
Glycerol consumption rate (mg / L · d)	-	265.69 ± 360.51	325 ± 140.91	166.97 ± 145.48	535.67 ± 456.75	1732.42 ± 1044.23	1749.16 ± 1050.55	1680.48 ± 381.96	116.21 ± 134.03
Sulfate consumption rate (mg / L · d)	-	400.21 ± 451.86	547.25 ± 197.05	276.14 ± 200.77	710.16 ± 653.76	2893.82 ± 1927.19	3103.52 ± 1845.44	2881.84 ± 510.86	178.81 ± 347.95

[a]This period was in batch mode, which lasted until the carbon source was depleted at day 9, then the continuous operation began.

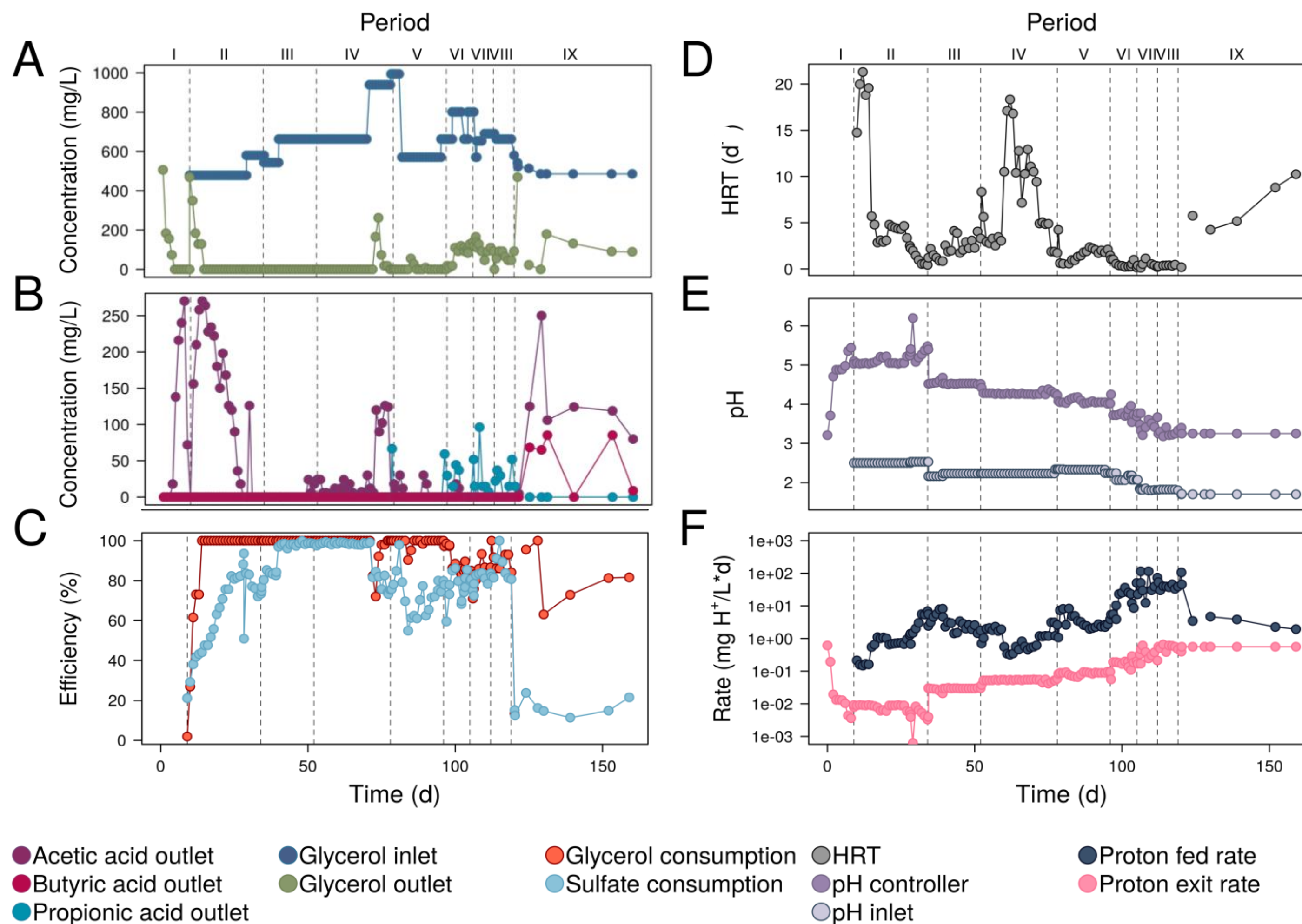


Figure 4.2 Profiles of the reactor during set-up (Period I) and continuous operation (Periods II-IX). A) Outlet of byproducts as result of glycerol oxidation. B) Glycerol concentration in the inlet and outlet. C) Consumption efficiency (%) of glycerol and sulfate, D) Hydraulic retention time (HRT), E) pH values in the reactor and in the feed media (inlet). F) Proton rate in the fed media (inlet) and in the exit of the continuous reactor.

Figure 4.3. shows that the sulfate and glycerol consumption rates increased as the pH decreased. During the periods at pH 3.75-3.25 (VI-VIII), the reactor achieved the highest glycerol and sulfate consumption rates, indicating the acidophilic character of the consortium. It is worth mentioning, that such consortium was obtained after more than 200 days of cultivation and selection, to ensure stability and reproducibility of the microbial activity (**Chapter 2**). There was an abrupt decrease in the consumption rates when the pH was controlled to 3.16 (Period IX, Figure 4.3).

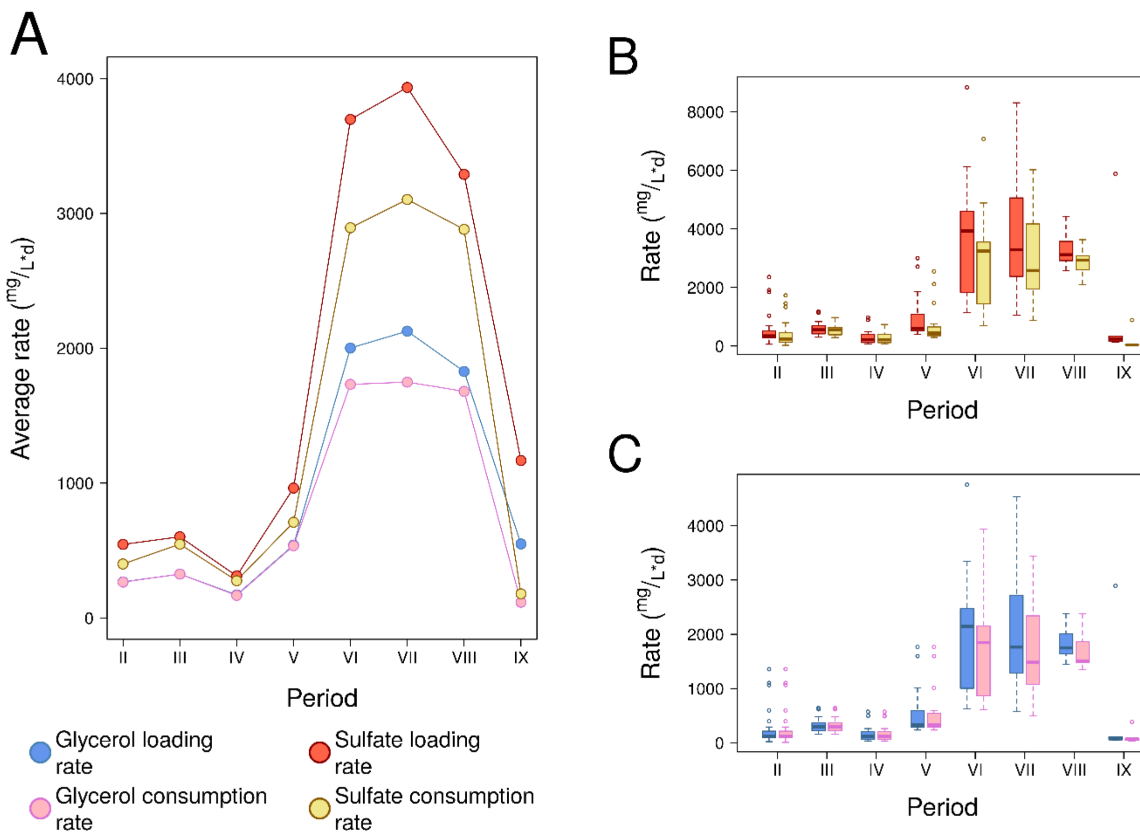


Figure 4.3. Loading and consumption rates A) Glycerol and sulfate average loading and consumption rates; B) and C) Box plots showing the sulfate and glycerol loading and consumption rates in each of the operational periods.

The sulfate consumption rates reached in Period VII (881 - 5108 mg/L·d) and in Period VIII (~2098 – 3633 mg/L·d) are comparable with those reported previously for low pH sulfidogenic bioreactors ~2017 mg/L·d pH inside the rector 2.8-4.5 (Nancucheo and Johnson 2014), ~1536 mg/L·d (Santos and Johnson 2017), and ~1421 mg/L·d (Santos and Johnson 2018). The rates of glycerol consumption presented the same tendency,

reaching high values in Period VI (pH 3.75) ~6.361 mM – 29.615 mM , Period VII (pH 3.5) ~5.142 – 35.816 mM, and Period VIII (pH 3.25) ~14.027 – 24.726 mM. The glycerol consumption rates achieved in this work are comparable to the rates previously reported ~800 - 1700 mg/L·d, at pH 2.8 to 4.5 (Nancucheo and Johnson, 2014). Being one of the reports with the highest consumption values of glycerol and sulfate reported so far under continuous acidic conditions.

The acetate production rates were ~10 mg/L·d in periods III to VI, whereas in periods VII and VIII acetate was not detected in the reactor (Table 4.1). This performance was ideal, because all the acetate produced was consumed, despite the low HRT and pH conditions, with high concentration of glycerol ~543.35 – 663.01 mg/L·d, and sulfate ~ 998.06 – 1200.25 mg/ L·d. At pH <5 (periods III- VIII) the sulfate-reducing reactor produced fermentation products that remained below 1 mM, even with a glycerol feed exceeding ~994.60 mg/L·d. This study not only succeeded in sustaining consortium activity but also confirmed the acidophilic nature of the consortium.

Although there are several studies that use acidic reactor influents such as 3.0, 2.1 or 1.8-1.3 (Hernández et al., 2022; Hernández et al., 2023; Salo and Bomberg, 2022) few are those that operate at pH <4 inside the reactor, under continuous conditions pH (<4). This may be because these conditions are limiting for the different members of the microbial community inside the reactor and few of them can not only withstand such limiting conditions but be active under these conditions. This shows that although it took time to obtain the sulfate-reducing consortium, it was worth the time invested to develop an acidophilic consortium (**Chapter 2 and 3**).

4.3.2 Microbial community and statistical analysis

Sulfate reduction is a microbially driven, and understanding the abundance and composition of the microbial community can enhance our comprehension of reactor performance. Using Illumina Miseq sequencing enables the generation of a vast amount of DNA data using a highly efficient sequencing-by-synthesis approach. This method allows the identification of thousands of OTUs to explore microbial diversity (Derakhshani

et al., 2016). Through this technology, we managed to acquire a total of ~450,000 reads, with ~200,000 in the solid phase and ~250,000 in the planktonic phase, distributed across 9 periods with an average of approximately 17,000 reads per period. The microorganisms identified encompass a significant portion of the diversity within the consortium across different time frames, as evidenced by the rarefaction curves (Figure 4.4B).

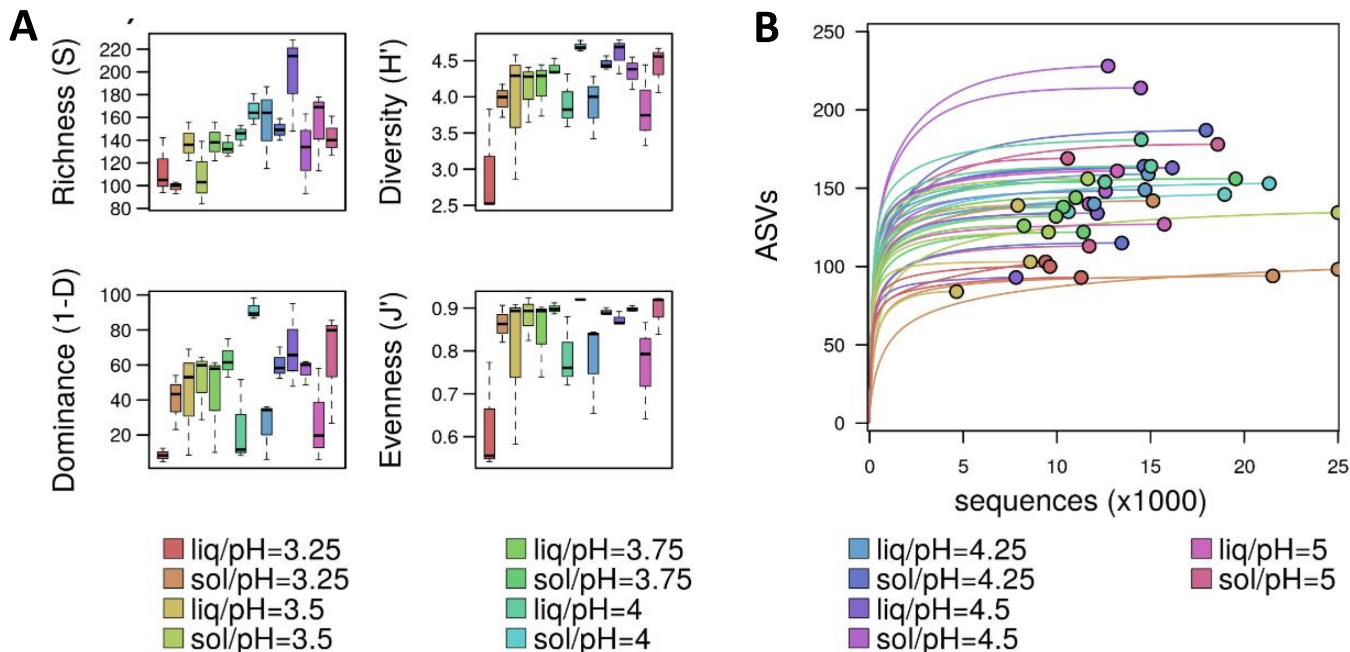


Figure 4.4 A) Diversity indexes of planktonic and biofilm communities developed during continuous operation of the reactor from Period II (pH = 5) to Period VIII (pH = 3.25). B) rarefaction curves of the planktonic and biofilm communities from amplicon sequence variants (ASVs), which provide a more precise resolution of microbial diversity by denoising raw amplicon data, resulting in distinct variants that capture finer taxonomic differences.

The analysis of the diversity indexes between the planktonic and biofilm communities showed that, in general, the species richness (S) was higher for the planktonic communities than those from the biofilms (Fig. 4.4A). The diversity (H') and evenness (J') did not show a clear tendency with the change in the pH, except for the liquid sample at pH 3.25 (Period VIII), which decreased compared to the previous two periods that were higher. Probably, the pH 3.25, affected the community where not all

microorganisms could resist these conditions (Fig. 4.4A). The biofilm communities showed a higher dominance than the planktonic ones; this trend was more consistent in the samples from pH 4.0 and lower values. Overall, the diversity indices pointed out that the microbial communities differed between the planktonic and biofilm.

In the planktonic phase the communities were mostly dominated by *Clostridaceae*, impacting the Simpson dominance index (1-D Fig. 4.5) but the richness and diversity indexes were similar regardless of the phase or period meaning that the community was resistant to acidic changes pH. From the pH point of view, the diversity indexes tended to decrease with the pH (Fig. 4.5B), surprisingly the richness (S) values at pH 4.5 resulted higher (220) than in the rest of the samples (S = 95 – 160). Probably, more members of the consortium were able to grow at pH of 4.5 and at lower pH not all microorganisms were capable to survive and be active. Samples from the period at pH 3.25 (Period VIII) showed the lowest richness values, meaning that the microbial community was selected by the pH. The Shannon-Wiener index (H) had similar median values except from sample at pH 3.25 which had the lowest value. Despite the confidence intervals, the dominance (1-D) and evenness (J’) resulted similar except for the sample at pH 3.25, which had the lowest value. The diversity indexes showed a clear difference between the highest pH value (5) and the lowest operating pH value (3.25) of the reactor. When pH decreases, the diversity of microorganisms can be impacted, as not all microorganisms are able to resist acidic conditions (Campos-Quevedo et al., 2021a; Nancucheo and Johnson, 2014).

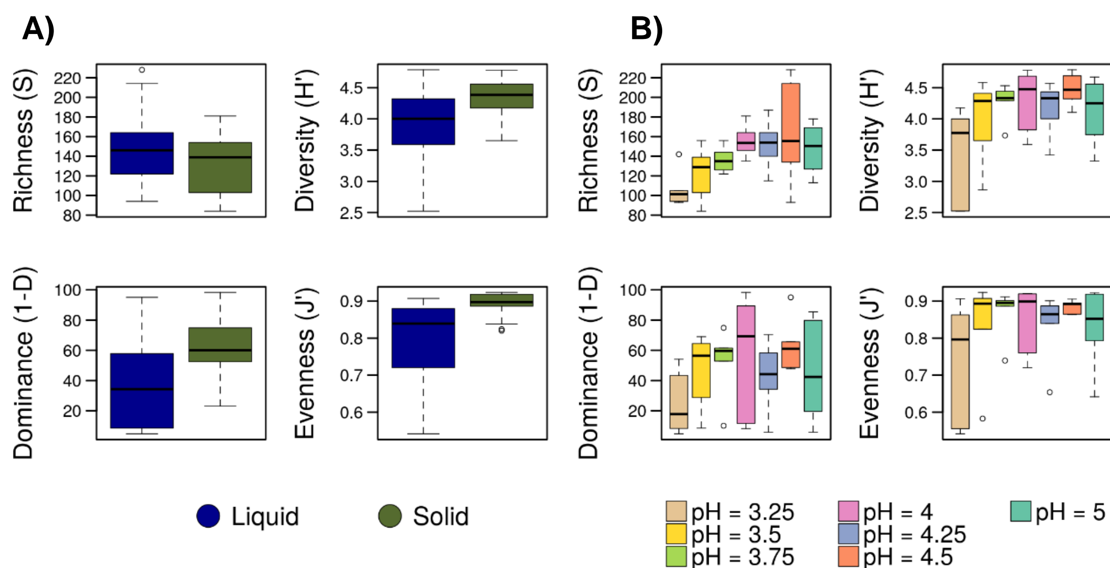


Figure 4.5 A) Diversity indexes of the planktonic (liquid) and biofilm (solid) communities during all the continuous operation. B) Diversity indexes at pH level.

This experiment was not the exception as it can be observed as the richness decreases (S= 100 and 160), and the diversity (H= 4 and 4.7) as the pH value is lowered within the reactor.

To evaluate the influence of the pH on the composition of the microbial communities, a statistical analysis through a multivariate homogeneity of groups dispersion (variances) was performed (Fig. 4.6). The composition of the microbial communities was different in all the pH values, depending on the type (biofilm or planktonic), except for the community at pH 4.25 (Period IV) where both communities were similar. Globally, the microbial communities obtained from the biofilm were statistically different from the planktonic communities (Fig. 4.7), which shows that the support material influenced the microbial community composition. The communities at the different reactor stages were statistically different compared with the samples obtained at pH 3.25 in period VIII (Figure 4.6). The samples that were statistically different in the planktonic and biofilm communities were the ones corresponding to the pH values of 4.5, 3.75, and 3.25 ($F<0.05$). Overall, these results indicate that the microbial community composition in the reactor varied with the pH imposed in each period.

The percentage of relative abundance of SRM in the planktonic phase is notably within the range of approximately 13.2% to 53%, while in the biofilm/solid phase, it falls between approximately 22% to 43%. These values represent some of the highest relative abundances of SRM documented to date under continuous acidic conditions (Montoya et al., 2013; Sato et al., 2019; Sun et al., 2014). Hessler et al., (2020) documented a significant decrease in the abundance of SRM, within a biological system designed for the mitigation of acid rock drainage, where biomass retention was implemented. Despite the system's characterization as a sulfate reducing reactor, the relative abundance of SRMs was found to be under ~5%. The Illumina high throughput sequencing results showed that the microorganisms composing the communities belong to five phyla: *Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria* and *Spirochaetae* mostly across all samples in solid and planktonic.

To have a better understanding of the microbial community we based our analysis at the family level and relative abundances (Figure 4.7). Overall, the communities contained 46 bacterial families.

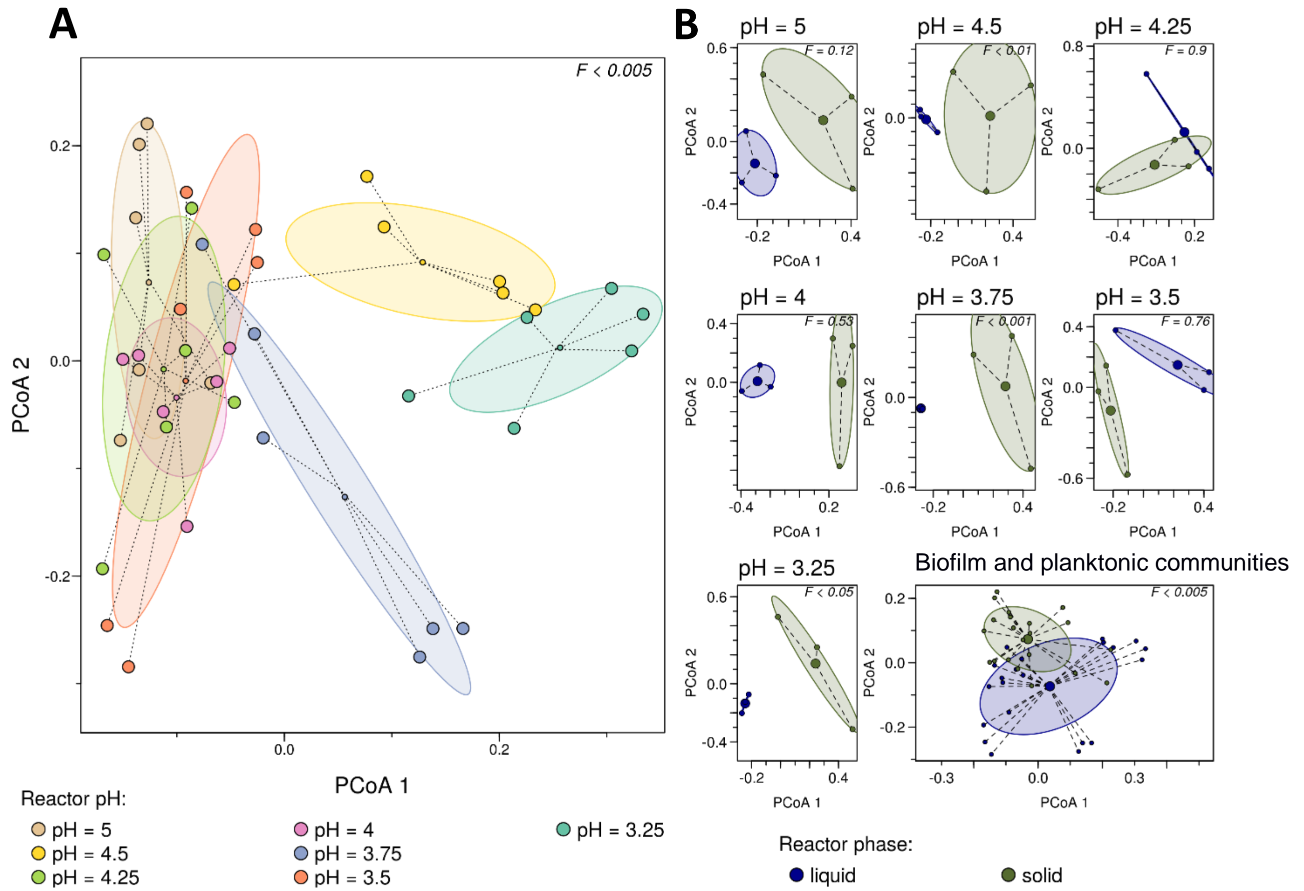


Figure 4.6. A) Statistical analysis achieved by Multivariate Homogeneity of Groups Dispersions (Variances) from B) the community samples evaluated at the different pH values, and retrieved from the biofilm (solid) or planktonic (liquid) communities.

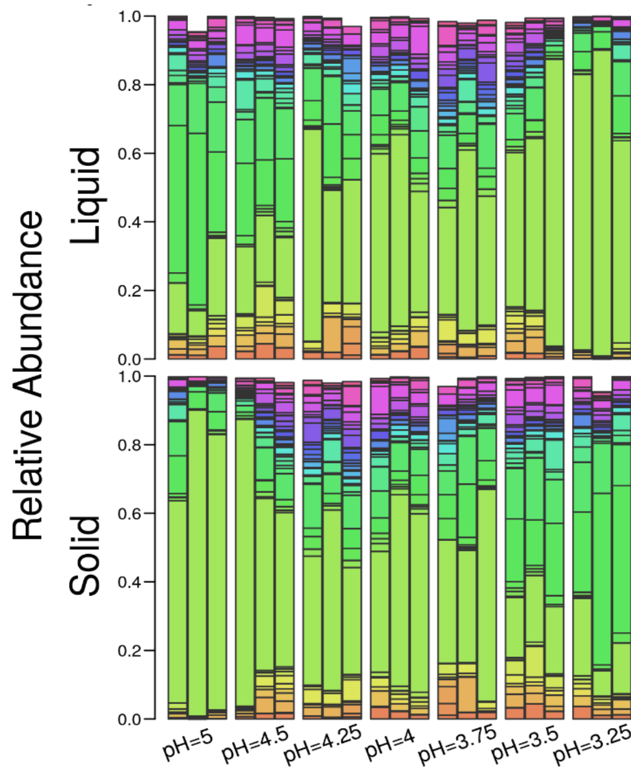


Figure 4.7. 16S rRNA based community composition at the family level and relative abundances at the different periods when decreasing the pH from 5 (Period II) to 3.25 (Period VIII).

It is also of particular interest to correlate the microbial community and their changes to each pH of the reactor. In this way, we can gain insight into which microorganisms could adapt and consume acetate at low pH; and extend the knowledge to subsequent development of technologies for environmental remediation (Giordani et al., 2019).

During pH decrease (Periods II-VIII), the community composition in the liquid (planktonic) changed more than the one in the solid phase (biofilm) (Fig. 4.7). At pH 5 (Period II), members of *Peptococcaceae* family (Order *Clostridiales*) dominated the communities of the liquid (38%) and solid (30%). As the pH decreased, members of this family remained in the biofilm but with lower relative abundances in periods III (~15 %) to VII (~16%). Some genera of sulfate reducers belong to this family such as *Desulfosporosinus*, *Desulfotomaculum*, *Desulfofundulus*, and *Desulfallas*. Most probably, members of *Desulfosporosinus* were responsible for the sulfate reduction at low pH in the reactor since this genera have been broadly detected in sulfate-reducing reactors operated at low pH (Nancucheo and Johnson, 2012; Sánchez-Andrea et al., 2014).

The dominance of the *Peptococcaceae* family was reported in enrichments aiming to remediate AMD. Dev et al., (2017) enriched a psychrophilic and acidophilic sulfate-reducing bacterial consortia from Arctic mine sediments, to treat AMD, were the *Peptococcaceae* family were involved in the initial glycerol co-fermentation to acetate (pH 6-8), which resulted a critical step for successful sulfate and metal removal in the proposed treatment process.

Clostridiaceae 1 was another family of the *Clostridiales* order enriched throughout the operation of the reactor. This family, constituted of spore-forming and fermenting bacteria, dominated the planktonic community (liquid) starting from a relative abundance of ~14 % in Period II reaching a maximum value of ~60%, in period VIII. In comparison with the biofilm, the relative abundance in Periods II to VII was less than 20% with a slight increase in Period VIII (~38.6 ± 10.02%). Alexandrino et al., (2014), showed that *Clostridiales*, such as *Clostridium spp.*, created favorable conditions for SRM activity by promoting sulfide production and pH increase through metabolic processes, enhancing the overall bioremediation efficiency. Additionally, *Clostridia* members have been described as potential acetate-oxidizing core communities under anoxic conditions (Hernández et al., 2022).

The microbial community plays a decisive role in the substrate consumption and byproducts formation such as acetate. These microbial communities should ideally

consume acetate and be resistant to acidic conditions for the successful treatment of acidic effluents using sulfate-reduction. Such microbial communities could be retrieved directly from naturally or anthropogenic acidic sites and exploited in continuous reactors. The selection and culturing should be done in such a way to obtain a robust community since the beginning and avoid bioaugmentation (Santos and Johnson 2017) or other strategies to control the acetic acid concentration.

In the planktonic community, the relative abundances of the SRM were between 13 ± 2% (Period VIII, pH 3.25), and 53 ± 8% (Period I, pH 5) and in the biofilm were between 22 ± 2% (Period VIII, pH 3.25) and 43 ± 6% (Period II, pH 4.5). Broadly, the relative abundance of SRM was higher in the biofilm than in the planktonic community at acidic pH 4.25 - 3.25 (Fig. 4.8).

Concerning the sulfur cycle microorganisms, the sulfate-reducing family *Desulfovibrionaceae* clearly showed a preference to remain in the biofilm since its relative abundance was much higher in the solid >10% than in the liquid <4.5% (Fig 4.7). *Desulfovibrionaceae* is a diverse family that was reported in freshwater lake sediments, rice roots, hydrocarbon-contaminated aquifers, terrestrial subsurface systems, river floodplains, and acid streams which shows the great adaptability of this family (Rabus et al., 2015). Sequences attributed to the genus *Desulfovibrio* were detected in nearly all periods, particularly within the biofilm, exhibiting the highest relative abundance during period III at 15 ± 3% (Fig 4.8). This observation highlights the resilience of *Desulfovibrio* towards a broad spectrum of pH fluctuations (Martins et al., 2009). Such findings are opposite from what was presented in **Chapter 3**, where *Desulfovibrio* predominated in the planktonic phase microorganisms, highlighting a different behavior in batch and continuous conditions.

Thermodesulfobiaceae was another sulfate-reducing family mostly enriched in the biofilm when the pH varied from 4 to 3.25 (periods V-VIII) (Fig. 4.7). Waite et al., (2020) reclassified the classes *Deltaproteobacteria* and *Oligoflexia*, and the phylum *Thermodesulfobacteria* into four novel phylum-level lineages, including the class Oligoflexia as a separate phylum named Bdellovibrionota. In 2012, Meier et al., enriched

batch cultures from acidic sediments of a pit lake, showed that at initial pH values of 3 and 4, sulfate-reduction and cell growth occurred only after an extended lag phase, however, at a higher rate than in the less acidic assays. At the end of the growth phase, enrichments were dominated by *Thermodesulfobium spp*. suggesting that these sulfate reducers were better adapted to acidic conditions. The *Thermodesulfobiaceae* family exhibited dominance (with a relative abundance of approximately 9% in the biofilm) within our reactor during Periods VII and VIII. This observation implies that *Thermodesulfobiaceae* effectively acclimated to the acidic conditions (Figures.4.7 and 4.8).

Thermodesulfobium genus was mostly abundant in the biofilm, especially in periods VII and VIII, which corresponded to the lowest pH values. Some of the strains affiliated to this genus are acid-tolerant and may grow at a broad pH interval 2.6-6.6. *Thermodesulfobium acidiphilum* (Frolov et al., 2017) and *Thermodesulfobium narugense* (Mori et al., 2003) were closely related to the sequences with ~97% similarity, respectively.

Desulfobacteraceae family was found throughout all the periods, mainly in the biofilm, in contrast to the planktonic phase, in the solid part *Desulfobacteraceae* was present ~7-10 % of relative abundance (Fig. 4.8). Members of this family can couple acetate oxidation with sulfate-reduction, *Desulfatirhbadium butyrativorans*, helping to avoid increasing concentrations of residual acetate; classified *Desulfobacteraceae* OTUs were closely related to environmental clones from oil sands tailings ponds, heavy metal contaminated wetlands, acid mine drainage, and cold lake sediments (Rezadehbashi and Baldwin, 2018).

The genus *Desulfosporosinus* reached the highest relative abundances in the planktonic and biofilm communities, 30 % and 13 % in Period II, respectively, and 23 % and 13 % in Period III, respectively (Fig 4.8). *Desulfosporosinus* is a SRM genus largely described in communities from acidic streams (Alazard et al. 2010; Sato et al. 2019). When analyzing the *Desulfosporosinus* sequences with similarities around 97%, the closest relative was *Desulfosporosinus acididurans*, isolated from sediment enrichments

can oxidize incompletely substrates with an optimal pH of 5.5 and was described in continuous reactors (Nancucheo and Johnson 2014; Santos and Johnson 2018).

The genus *Desulfofarcimen* was the second most abundant SRM in both communities, with relative abundances ~9.50% (periods II and IV) in the planktonic community (Fig. 4.8). Whereas in the biofilm, the relative abundances reached ~11.00 ± 2.62 (periods II, IV, and V). Looking for its closest relative, we found similarities of ~94% with *Desulfofarcimen acetoxidans* and ~95% with *Desulfofarcimen intricatum*, which may indicate the presence of a new member of this reclassified genus (*Desulfofarcimen*) formerly classified as *Desulfotomaculum* (Watanabe et al., 2013). Probably, microorganisms belonging to this genus tolerated the acidic pH and contributed to consuming acetic acid on Periods IV-VIII (pH 4-3.25). Especially *Desulfofarcimen* managed to increase its relative abundance in the biofilm particularly on Period VIII (pH 3.25) with ~5.49% relative abundance, which may be one of the acetate-oxidizing SRM present in the community

Sequences resembling *Desulfobacter,* with a similarity to the closest relative at the species level of ~ 99 % to *Desulfobacter postgatei* were also present in the samples. The relative abundance of *Desulfobacter* increased in the biofilm at pH 3.5. Usually, *Desulfobacter* is described as a neutrophilic mesophilic SRM, although it can also tolerate pH 5; SRM belonging to this genus are acetotrophic (Crine et al. 1999), and could also contribute to acetate consumption.

Other families harboring sulfur cycle microorganisms presented relative abundances below 1%, such as *Sulfurospirillaceae, Sulfurovaceae, Desulfobulbaceae, Desulfomicrobiaceae, Desulfurellaceae,* and *SRB2*.

The rest of the SRM, *Desulfofundulus*, *Desulfallas*, *Desulfobulbus*, *Desulfoprunum*, and *Desulfomicrobium*, were present in relative abundances below 1% in the planktonic and biofilm communities in all periods (Fig. 4.8), and together could help the maintenance of the sulfate-reducing community mostly.

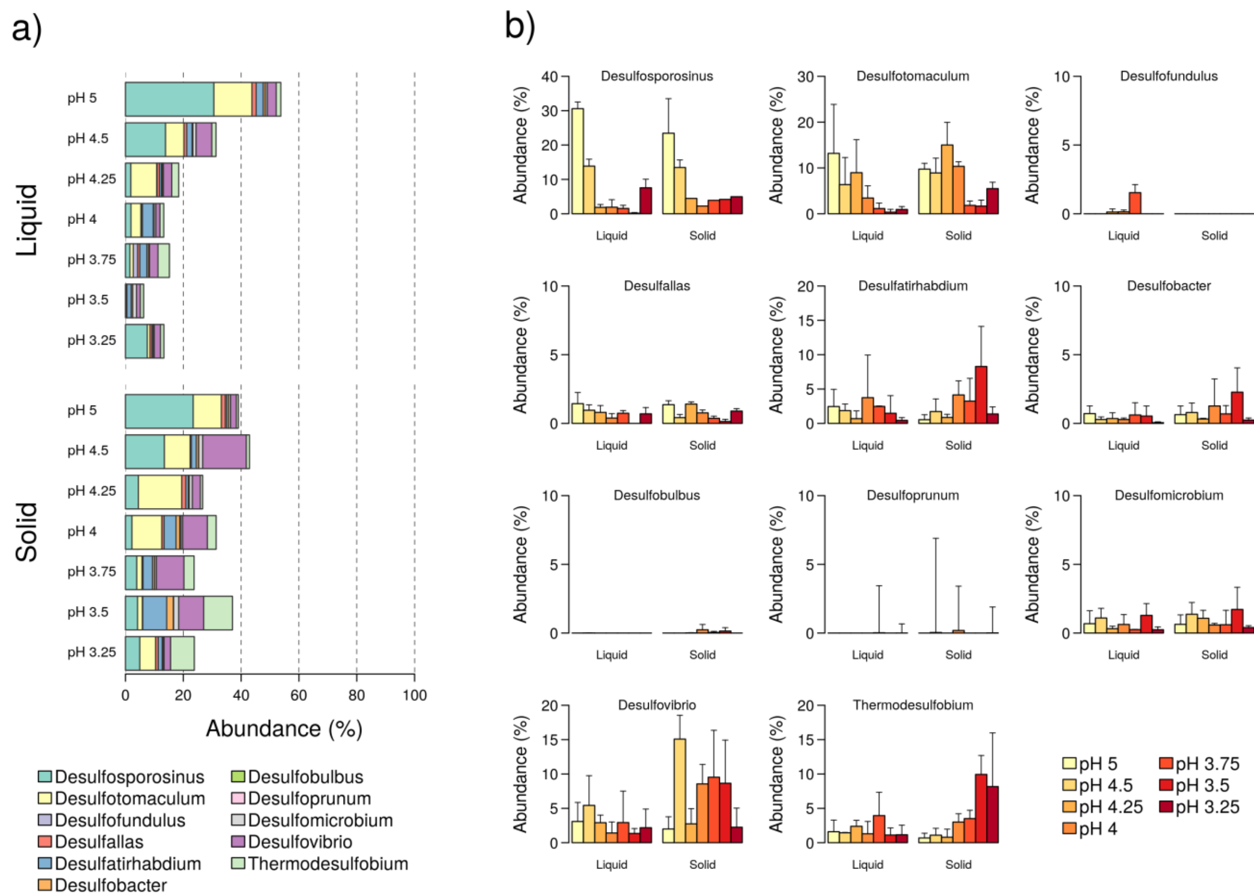


Figure 4.8. a) Relative abundances of the SRM found in each sample at different pH values in the solid/biofilm and liquid/planktonic phases. b) relative abundances of each of the SRM at different pH values in the liquid and solid phase.

Conclusions

We operated successfully a sulfidogenic continuous reactor at acidic conditions, controlled with an acidic feed media (pH ~2.3-1.7) with low to no acetate production. The sulfate-reducing efficiencies of Periods II-IV were ~99% (pH inlet of ~2.3), and in the most acidic Periods V-VIII were ~70% (pH inlet of ~2.3-1.8), representing one of the most efficient sulfidogenic reactors reported so far. Accumulation of acetic acid in acidic sulfidogenic reactors is the main drawback to microbial communities and, in this work, acetic acid was totally consumed at extremely acidic conditions (pH 3.77-3.27). The hydraulic retention time of the reactor was dictated by the efficient microbial activity of the

acidophilic consortium. The hydraulic retention time is one of the shortest reported so far (~ 0.5 d), with a controlled pH ~ 3.75. In this work, we used a microbial community developed and cultured under acidic conditions over four years. We observed the enrichment and permanence of different sulfate-reducing microorganisms, identifying 11 genera in the planktonic phase and biofilm. From these, three genera are complete acetate oxidizers (sequences closest to *Desulfofarcimen*, *Desulfatirhabdium*, and *Desulfobacter*). *Desulfatirhabium* and *Thermodesulfobium* increased their relative abundance during the reactor operation, while the non acetotrophic *Desulfovibrio* maintained its presence along the reactor run in the biofilm and liquid. The enrichment of acetotrophic SRM within the reactor was advantageous because their activity avoided acetate to accumulate, increasing the efficiency of the process and avoiding the toxicity of acetate at acid pH.

Chapter 5

General discussion, conclusions, and perspectives

The primary objective of this thesis was to focus on developing processes involving sulfate-reducing consortia and examining their adaptability in functioning effectively within acidic environments. This was achieved using an adapted and enriched consortium sourced from an abandoned sulfur mine in Mexico, as elaborated upon in **Chapter 2**. Furthermore, **Chapter 3** delves into the detailed process of adapting the selected consortia onto various inert support materials in a batch setting. Additionally, **Chapter 4** presents an in-depth analysis of the process involving the inoculation of a continuously stirred reactor with an extremely acidic inlet pH (3.5-1.3). A descriptive diagram of the chapters of this thesis are shown in Figure 5.1

5.1 The pH as a selective pressure factor

In many environments, toxic compounds or harsh conditions, such as dissolved metals or acidic pH, define the community composition. Particularly, at low pH, microorganisms are exposed to a high concentration of protons, which might enter the cells and lower the cytoplasmic pH, damaging cellular structures and inhibiting biological processes, subsequently leading to cell death (Guan & Liu, 2020). It is challenging to determine which specific conditions promote the prevalence of microorganisms in the communities, allowing them to grow and to remain active in harsh or extreme environments. In nature, selective pressures are biogeochemical determinants that drive changes in microbial community composition based on variations in the relative fitness of microorganisms (Carlson et al., 2019).

The pH level plays a critical role as a selective pressure factor in the remediation of acid mine drainage (AMD) using sulfate reducing microorganisms (SRM). The effectiveness of sulfate reducers in treating AMD is significantly influenced by the pH of the environment, which affects both the microbial activity and the chemical processes involved in sulfate reduction and metal precipitation (Neto et al., 2018; Santos and Johnson, 2017).

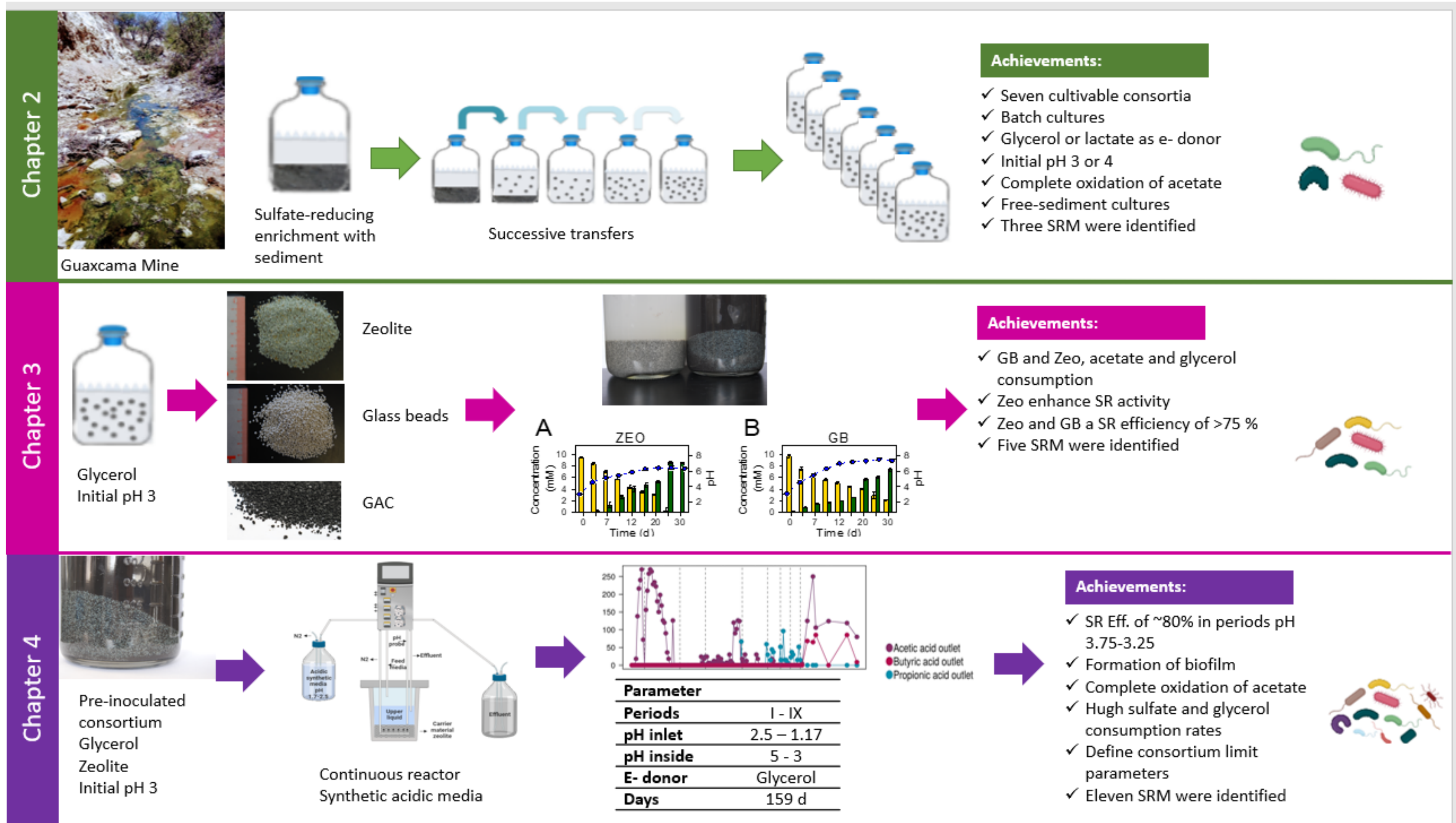


Figure 5.1 General diagram of the development of this thesis

Laboratory experimental approaches evaluating microbial fitness in biogeochemical gradients can be very useful in identifying likely selective pressures (Carlson et al., 2019). In the **Chapter 2** of this thesis, the pH was a selective pressure for the successful enrichment of the seven obtained consortia; the depletion of sediment from the cultures were identified as a leftover so its depletion might not press to obtain the planktonic seven consortia. Time was also decisive in obtaining successful and reproducible (sulfate-reducing, acetate-consuming) cultures. After 245 days, we obtained cultures free of sediment, eliminating the endogenous noise of having sediment in the consortia in the experiments in batch with the carrier materials and posteriorly in the continuous reactor.

Having a reproducible inoculum that consistently yields the same results is crucial for ensuring the reproducibility of research findings. This reliability allows for accurate comparisons between different experiments and enhances the credibility of scientific studies. By using a consistent inoculum, researchers can confidently replicate their experiments multiple times, leading to more reliable and robust conclusions. Therefore, the importance of maintaining a reproducible inoculum cannot be overstated in scientific research to ensure the consistency and validity of results (Sandoval-Espinola et al., 2015).

On the other hand, resilience is crucial for maintaining sulfate reduction efficiency in acidic environments, where incomplete oxidation of substrates can lead to acetate accumulation (Griffiths & Philippot, 2012). According to some authors, the time or duration in which the microbial community is adapted to the new circumstances, may vary from a matter of days during laboratory incubation, or even mere minutes for certain physical measurements (Zhang et al., 2005), extending to several years for observations conducted in the field, and is predominantly associated with the characteristics of the disturbance. The resilience of microorganisms composing the seven consortia was evident within the cultured communities due to the prosperity of microorganisms when exposed repeatedly to acidic pH (3 or 4), and to the consumption of the electron donor and its byproducts (Shade et al., 2012) **(Chapter 4)**, especially in the most acidic periods (VI-VII) with a pH inside the reactor of 3.25-3.75 and an HRT of ~0.5d. The ability of the

microbial community to withstand the harsh acidic pH may be attributed to the existence of numerous acidophilic members, such as *Clostridia*, *Desulfobacteraceae*, and *Thermodesulfobium*. It is important to note that these members did not constitute the entire community, as other microorganisms were also present in the consortia, denoting that the microbial community is tolerant to acidic conditions. Acidophiles, microorganisms that thrive in extremely low pH environments, have been the focus of research due to their potential applications in biotechnology. Santos and Johnson (2017) and Rüffel et al., (2018) both highlight the role of acidophiles, particularly sulfate-reducing microorganisms, in the treatment of acidic effluents.

The source of sulfate-reducing acidophiles could be natural or anthropogenic sites, including sulfidic mine areas and marine volcanic vents (Baker-Austin & Dopson, 2007; Jameson et al., 2010). Contrary to expectations, utilizing the inoculum from wastewater systems, with or without sediment, was widespread, hinting at its potential effectiveness (Nancucheo and Johnson, 2012).However, replicating the experiment under identical conditions proved unattainable, highlighting the need for a standardized and reproducible adapted inoculum, which must be preserved effectively to maintain its functionality over time (Nancucheo & Johnson, 2014; Santos and Johnson, 2017). In this thesis, the primary source of the seven consortia was the acidic leachate from the tails of an abandoned sulfur mine (Moreno-Perlin et al., 2019). Similarly to neutrophiles, acidophiles require a circumneutral intracellular pH. However, the difference between neutrophiles and acidophiles is that acidophiles tolerate pH gradients several orders of magnitude greater than neutrophiles. Acidophiles employ various mechanisms to maintain pH homeostasis, including restricting proton entry and pumping out protons (Baker-Austin and Dopson, 2007). These mechanisms involve changes in membrane lipid composition (Chong, 2024), increased activities of dehydrogenases (Jain et al., 2013), and the coordination of proton pump activity (Chen, 2021). Acidophiles also utilize active and passive mechanisms for acid resistance, such as proton efflux and consumption systems, DNA and protein repair systems, and chemotaxis and cell motility (Chen, 2021). The gradual decrease in pH over time has played a vital role in enhancing the adapted sulfate-reducing

consortia, facilitating their growth, and facilitating the efficient consumption of electron donors and byproducts (Salo and Bomberg, 2022).

All microbial communities, even acidophilic ones, have a pH limit. The continuous experiments described in **Chapter 4** revealed that the consortium at a controlled pH of 3.25 inside reactor, was the limit pH, because when this extremely low pH inside the reactor was changed to pH 3 it took to the edge of the microbial community and finally collapsed. Overall, the continuous experiments led us to know the operational limits of the acidophilic consortium.

5.2 Searching for acidophilic sulfate-reducing microorganisms is not an easy task

Reports describing the microbial communities of extremely acidic environments in many parts of the world abound, either in natural or anthropogenic ones (Johnson and Aguilera, 2015; Johnson and somg-Andrea, 2019). The microbial communities in acidic anthropogenic environments such as mining effluents, coal sewage, and domestic sewage have received increasing attention in recent decades due to the hazards that such effluent pose (Papirio et al., 2013).

Extreme sulfate-reducing communities have been identified as potential tools for bioremediation and environmental biotechnologies due to their ability to resist harsh conditions (Florentino, 2017; Johnson and Sanchez-Andrea, 2019; Zampieri et al., 2021). The composition of sulfate-reducing communities significantly impacts the degradation of organic matter and their ability to resist specific conditions. For example, Zhang et al., (2022)identified significant ecological variables that impact sulfate reduction, including the ratio of chemical oxygen demand to sulfate, the rate of sulfate loading, pH levels, oxidation-reduction potential, and alkalinity. The works of Frank et al., (2015) and Kwon et al., (2016) underscore the impact of temperature, pH, and electron donors on the rates of sulfate reduction. Meanwhile, Sánchez-Andrea et al., (2012) emphasize the contribution of sulfate-reducing bacteria in mitigating acid mine drainage. Ling et al., (2015) highlighted the adaptability of sulfate-reducers to adapt to variations in salinity and tidal conditions. Kikot et al., (2010) observed a detrimental impact on sulfate reduction when pH levels decreased from 7 to 5, inhibiting the process within individual bacterial

strains and the microbial community. However, the presence of heavy metals such as copper, and their interactions with other metals (e.g., pH-Cu(II), Zn(II)-Cr(III)), demonstrated an inhibitory effect on sulfate reduction by bacterial strains, although not significantly affecting the original microbial community due to the existence of metal-resistant organisms. In a research conducted by Bijmans et al., (2010), pH was identified as the predominant factor influencing sulfate reduction, achieving high-rate sulfate reduction at pH 4; showing that pH has a greater influence than substrate supply on the ratio of iron to sulfate reduction. In the context of our consortium, pH levels, and residual acetate concentrations were established as the primary ecological parameters determining the microbial composition, thereby playing a crucial role in enhancing a resilient consortium.

Although there are many potential sources of acidophilic communities, one of the main challenges is the lack of identified acidophilic SRM species. Most known SRM are merely acidotolerant rather than true acidophiles, which limits their effectiveness in extremely acidic conditions (Egas, 2024). For instance, the novel strain *Acididesulfobacillus acetoxydans* was identified as a moderately acidophilic SRM capable of complete oxidation of organic acids at low pH, a rare trait among SRM that may serve as an acid stress resistance mechanism (Sánchez-Andrea et al., 2022). This highlights the difficulty in finding SRM that can thrive and function optimally in such harsh environments. Moreover, the development of effective SRM consortia requires careful selection and cultivation under controlled conditions. Research has shown that culturing consortia from natural acidic sources can lead to successful sulfate reduction and acetate consumption, but this process involves multiple transfers and adjustments of electron donors and pH levels to optimize performance like is shown in **Chapter 2.**

How these microorganisms are cultivated and sampled is crucial to obtain successful cultures. Here is where the techniques used to enrich and select the microorganisms of interest play a relevant role. The traditional microbiology technique based on successive transfers is tedious and time-consuming. However, the results are worth it since it is possible to obtain reproducible cultures, both in growth and activity. The reproducibility of the seven consortia was assessed by the performance and the microbial

community composition of these consortia. Throughout this thesis **(Chapters 2-4)**, the microbial community exhibited similarities, as indicated by the relative abundance of the microorganisms comprising the consortium; however, when conducting the continuous experiments **(Chapter 4)**, the presence and activity of the SRM reached a higher level compared with the batch experiments. Several SRMs that were underrepresented (*Desulfofundulus*, *Desulfobacter*, and *Desulfallas)* in the batch cultures and in continuous conditions became detectable under constant acidic pH conditions. This result could be due to the preference of the microbial community for acidic pH, highlighting the importance of tracking the microbial community throughout the continuous experiments and acquiring better control and knowledge of the reactor performance.

The initial consortium was chosen for inoculation purposes of the continuous reactor, as the inoculum comprised three dominant sulfate-reducing genera, namely *Desulfovibrio*, *Desulfotomacolum*, and *Desulfatirhabdium*. Several reactor configurations for the treatment of acid streams rely on biomass retention, and the sulfate-reducing community must possess the ability to adhere to the carrier material and establish a biofilm (Sánchez-Andrea et al., 2014). Various types of carrier materials have been employed in the creation of sulfate-reducing biofilms; in a previous study (**Chapter 3**), zeolite was identified as a suitable carrier material for our sulfate-reducing consortium. Subsequent attachment of this consortium to zeolite resulted in the detection of five sulfate-reducing genera, encompassing *Desulfovibrio, Desulfotomacolum, Desulfatirhabdium, Thermodesulfobium*, and *Desulfosporosinus*. Notably, as the experiment transitioned into a continuous phase, the sulfate-reducing genera further proliferated to an impressive count of eleven taxa, including *Desulfovibrio, Desulfatirhabdium, Desulfobacter, Desulfomicrobium, Desulfobulbus, Desulfopronum, Desulfofarcimen, Desulfosporosinus, Desulfofundulus, Desulfallas*, and *Thermodesulfobium* showing the metabolic versatility and environmental adaptability of the consortium. *Desulfosporosinus* and *Thermodesulfobium* both genera are also part of the phylum *Firmicutes*, and usually reported in extreme pH conditions and temperature. Remarkably, within this cohort of eleven sulfate reducer taxa, three members with known acetate-consuming characteristics, specifically *Desulfatirhabdium, Desulfofarcimen*, and

Desulfobacter, consequently playing a crucial role in averting residual acetate as a byproduct (**Chapter 4)**.

The microbial composition plays a key role in the consumption of the substrate and its byproducts. In sulfate-reducing systems, acetate is a non-desired byproduct because its presence reduces the organic matter removal efficiency, and its toxic at acidic pH (Kaksonen et al., 2004c). Several strategies have been used to solve the presence of acetate in continuous reactors. For example, Santos and Johnson (2017) bioaugmented an acetoclastic acidophile (*Acidocella aromatica*) to a continuous reactor. Bioaugmentation would not have been necessary if the original inoculum had microorganisms able to oxidize acetate. In contrast, since the beginning of this work, we aimed to solve the bottleneck that acetate represents in sulfate-reducing reactors and invested time and efforts to obtain acidophilic acetate-consuming sulfate-reducing communities. Bioaugmentation can solve an immediate need; however, in the long term, the augmented microorganism will decrease its relative abundance because it was not originally a member of the microbial community and finds competition with other microorganisms and finally is displaced from the community (Radwan et al., 2019).

5.3 Carbon source suitable for sulfate-reduction at low pH

Acid mine drainage usually contains low concentrations of organic substrates. Therefore, the addition of suitable carbon sources and electron donors for sulfate reduction is often necessary to promote biogenic sulfide production, especially at low pH (<5) (Kaksonen et al., 2004b). Usually, lactate and ethanol are adequate substrates to enrich SRM at neutral pH. Still, when it comes to acidophilic sulfate reduction, the selection of the substrate could be a challenging area. When we talk of an acid effluent such as AMD, we are looking for substrates that are not ionizable and do not produce toxicity to the SRM communities. Using lactate or ethanol as substrates at acidic pH for sulfate reduction has the inconvenience that if incomplete oxidation occurs, the efficiency of substrate oxidation via sulfate reduction is lower because acetate remains as a byproduct (Barbosa et al., 2014).

Sulfate reduction can indeed be a pH-neutral reaction, as demonstrated by the active sulfate reduction in acidic sediments of gold mine tailings (Pimenov et al., 2015). This process is crucial for metal precipitation and the natural purification of dissolved metal ecosystems. However, the sulfate reduction rate in acidic lakes is regulated by a fragile equilibrium between proton flux and buffering reactions (Koschorreck et al., 2004). Although we managed to cultivate consortia that completely oxidized lactate and glycerol, and their byproducts (acetate, propionate), at pH 3 or 4 **(Chapter 2)**, for sulfate reduction at acidic pH, the most opted option is to use glycerol as the electron donor.

Glycerol, classified as a non-ionizable organic compound, stands in stark contrast to certain other substrates, such as glucose, in that it retains its molecular integrity without dissociating into ions when introduced into a solution. The presence of non-ionizable substrates results in a notable advantage as they do not exert any influence on osmotic conditions or pH levels in the course of metabolic processes. Furthermore, it is worth noting the carbon content of glycerol as a substrate, which results in a higher energy yield than alternative substrates, as indicated in the study by Santos and Johnson (2018). In this study, it was discovered that a maximum of 4 mM of sulfide originated from the decomposition of around 15 mM of glycerol. This indicates that the conversion of glycerol was not entirely efficient within the specified parameters. In contrast, in our sulfate-reducing system, 7.25 mM of sulfide was produced from the breakdown of roughly 5.71 mM of glycerol, accompanied by a sulfate generation rate of approximately 72%.

There are three potential byproducts that can be obtained from the oxidation of glycerol with the reduction of sulfate, depending on whether the oxidation is complete or not: carbonic acid/bicarbonate, hydrogen sulfide (uncharged or anionic), and acetic acid/acetate (Johnson & Sanchez-Andrea, 2019). Although acetate was produced from the incomplete oxidation of the substrates, the potential toxicity was not evident because the consortia consumed this byproduct **(Chapters 2-4)**. In addition, to get rid of the sediment, glycerol was the only substrate that allowed recovery of the consortium from the cultures with sediment at an initial pH of 3 **(Chapter 2)**.

Several researchers have employed acetate as the primary substrate to enrich acetotrophic sulfate-reducing microorganisms. Various attempts have been undertaken

to enhance the utilization of acetate, focusing on reducing the formation of this compound. The accumulation of acetate within microbial systems has the potential to disrupt numerous cellular processes severely (inhibit cell growth and accumulation of volatile fatty acids), thereby impeding the overall growth of these microorganisms (Li et al., 2022b). In sulfate-reducing microbial communities, the effective management of acetate levels emerges as a critical task for sustaining a harmonious and well-balanced ecosystem (Sánchez-Andrea et al., 2022).

The inoculum lacked acetate-consuming sulfate-reducing microorganisms capable of preventing the build-up of acetate. It is worth noting that the process of acetate oxidation is characterized by being less energetically advantageous when compared to other electron donors (Sahinkaya et al., 2015). Furthermore, it should be highlighted that microorganisms engaged in the oxidation of acetate exhibit a slower growth rate in comparison to their counterparts unable to use acetate, thereby potentially leading to a scenario where there is a competitive interaction for the electron acceptor, which in this case is sulfate (Plugge et al., 2011).

5.4 Carrier materials suitable for SRM attachment at low pH

An inert carrier material refers to a substrate that does not actively participate in microbial metabolism; it serves as a support structure for microbial attachment (Flayac et al., 2017). The employment of an inert carrier material has the potential to augment the metabolic activity of microorganisms attached to it, mitigate the risk of washout in continuous reactors, and protect the microorganisms against severe environmental conditions (Silva et al., 2006). Numerous prior research endeavors have documented the utilization of various carrier materials in sulfidogenic reactors, particularly under conditions of low pH (Bratkova et al., 2011; Nancucheo and Johnson, 2012; Silva et al., 2006)

Communities derived from natural habitats may become attached within sediments or soils which act as a “carrier material” in their natural environment. Sediments exhibit significant variations in characteristics across different ecosystems, posing a challenge in regulating experimental parameters due to the inherent variability in sediment conditions.

Thus, achieving reproducibility in the activity of inoculums combined with sediments in batch or reactor setups poses an increasing difficulty (Shrivastava et al., 2020). Therefore, we focus in this thesis in exploring carrier materials for our low pH sulfidogenic process.

The assessment of diverse carrier materials tailored for a specific microbial community has often been disregarded, a practice that is not advisable given the established fact that the choice of carrier material plays a pivotal role in shaping the composition of the microbial community that emerges and adheres to the surface of the carrier material (Bratkova et al., 2011; Sánchez-Andrea et al., 2012).

Several varieties of carrier materials have been employed to form sulfate-reducing biofilms. For instance, glass beads have been employed in percolating columns to evaluate the resistance to acidic conditions (pH 2.5–4.0) and to determine the efficiency of sulfate reduction using various carbon sources (such as glycerol, lactate, and ethanol) through the enrichment of acidophilic and neutrophilic SRM (Kolmert and Johnson, 2001). Glass beads have also been utilized as carrier material in continuous biofilm reactors to eliminate sulfate from highly acidic synthetic groundwater (pH 1.6–3.0) by utilizing glycerol as the primary substrate (Nancucheo and Johnson, 2014). Kim et al., (2015) aimed to develop a novel biocarrier using zeolite and sulfate-reducing bacteria to remove heavy metals from seawater, demonstrating high removal efficiency, achieving 98.2% for Cu^{2+}, 90.1% for Ni^{2+}, and 99.8% for Cr^{6+} at a concentration of 100 ppm,

The impact of the carrier material may manifest negatively, exemplified by instances using GAC, which results in the incomplete consumption of acetate (**Chapter 3**). Conversely, only in the assays with zeolite there was no sulfate at day 30, and the sulfide produced (9 mM) was the highest produced across incubations. Furthermore, in the scenario involving glass beads, the performance was comparable to that of the consortium operating without any support material, as elucidated in **Chapter 3**.

Continuous cultivation of sulfate-reducing consortia offers a more consistent and regulated setting for their proliferation, and efficiency, as well as reduced operational interruptions in comparison to batch cultivation. As demonstrated by Celis et al., (2009

and 2013) and Montoya et al., (2013), the continuous operation of the bioreactor throughout biofilm development facilitated the adherence of microorganisms to the polyethylene carrier material, excluding those incapables of thriving under the specified conditions. We emulate the start-up strategy from these authors which entails a 9-day batch period, along with our in-depth knowledge of the reproducible microbial consortium.

Examining this data leads us to deduce that the benefits of continuous systems entail a consistent supply of nutrients to the biofilm.

Operating at acidic pH in a sulfate-reducing reactor, such as a continuous reactor, is relevant for several reasons, particularly in the context of treating acidic mine drainage and other acidic effluents. The ability to function effectively at low pH is crucial for the bioremediation of environments where neutralization is not immediately feasible. Firstly, operating at acidic pH allows for the direct treatment of acidic mine drainage, which typically has a low pH and high concentrations of metals. This is significant because it reduces the need of pre-neutralization steps, thereby simplifying the treatment process and potentially lowering the costs (Nancucheo and Johnson, 2012).

Also contributes with continuous availability of nutrients supports the growth of microorganisms and the establishment of biofilms. Under continuous conditions, biofilms display higher attachment rates when contrasted with batch cultures. The constant flow aids in maintaining microbial adhesion, thereby preventing the washout risk. Furthermore, adaptation and stability are significant advantages, as microorganisms acclimate to the flow conditions and optimize their metabolic processes for sustained growth. With time, well-established biofilms can form, ultimately enhancing the effectiveness of the treatment process (Mohan et al., 2007). Nevertheless, discrepancies emerge when examining sulfate-reducing consortia operating under acidic conditions. Acidophilic microorganisms, including SRM, face challenges attributed to disrupting biological macromolecules and the denaturation of proteins at acidic pH levels. Maintaining an elevated pH within the cytosol necessitates energy expenditure, potentially influencing the viability of SRM.

The efficiency of SRM activity in acidic environments is dictated by the particular microbial population and the prevailing conditions within the bioreactor (Sánchez-Andrea

et al., 2014; Zambrano-Romero et al., 2022). Some of the examined communities in prior research on reactors have not demonstrated the ability to fully decompose acetate. The accumulation of acetate has the potential to impede the efficiency of the process, resulting in the presence of residual organic substances in the effluents (Celis et al., 2009; Nancucheo and Johnson, 2014; Patel et al., 2020). Johnson and colleagues (2018; 2022; 2012; 2014; 2017), examined various factors, such as pH, temperature, and metal concentrations, under continuous acidic conditions ranging from pH 2.3 to 5. They utilized glass beads as a carrier material and glycerol as the electron donor, resulting in acetate being produced as a byproduct in all instances, consequently diminishing the efficiency and success of the entire process.

The effectiveness of our acidophilic sulfate-reducing bioreactor **(Chapter 4)** relied on the consistent removal of hazardous by-products, such as acetate. Starting with **Chapter 2**, the consortium illustrated the complete consumption of the substrate and its associated by-products. This is why the consortium was able to avoid the accumulation of toxic by-products and maintain high removal efficiencies.

5.5 Sulfate-reducing communities and the description of novel species

The use of a sulfate-reducing consortium, as opposed to a pure culture, in the remediation of AMD presents numerous benefits, as demonstrated by recent investigations (Kolmert and Johnson, 2001; Yu et al., 2022). A consortium, defined as a collective of diverse microbial species, has the potential to augment the stability and efficacy of the bioremediation procedure. This enhancement is chiefly attributable to the varied metabolic capabilities and interactions among the distinct microbial species that comprise the consortium (Oliveira et al., 2003). Furthermore, consortia possess the ability to acclimatize to a broader spectrum of environmental conditions, such as fluctuating pH and temperature, which are frequently encountered in AMD locations. These advantages render consortia a more resilient and proficient alternative for bioremediation in the unpredictable and often severe conditions characteristic of acid mine environments.

However, when analyzing the microbiological community, it is important to note the importance of isolating microorganisms of interest. The isolation of acetate-oxidizing sulfate reducers at acidic pH is relevant for several reasons, primarily related to environmental remediation and bioprocess efficiency. In acidic environments, such as acid mine drainage, SRM play a vital role in mitigating extreme conditions by precipitating metals as sulfides and neutralizing acidity through proton consumption (Sánchez-Andrea et al., 2022). However, a significant challenge in these environments is the incomplete oxidation of substrates, which can lead to the accumulation of acetic acid, thereby reducing process efficiency. The novel species *Acididesulfobacillus acetoxydans*, capable of growing at pH 3.8, demonstrates the ability to completely oxidize organic acids to CO_2, which is uncommon among acidophilic SRB. This complete oxidation is particularly beneficial as it reduces the toxicity associated with protonated organic acids at low pH, thus serving as an acid stress resistance mechanism (Baker-Austin and Dopson, 2007).

During the experiments, the microbial community became more specialized with time. The planktonic consortium that was used as the inoculum at the beginning **(Chapter 2)** had ~15% relative abundance of sulfate-reducing microorganisms. The planktonic inoculum that was attached to the carrier materials **(Chapter 3),** it had up to ~30% relative abundance of sulfate reducers, and at the end, the consortium(inoculum) + zeolite (carrier material) used to inoculate the reactor contained ~53% relative abundance of sulfate-reducing microorganisms in the liquid and ~38% in the biofilm **(Chapter 4)**. The remarkable levels of efficiency attained within the reactor can be attributed to the notably high relative abundances of sulfate-reducing microorganisms present. It is noteworthy to highlight that the microbial community in question exhibits the most elevated proportion of sulfate-reducing microorganisms documented thus far, particularly when considering that this consortium is a mixed community formed by sulfate-reducers and fermenters, among others. Moreover, considering the changes in pH within the reactor, as well as in the composition of the feed medium, our investigations have allowed us to confirm the resilience and adaptability of the microbial community. This is evidenced by their ability

to thrive under conditions of sustained low pH, leading to exceptional efficiencies and the successful complete oxidation of acetate.

In **Chapter 4**, in the sulfidogenic reactor, it was possible to obtain sequences resembling eleven genera with sulfate-reducing microorganisms as members and three of them have members which are complete acetate oxidizers (*Desulfofarcimen*, *Desulfobacter* and *Desulfatirhabdium*). The relative abundances of sulfate-reducing microorganisms during the reactor operation were quite high (~43 to 23%), in addition to be able to form biofilms on the support material (zeolite).

Isolation of sulfate-reducing microorganisms that could tolerate acidic pH and oxidize acetate is relevant do develop biotechnological processes for the treatment of acidic effluents that contain metals and high sulfate concentration, for example those produced from acid mine drainage, acid rock drainage or papermill.

So far, only eleven species of acidotolerant or acidophilic sulfate-reducing microorganisms are fully described and validated, of which four are archaea and seven are bacteria (*Thermodesulfobium narugense*, *Desulfosporosinus acidiphilus*, D. *acididurans* D. *metallidurans, Desulfothermobacter acidiphilus, Acididesulfobacillus acetoxydans* and *Thermodesulfobium acidiphilum*) (Egas, 2024). All thoroughly characterized SRM belong to the genera *Thermodesulfobium, Desulfosporosinus, Desulfothermobacter*, and *Acididesulfobacillus*. Fully characterized SRM exhibit they are moderate thermophilics or mesophilics, with variations noted in their respective temperature ranges and optimal conditions for growth. Conversely, the pH range established for all characterized SRB bacteria exhibits considerable uniformity. Due to the low number of acidophilic sulfate-reducing isolates, we do not have enough information about their metabolic capabilities, the mechanisms they might be using in specific conditions in batch or reactor cultures, or what role they play in the site from which the enrichments were obtained. Further investigations are needed to complete the gap of knowledge related to acidophilic sulfate-reducing microorganisms.

5.6 Concluding remarks and perspectives

Acidic streams are often identified and defined by having a low pH level (< 4), as well as notably elevated levels of sulfate and heavy metals within their composition (Jameson et al., 2010). The presence of such components contributes to making these streams highly toxic, thereby posing a significant detrimental impact on the surrounding environment, particularly on the quality of water and the ecosystem's biodiversity. Reproducibility in sulfate-reducing reactors is crucial for ensuring consistent and reliable performance in bioremediation process. This thesis significantly adds to the existing knowledge base by focusing on the enrichment of seven planktonic distinct cultivable consortia, each of which has been shown to be reproducible and capable of completely consuming specific substrates such as glycerol or lactate, especially when introduced at an initial acidic pH level of 3 or 4. The cultivation of these consortia was achieved through the meticulous application of the technique of successive transfers over a period spanning 245 days. The findings of this study underscore the importance of dedicating sufficient time and resources to the cultivation of sediment-free cultures, as well as the necessity of establishing reproducible cultures that can be reliably used for future applications and research endeavors. These planktonic consortia can be more easily manipulated in laboratory settings, allowing for the isolation and study of specific SRM strains with desirable traits, such as high sulfide production or metal immobilization capabilities.

In this thesis, it was possible to conduct a deeper analysis of the importance of testing different carrier materials and their effect on the microbial community. We were able to observe the changes in the microbial community depending on the carrier material and in the community composition of the biofilm or planktonic communities. When conducting experiments with carrier materials, including abiotic controls, it is of utmost importance to assess the suitability of the carrier for the microbial community to preserve its activity.

Additionally, it was feasible to operate an acidophilic sulfidogenic reactor in continuous mode (pH 5 to 3.25), having high sulfate-reducing efficiencies, even in the most acidic periods with very low HRT (1-0.5 days). In the sulfidogenic reactor, the continuous acidic conditions facilitated the growth of 11 genera containing sulfate-

reducing microorganisms found in both planktonic and biofilm communities. Among these genera, *Desulfofarcimen*, *Desulfatirhabdium*, and *Desulfobacter* are known for acetate oxidization. Over the course of reactor operation, *Desulfatirhabium* and *Thermodesulfobium* saw an increase in their relative abundance, while *Desulfovibrio* remained consistently present.

This work allowed us to describe the importance of searching for communities that can completely oxidize acetate from natural sites and to culture them without the interference of the sediment. However, time can be a drawback, which should be taken into consideration. An "omics" analysis could be helpful to get an idea if the microorganisms of interest are present in a given sample. In this way, the classical microbiology techniques (successive transfers) can be made time efficient.

From a perspective standpoint, identifying and isolating additional acetate-oxidizing sulfate-reducing microorganisms that may coexist in the consortia could significantly contribute to the advancement of knowledge regarding acidophilic sulfate-reducing microorganisms. These isolates will help to understand the survival mechanisms they use in extreme conditions, compared with what is already known, or possibly find a new metabolic pathway. The composition of microbial communities that emerge during continuous operational processes offers a promising inoculum for the targeted extraction of such microorganisms, particularly as their prevalence shows a marked increase compared to batch cultures.

Using different acceptor donors could help to minimize time when we try to obtain acidophilic microorganisms of interest and explore new options for enriching a microbial community.

Another area of opportunity that warrants exploration involves acquiring the entire genetic makeup of the newly discovered acetotrophic sulfate-reducing microorganism delineated within the pages of this scholarly thesis. Such an effort would notably advance our insight into the metabolic pathway through which acetate becomes part of its cellular structure, and also help pinpoint specific genetic components that might provide it with the capability to survive in acidic surroundings.

The exploration of selective precipitation of metals may also be undertaken, capitalizing on the extensive range of pH levels in which the sulfate-reducing consortium exhibits activity under sustained operational circumstances. Within the reactor, the efficiency of the sulfate-reducing process was notably observed within the pH range spanning from 5 to 3.25, coupled with an influent pH level surpassing the threshold of >1.8-2.5, thereby delineating a substantial operational window conducive to the selective precipitation of metals. And to know which metals can be successfully precipitated and which metals can be toxic to the consortium.

Most importantly, it is crucial to maintain the consortia obtained during this thesis. This will ensure preserving the activity; otherwise, the cultures and activity could be lost.

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