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Anaerobic arsenotrophy linked to iron and sulfur biotransformations: implications on the fate of arsenic in solid-liquid interfaces

Tesis que presenta

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Doctora en Ciencias Ambientales

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Dra. María de Lourdes Berenice Celis García
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Dedicatorias

A mis padres, por todo su amor y apoyo incondicional

A mis hermanos, por su cariño y compañía

A mis sobrinos, que me contagian su alegría e inquietud

Gracias por estar conmigo en las buenas y en las malas. Ustedes son la fuerza y motivación para lograr mis propósitos en la vida.

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List of Abbreviations

AAS	Atomic absorption spectroscopy
aio	Operon of heterotrophic arsenite oxidase
arr	Operon of arsenate respiratory reductase
ars	Operon of arsenic resistance by detoxification
arx	Operon of autotrophic arsenite oxidase
CB	Artesian hydraulic complex Cerrito blanco
CH_2O	Organic mater
CSE	Chemical sequential extraction
CT	Spring club de tiro
DARB	Dissimilatory Arsenate-reducing bacteria
DNA	Deoxyribonucleic acid
ΔG°´	Standard Gibbs free energy
EDS	Energy dispersive spectroscopy
Eh	Redox potential
e^- meq	Electron milli equivalents
ESEM	Environmental scanning electron microscopy
FISH	hibridación in situ fluorescente
ICDD	International Center of Diffraction Data
ICP	Inductively coupled plasma
Kev	Kilo electron volts
Log K_{sp}	Logarithm of the solubility product
mM	Millimolar
nm	Nanometers
pH	Hydrogen potential
SEM	Scanning electron microscopy
SRB	Sulfate-reducing bacteria
TEM	Transmission electron microscopy
ESEM	Environmental scanning electron microscopy
PCR	polymerase chain reaction
VSS	volatile suspended solids
XRD	X-ray diffraction
µm	Micrometers

RESUMEN

Arsenotrofía anaerobia vinculada a las biotransformaciones de azufre y hierro: implicaciones en el destino del arsénico en interfases sólido-líquido

Palabras clave: Arsénico, comunidades microbianas, ciclos biogeoquímicos, bioprocesos, As(V)-reducción, sulfato-reducción, Fe(III)-reducción

El arsénico (As) es un metaloide clasificado dentro del grupo de los metales pesados por su toxicidad a bajas concentraciones (µg/L). A escala global, el papel del arsénico como contaminante prioritario se deriva de su presencia en agua subterránea que se usa para consumo humano. El origen del As acuoso se puede se puede atribuir a fuentes naturales (composición geológica de acuíferos) y/o antropogénicas (minería). En gran medida, la interconexión entre los ciclos biogeoquímicos del As, C, Fe, S y N determina el destino de este contaminante en cuerpos de agua. Dentro de este marco, los procesos microbianos son uno de los principales actores en la especiación química y trasporte del arsénico. Se han efectuado estudios detallados con el fin de explicar los procesos de movilización/inmovilización de As y su remediación. Sin embargo, sigue siendo un reto el escalamiento de los sistemas de laboratorio para su aplicación en ambientes naturales. La investigación en este campo puede contribuir al entendimiento de los procesos biogeoquímicos implicados en el ciclaje de arsénico. Asimismo, el conocimiento generado es útil para hacer predicciones del alcance de los procesos microbianos, valoración del riesgo ecológico, e implementación de estrategias de biorremediación apropiadas. El objetivo general de esta tesis fue explorar la arsenotrofía anaerobia vinculada a las biotransformaciones de hierro y azufre con un enfoque en la remediación de acuíferos contaminados con arsénico.

Como una primera aproximación, se colectaron dos sedimentos superficiales contaminados con As (CB y CT), se investigó la distribución de As en las distintas fracciones minerales de los sedimentos y se determinó que más de 77% de este se encuentra altamente biodisponible. El segundo paso fue conocer la estructura y función de la comunidad microbiana nativa de los sedimentos, se encontró que los altos niveles de arsénico no limitan la diversidad bacteriana, siendo el sedimento CT el más contaminado (2263.1 ± 167.7 mg de As/kg) y el que presentó la mayor diversidad (índice de Shannon 7.4). La microbiota de este sedimento sufrió un proceso de adaptación, lo cual se corroboró con la amplificación del gen *arrA* en el sedimento fresco, este gen es responsable de codificar la enzima arseniato reductasa para la respiración de arseniato (As(V)).

Los sedimentos se usaron como inóculo para enriquecimientos en microcosmos anaerobios y mediante el acoplamiento de los bioprocesos de reducción de As(V) y sulfato se obtuvieron minerales biogénicos de arsenito (As(III))/sulfuro (S(-II)), que fungieron como sumideros de As. El género bacteriano *Desulfosporosinus* se identificó como el miembro dominante de la microbiota vinculada a los biominerales de los enriquecimientos de sedimentos, alcanzando abundancias cercanas a 75%. Este género es metabólicamente flexible, puede respirar As(V), sulfato, Fe(III) y promueve la precipitación de sulfuros de arsénico.

Partiendo de los enriquecimientos de sedimentos, por un proceso de transferencias sucesivas se obtuvieron consorcios bacterianos As(V)/sulfato-reductores. Los consorcios esencialmente se conformaron por bacterias reductoras de As(V) y formadoras de esporas. Cabe destacar que durante la propagación de los consorcios se identificaron presiones selectivas importantes: 1) La remoción del sedimento (una fuente de protección y nutrientes), 2) las altas concentraciones de As(III) acuoso (10 mM) y 3) la inactivación de células por atrapamiento en la matriz del biomineral (mineralización de células).

Los consorcios bacterianos de ambos sedimentos mediaron la formación de nanofibras de As(III)/S(-II). No obstante, los biominerales presentaron distinta mineralogía dependiendo de las tasas de reducción de As(V) y sulfato. El biomineral del consorcio CB, que presentó una mayor tasa de sulfato-reducción, se identificó como bonazziita y biomineral del consorcio CT, donde la tasa de As(V)-reducción fue ~5 veces mayor que la tasa se sulfato-reducción, se identificó como rejalgar (fase más cristalina). En ambos biominerales el 90% de los géneros bacterianos (*ej. Desulfosporosinus, Sedimentibacter, Exiguobacterium, Fusibacter)* incluyen especies portadoras del gen *arrA.* La diferencia más evidente entre las comunidades de los biominerales fue el predominio de *Desulfosporosinus* (34%, un sulfato-reductor) en el biomineral CB y de *Pseudomonas*, (36%, un As(V)-reductor) en el biomineral CT, esta diferencia repercutió en la actividad reductora de As(V) y sulfato.

Empleando el consorcio CT como inóculo, se estudió la influencia de bioprocesos de reducción de As(V), Fe(III), y sulfato en la disolución de As(V) coprecipitado con calcita, yeso, y ferrihidrita cuando dichos minerales se usaron como aceptores de electrones. Se encontró que con calcita-As(V) la reducción de As(V) promueve la acumulación de As(III) acuoso, mientras que con yeso-As(V) el sistema presentó un mecanismo de autorregulación ya que el As(III) acuoso se remineralizó como sulfuros de arsénico. Respecto al sistema ferrihidrita-As(V), la sulfato-reducción tuvo un impacto negativo propiciando la movilización de As(III). Cada mineral propició diferentes trasformaciones redox lo que a su vez impactó la estructura de comunidad bacteriana y en el destino final del arsénico.

Con el fin de enriquecer los hallazgos de esta investigación algunas perspectivas se discutieron, por ejemplo, la necesidad de identificar y cultivar nuevas bacterias reductoras de As(V) y a la par corroborar en estas especies la capacidad de metabolizar y/o detoxificar As, usando marcadores moleculares (ej. *arsC* gene, *arrA* gene). Se recomienda evaluar la estabilidad de los sulfuros de As biogénicos bajo oxidación microbiana, en un ambiente con limitado en carbón orgánico, donde el As(III) y el S(-II) podrían ser utilizados como donadores de electrones en presencia de un aceptor favorable como el nitrato.

SUMMARY

Anaerobic arsenotrophy linked to iron and sulfur biotransformations: implications in the fate of arsenic in solid-liquid interfaces

Keywords: Arsenic, microbial communities, biogeochemical cycles, bioprocesses, As(V)-reduction, sulfate-reduction, Fe(III)-reduction

Arsenic (As) is a metalloid classified in the heavy metal group as it can induce toxicity at low concentrations (µg/L). On a global scale, the recognition of arsenic as a priority pollutant derives from its occurrence in groundwater for human consumption. The origin of the aqueous arsenic can be due to natural (geological composition of aquifers) and/or anthropogenic (mining) sources. In great extent, the interconnection among the biogeochemical cycles of As, C, Fe, S, and N determines the fate of this toxic element in water bodies. Within this framework, microbial processes are one of the main actors in chemical speciation and arsenic transport. Detailed studies have been carried out in order to explain the processes of arsenic mobilization/immobilization and remediation. However, the scaling of laboratory systems to natural environments is still a challenge. Research in this field can contribute to the understanding of biogeochemical processes involved in arsenic recycling and the generated knowledge will allow making predictions of the extent of the microbial processes, ecological risk assessment, and implementation of appropriate remediation strategies. The global aim of this dissertation was to explore the anaerobic arsenotrophy linked to iron and sulfur biotransformations, with a focus on arsenic remediation in As-polluted aquifers.

As a first approach, two surface As-polluted sediments (CB and CT) were collected, the partitioning of arsenic in the mineral fractions of the sediments was investigated and it was determined that more than 77% of arsenic is highly bioavailable. The second step was to investigate the structure and function of the native microbial communities of the sediments, it was determined that the high levels of arsenic did not impact negatively the bacterial diversity, the sediment CT was the most contaminated (2263.1 ± 167.7 mg of As/kg) and presented the greatest diversity (Shannon 7.4 index). The microbiota of this sediment already suffered an adaptation process, which was corroborated by the amplification of the *arrA* gene in the fresh sediment, this gene is responsible for encoding the enzyme arsenate reductase for arsenate (As(V)) respiration.

The sediments were the source of microorganisms for enrichments in microcosms. Through the coupling of As(V)- and sulfate-reduction were obtained biogenic minerals of arsenite (As(III))/sulfide (S(-II)) which served as arsenic sinks. The genus *Desulfosporosinus* was identified as the dominant member of the microbiota linked to biominerals from sediment enrichments, reaching abundances close to 75%. This bacterial genus is metabolically flexible, can respire As(V), sulfate, Fe(III) and promotes arsenic sulfide precipitation.

As(V)/sulfate-reducing bacterial consortia (Sediment-free) were obtained after 8 months of sub-culturing of sediment enrichments. The consortia essentially composed by spore forming and As(V)-respiring bacteria. Remarkably, significant

selective pressures were identified during consortia propagation: 1) sediment removal (a source of protection and nutrients), 2) high concentrations of aqueous As(III) and 3) cell inactivation by trapping in the matrix of the biomineral (cell mineralization). Bacterial consortia of both sediments precipitated nanofibers of As(III)/S(-II). However, each biomineral presented different mineralogy and crystallinity, the biomineral CB was identified as bonazziite and the biomineral CT as realgar. The changes in the mineralogy were related to the rates of As(V)- and sulfate-reduction. In the consortium CT, where the rate of As(V)-reduction was about 5 times higher than the rate of sulfate-reduction, realgar (the most crystalline phase) was formed. About 90% of the bacterial genera (*e.g. Desulfosporosinus, Sedimentibacter, Exiguobacterium, Fusibacter)* present in the biominerals from consortia gathered *arrA*-carrier species. The most evident difference in the bacterial communities of the biominerals was the dominance of *Desulfosporosinus* (34%, a sulfate-reducer) in the biomineral CB and the dominance of *Pseudomonas*, (36%, an As(V)-reducer) in the biomineral CT, this difference impacted the As(V)- and sulfate-reducing activities. Using the CT consortium as inoculum, the influence of bioprocesses of As(V)-, Fe(III)- and sulfate-reduction in arsenic mobilization was studied when calcite, gypsum, and ferrihydrite coprecipitated with As(V) were the electron acceptors. It was found that in the calcite-As(V) system As(V)-reduction culminates with the accumulation of aqueous As(III), while the gypsum-As(V) system presented a self-regulation mechanism, and the aqueous As(III) was remineralized as arsenic sulfides. In the case of ferrihydrite-As(V) system, sulfate-reduction had a negative effect promoting the mobilization of As(III), despite iron secondary minerals formation that also adsorb arsenic (*i.e.* siderite and iron sulfides). Each mineral led to different redox transformations, which in turn impacted the structure of the bacterial community and the final arsenic fate.

In order to enrich the findings of this research, some perspectives were discussed, for instance, the need to identify and cultivate novel As(V)-respiring bacteria, and at the same time corroborate their ability to metabolize and detoxify arsenic, through the use of molecular markers (*e.g. arsC* and *arrA* genes). We recommend evaluating the stability of biogenic arsenic sulfides, under microbial oxidation, in an environment depleted in organic carbon, where As(III) and S(-II) could be used as electron donors in presence of a favorable electron acceptor such as nitrate.

CHAPTER 1

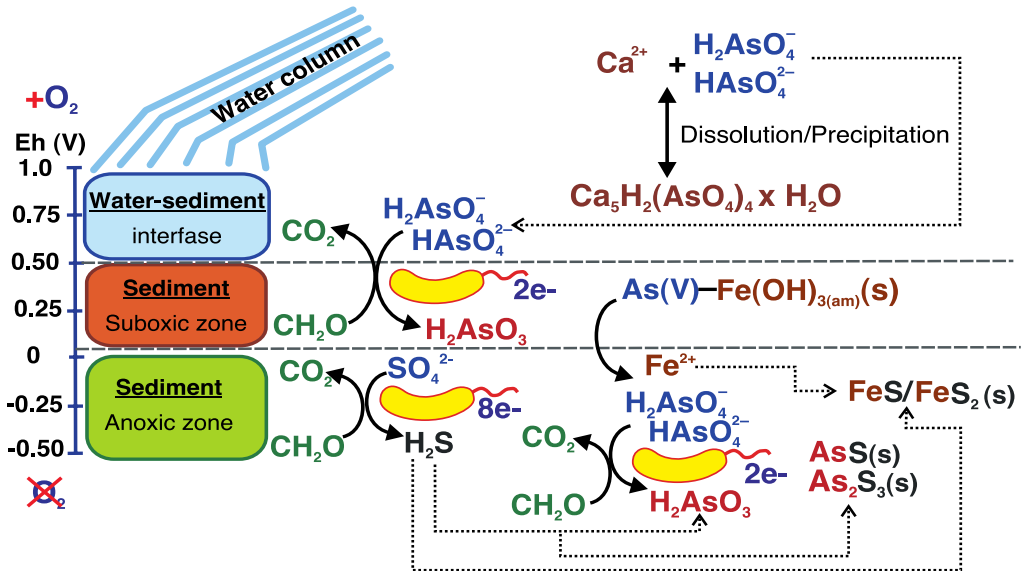
General Background

Microbial anaerobic arsenotrophy in arsenic-polluted freshwater sediments

Highlights

- The integrated study of the biogeochemical cycles of As, S, Fe, N, and C is an important tool to determine the chemical speciation, fate, and mechanisms of arsenic attenuation in polluted-sites.
- Bioremediation of arsenic contaminated sediments is governed by three main challenges: microbial metabolism at the water-solid interface, formation of biominerals as arsenic sinks, and their stability against environmental parameters and microbial activity.
- Diverse microbial phylogenetic groups (autotrophic and heterotrophic) are involved in arsenic metabolism in freshwater sediments.

Graphical Abstract



CHAPTER 1

ABSTRACT

High concentrations of arsenic (As) have been reported in groundwater destined for human consumption on a global scale; consequently, arsenic is of primary concern in aquifers worldwide due to its great eco-toxicological potential. The co-occurrence of arsenic with iron (Fe) and sulfur (S) minerals is very common in subsurface environments, and the interaction of As, S, and Fe cycles is relevant to understand the distribution of this metalloid in water bodies. The presence or absence of aqueous arsenic depends on biogeochemical processes such as precipitation and dissolution of As-bearing minerals, which mainly involve microbial activity. Microorganisms are responsible for catalyzing multiple redox reactions that positively or negatively impact the stabilization of arsenic. For instance, according to the extensive background of investigations, microbial sulfate- and Fe(III)-reduction can mitigate or trigger the accumulation of aqueous arsenic, depending on the geologic features and physicochemical parameters (pH, redox potential) of the polluted-sites. Under this perspective, three main aspects need further investigation: 1) Deciphering of the mobilization/immobilization mechanisms of arsenic under scenarios with biogeochemical heterogeneity, 2) understanding of the interaction biomineral-microorganisms in distinct microenvironments, and 3) study the influence of the sediment mineralogy in the microbial community behavior and arsenic fate. The new knowledge generated in these areas will be an essential tool for the evaluation of ecological risk, remediation of polluted environments, and even to assess the autoregulation capacity that some systems could have.

Overall, this chapter summarizes the current state of knowledge concerning biogeochemical processes involved in the speciation and distribution of arsenic in polluted freshwater sediments. This chapter also underlines the anaerobic arsenotrophy in heterotrophic and autotrophic scenarios.

Keywords: Arsenic, anaerobic arsenotrophy, redox reactions, microbial activity, metabolism, detoxification, bioremediation

CHAPTER 1

1.1 INTRODUCTION

The release of arsenic (As) from sediments into groundwater is a global concern that has been highlighted in numerous studies; arsenic represents a latent risk for the human health, it is classified as a poison, carcinogen, teratogen and clastogenic (Gorny et al., 2015; Huang, 2014; Slyemi and Bonnefoy, 2012). According to the World Health Organization (WHO), the high threat of arsenic to public health is in the use of polluted water for drinking, food preparation, and irrigation of food crops. More than 200 million people worldwide are exposed to drinking water containing arsenic concentrations exceeding the guideline value established by the WHO, 10 mg/L (Alam and McPhedran, 2019). Therefore, the study of chemical, geologic, and biological processes involved in the transformation of this metalloid in freshwater systems is highly relevant.

In Mexico, the majority of the aquifers with high content of arsenic are located in arid zones, where water is limited and the supply to the people mainly depends on groundwater. In most of the cases, the origin of the high arsenic concentration has been attributed to the geologic nature of the aquifers and mining exploitation (Reyes-Gómez et al., 2013). The incidence of arsenic in groundwater exceeding the maximum permissible levels of the Mexican regulation (25 µg/L) has been reported in 16 states of the Mexican Republic (Aguascalientes, Chihuahua, Coahuila, Baja California Sur, Durango, Guanajuato, Hidalgo, Jalisco, Nuevo León, Oaxaca, Puebla, San Luis Potosí, Sonora, Jalisco, Morelos and Zacatecas). Currently, there is information about the hydrogeochemical processes that promote the presence of arsenic in water in the mentioned sites. However, the impact of microbiological processes over arsenic speciation and distribution has received little attention.

1.2 Arsenic, an overview

Arsenic (As) is a metalloid, thereby reacts rapidly to form oxyanions and their corresponding salts, forming organic and inorganic compounds. In nature the predominant oxidation states are arsenate (As(V) or As^{5+}) and arsenite (As(III) or

As^{3+}), these species are not free and are always forming part of oxyanions. Less frequently, in natural waters the oxidation states of arsenic could be -3 or 0 (Gorny et al., 2015). Arsenate predominates in oxidizing conditions, while arsenite predominates in reducing conditions (Cheng et al., 2009). The speciation also depends on pH, at the pH values commonly found in sedimentary environments (between 5 and 9), the following free inorganic species predominate $H_2AsO_4^-$, $HAsO_4^{2-}$, and H_3AsO_3 (Gorny et al., 2015).

The arsenic concentrations in uncontaminated soil and sediments usually range from 0.1 to 50 mg/kg, higher levels of arsenic can be due to natural contamination (*i.e.* caused by the geologic composition) or human activities (*e.g.* mining). High concentrations of aqueous arsenic is related to the arsenic release from the solid phase to the pore- and column-water (Halter et al., 2011). The occurrence of arsenic in natural water bodies is defined by complex interactions of geological, hydrological, and biological processes, which in turn are related to water chemistry, pH, redox potential (Eh), oxidation state, and to reactions of adsorption/desorption, precipitation/dissolution and ion exchange (Cheng et al., 2009).

1.3 Arsenic mineralization

Arsenic is the 20th most abundant element in the earth crust, in nature more than 300 arsenic minerals exist, from these about 60% are arsenates, 20% are sulfides and sulfosalts, 10% are oxides and the rest are arsenites, arsenides, and native arsenic metal alloys (Drahota and Filippi, 2009).

In sedimentary reducing environments, arsenic can precipitate as arsenopyrite (FeAsS), cobaltite (CoAsS), enargite (Cu_3AsS_4), orpiment ($A_{s2}S_3$) or realgar (As_4S_4), these are the most common primary arsenic minerals (Drahota and Filippi, 2009). Also, arsenic can be sequestered either by co-precipitation or adsorption into multiple minerals. Iron sulfides: pyrite (FeS_2), Pyrrhotite (Fes), greigite (Fe_3S_4), and mackinawite (FeS); carbonates: siderite ($FeCO_3$), vaterite/calcite ($CaCO_3$), dolomite ($MgCO_3$); sulfates: gypsum ($CaSO_4 \cdot 2H_2O$), jarosite ($KFe_3(SO_4)_2(OH)_6$), barite ($BaSO_4$); iron-oxides/(hydr)oxides: ferrihydrite

($Fe(OH)_3$), goethite (α-FeO(OH)), hematite (Fe_2O_3), magnetite (Fe_2O_4); aluminum (hydr)-oxides: gibbsite $Al(OH)_3$. The arsenic content reported in these minerals widely oscillates, depending on the retention capacity of the mineral and the degree of arsenic contamination of the sites. Fe- and Al-(hydr)oxides are the main host phases for As(V) adsorption in oxidizing sediments (up to 76,000 mg/kg Fe (hydr)oxides) and pyrite in reducing sediments (10-77,000 mg/kg) (Gorny et al., 2015; Smedley and Kinniburgh, 2002). Although minerals such as carbonates and sulfate usually have low arsenic content (less than 12 mg/kg), these are extensively distributed in sediments playing a key role in the control of arsenic fate (Meng et al., 2016; Smedley and Kinniburgh, 2002).

The stability of arsenic, incorporated into specific mineral phases, is subject to the mineral solubility, redox conditions, pH, and chemical mechanisms of incorporation. These mechanisms comprise adsorption, by the formation of inner-sphere complexes (coordinated covalent bonds) and outer-sphere complexes (electrostatic interactions), and coprecipitation that includes isomorphic substitution (*i.e.* replacement of one atom by another in a crystal lattice conserving the structure of the mineral). For instance, arsenic forms strictly inner-sphere complexes with Fe(III)-oxide minerals surfaces, which means it is highly stable. Alternatively, As(V) can be incorporated in lattice structures of minerals such as calcite and gypsum by isomorphic substitution, being stable while As(III) mainly form outer-sphere complexes with these minerals, these bonds are less stable and the arsenic could be easily mobilized (Alexandratos et al., 2007; Zhang et al., 2015).

1.4 Arsenic uptake and toxicity for microorganisms

Arsenic causes multiple adverse effects on human health. However, many prokaryotic organisms can uptake this element either by detoxification mechanisms or metabolism (*i.e.* reactions of energy conservation) (Oremland and Stolz, 2003).

The species of arsenate ($H_2AsO_4^-$ and $HAsO_4^{2-}$) are analogs to phosphate and can enter to microbial cells via phosphate transporters (*Pit/Pst*) (Figure 1.1); once

inside, arsenate can uncouple the oxidative phosphorylation by replacing phosphate resulting on the disruption of ATP synthesis (Cavalca et al., 2013; Slyemi and Bonnefoy, 2012). Meanwhile, arsenite (H_3AsO_3) at pH lower than 9.3 is an uncharged molecule and is structurally similar to glycerol, therefore it can enter microbial cells via glycerol transporters (*GlpF*) such as aquaglyceroporins (Figure 1.1) (Oremland and Stolz, 2003). Arsenite is extremely toxic, due to its high affinity for sulfhydryl groups such as the cysteine residues of proteins; it can affect both the structure and the activity of numerous enzymes, receptors, and transcription factors. In humans, As(III) results carcinogenic because it can oxidize reduced glutathione, which is the major cellular antioxidant (Cavalca et al., 2013; Slyemi and Bonnefoy, 2012). Prokaryotes can use different redox mechanisms to resist high arsenate and arsenite concentrations. Figure 1.1 shows the main strategies that prokaryotes have developed to transform arsenic, including arsenate-reduction (detoxification and metabolism), arsenite-oxidation (detoxification and metabolism) and arsenic methylation.

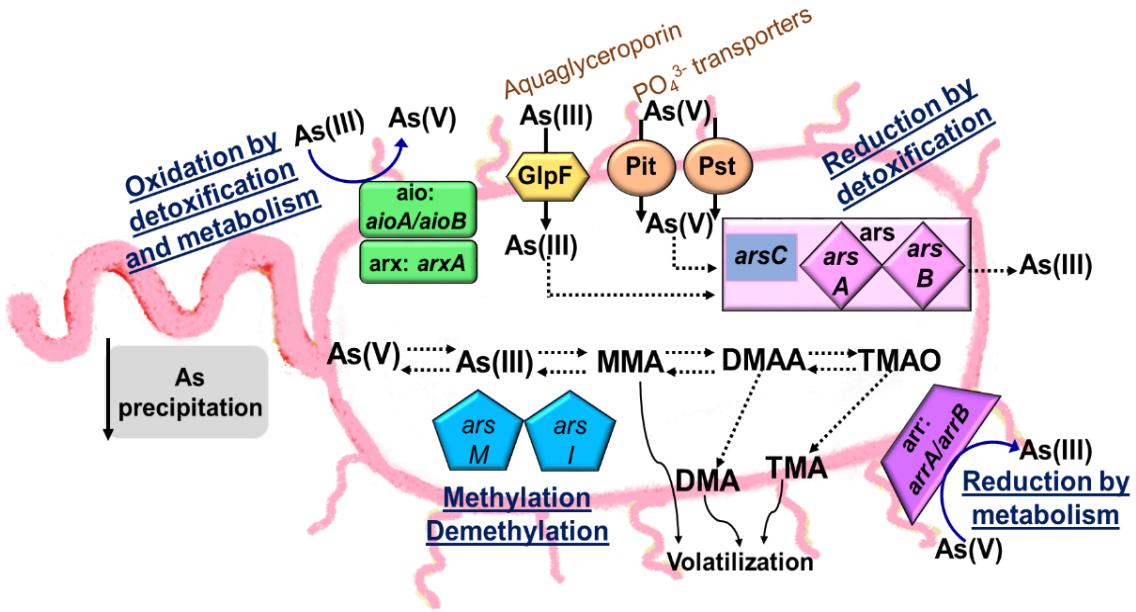


Figure 1.1. Schematic representation of the different processes used by prokaryotes to deal with arsenic. Molecular markers that allow identifying metabolic pathways are shown in italics: As(III)-oxidation by respiration with O_2 or NO_3^- (*aioA/aioB*) and detoxification (*and arxA*); dissimilatory As(V)-reduction (*arrA/arrB*) and As(V)-reduction by detoxification (*arsC* and *arsA/arsB*); and As-methylation (*arsM and arsI*).

1.4.1 Microbiology of As(V)-reduction by metabolism or detoxification

Microbial reduction of As(V) can be carried out by two mechanisms (Figure 1.1). The most common mechanism in bacteria is the arsenic resistance based on arsenic detoxification (*ars* system), in which As(V) is reduced intracellularly to As(III) by *arsC* a small protein (13-16 kDa); then, As(III) is excreted outside of the cell by an efflux pump named *arsB/ACR3*. This process requires energy investment as ATP, which is the function of *arsA* (an ATPase). As(V)-detoxification is more common in aerobic environments(Cavalca et al., 2013; Oremland and Stolz, 2003). The second mechanism is the dissimilatory As(V)-reduction (*arr* system), it is a form of anaerobic respiration where As(V) is the final electron acceptor of the respiratory chain. The *arr* system is encoded by *arrA/B* genes, the *arrA* gene (large subunit, 100 kDa) codifies for enzyme arsenate respiratory reductase and the *arrB* gene (small subunit, 30 kDa) for a protein that transfers electrons from the electron transport chain to arsenate respiratory reductase (Andres and Bertin, 2016; Escudero et al., 2013; Kruger et al., 2013). The *arrA* gene is outside the cytoplasm either attached to the cytoplasmic membrane in Gram (+) bacteria or within the periplasm in Gram (-) bacteria (Osborne et al., 2015). The representative genes of *arr* system have been characterized in few bacterial strains from the genera *Shewanella* (Malasarn et al., 2004), *Desulfitobacterium* (Kim et al., 2012), *Anaerobacillus* (Afkar et al., 2003), *Desulfosporosinus* (Pérez-Jiménez et al., 2005), *Chrysiogenes* (Krafft and Macy, 1998), *Sulfurospirillum* (Lear et al., 2007), *Wolinella* (Stolz et al., 2006), *Geobacter* (Ohtsuka et al., 2013) and *Desulfuromonas* (Osborne et al., 2015). Bacteria that carry the *arsC* gene can reduce only soluble As(V) while bacteria that carry the *arrA* gene can reduce either soluble or mineralized As(V).

1.4.2 Microbiology of As(III)-oxidation by metabolism and detoxification

The bacterial As(III)-oxidation was described first in heterotrophic arsenite-oxidizers (*aio* system) (Anderson et al., 1992) and more recently it was described

in autotrophic arsenite-oxidizers (*arx* system) (Zargar et al., 2010). In the majority of heterotrophic bacteria, the microbial As(III)-oxidation is a process of detoxification, while in autotrophic bacteria As(III) may serve as an electron donor for respiration either under oxic or anoxic conditions using O_2 or NO_3^- as electron acceptors (Cavalca et al., 2013; Costa et al., 2014). Oxic As(III)-oxidation is catalyzed by arsenite oxidase, which uses O_2 as terminal electron acceptor, and is encoded by *aioA/B genes*, where *aioA* is a large subunit and *aioB* is a small subunit (Costa et al., 2014). Strains of the genera *Rhizobium*, *Achromobacter*, *Agrobacterium,* and *Gallionella,* can grow autotrophically using As(III) as the sole electron donor (metabolism) or as a mechanism of As-resistance (detoxification) or both (Cai et al., 2009; Sarkar et al., 2013).

In anaerobic conditions, autotrophic microorganisms use the *arxA/B* system to catalyze the As(III)-oxidation which is either coupled with nitrate respiration or integrated into the electron transport chain of anoxygenic photosynthesis (Andres and Bertin, 2016; Kruger et al., 2013). The *arxA* is a gene that codifies for a type of oxidase that uses arsenite as the sole electron donor. This gene has been characterized only in three autotrophic microorganisms, in *Alkalilimnicola ehrlichii MLHE-1* coupled with nitrate-reduction, and in *Ectothiorhodospira sp. BSL-9* and *Halorhodospira SL1* coupled with anoxygenic photosynthesis (Zargar et al., 2012, 2010). However, several reports have demonstrated the use of As(III) as electron donor in anoxic environments (Zargar et al., 2012; Zhang et al., 2015), thus an opportunity for future investigations into the diversity of microbes that may use *arxA*-type arsenite oxidases.

1.5 Arsenic-rich sediments

Sediments, as part of freshwater bodies, are reservoirs of organic matter housing a substantial fraction of the Earth's living biomass (Castelle et al., 2013). Depending on the location and origin of each water body (*i.e.* aquifers, rivers, lakes), sediments have different geochemical composition, vertical redox stratification, availability of organic matter, and highly diverse indigenous microbiota. Therefore,

in freshwater sediments, numerous biogeochemical interactions can occur, controlling the speciation and transport of metals and metalloids, including contaminants such as arsenic (Castelle et al., 2013). Within this framework, geochemical composition, redox potential (Eh), and deposition of organic matter govern the microbial processes in sediments; these parameters allow different redox reactions to take place at the same time (Figure 1.2) (Gorny et al., 2015).

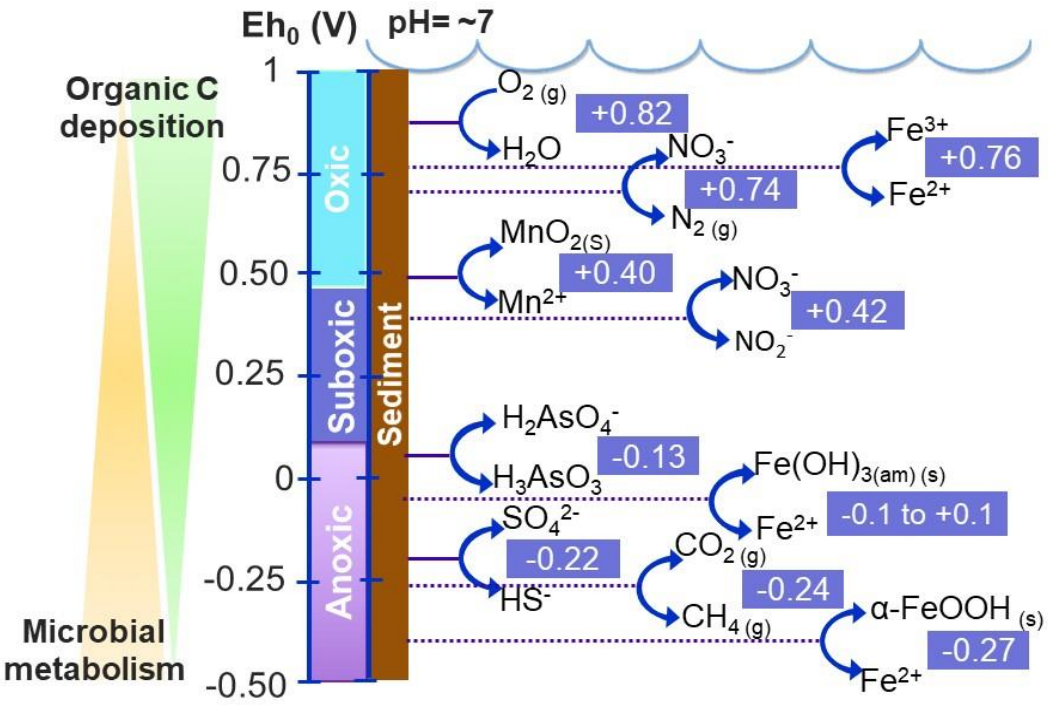


Figure 1.2. Environmental relevant redox couples in sediments.

The coupling of biogeochemical cycles comprises the co-occurrence of microbial processes, which modify the chemical speciation and saturation conditions of different compounds, promoting the fast precipitation and dissolution of distinct mineral phases (Lièvremont et al., 2009).

Particularly, the distribution of arsenic in sedimentary environments is closely associated with sulfur (S) and iron (Fe) biogeochemical cycles, because S and Fe minerals are widely distributed in sediments and arsenic frequently is part of these minerals. For instance, in oxic and suboxic environments, iron oxides are known as the main arsenic sorbents, whereas arsenic is mainly associated with

iron sulfides (pyrite) in anoxic environments (Smedley and Kinniburgh, 2002). Microbial activity catalyzes multiple redox reactions that strongly impact the chemical speciation of As, Fe, S and therefore play a key role in arsenic cycling in water systems.

1.6 Anaerobic arsenotrophy

Arsenotrophy refers to microbial arsenic-dependent growth (*i.e*. energy-conserving reactions), it can be autotrophic or heterotrophic (Oremland et al., 2017). Energy-conserving reactions involving arsenic metabolism can be oxidative, where arsenite is the electron donor with oxygen or NO_3^- as the final electron acceptors, or reductive, where arsenate is the terminal electron acceptor and organic matter is the electron donor. These redox reactions are catalyzed by three different types of operons: *arx/aio* (As(III)-oxidation) and *arr* (As(V)-reduction). In anaerobic conditions, dissimilatory arsenate-reduction is an heterotrophic process while As(III)-oxidation (with NO_3^-) is an autotrophic process, these reactions can occur in the same ecosystem, which confirms the importance of both processes in the arsenic cycle (Hoeft et al., 2010).

1.6.1 Anaerobic arsenotrophy linked with other reducing bioprocesses

In anaerobic systems when organic matter is available, microorganisms can interact with the arsenic cycle through a variety of heterotrophic bioprocesses including arsenate-reduction, sulfate-reduction, iron-reduction. Dissimilative anaerobic microorganisms are capable of obtaining energy by using inorganic compounds such as As(V), sulfate, and Fe(III) as terminal electron acceptors and organic carbon as electron donor, the occurrence of this bacteria is ubiquitous in sedimentary systems (Huang, 2014). Table 1.1 shows the stoichiometry of the As(V)-, Fe(III)-, and sulfate-reduction reactions, with lactate as the organic compound.

Table 1.1. Redox reactions of incomplete lactate oxidation to acetate using As(V), sulfate, and Fe(III) (shown as ferrihydrite) as electron acceptors.

Metabolism	Reaction	$\Delta G^{\circ\prime}_{pH=7}$ (kJ/mol)	$Eh^{\circ\prime}$	Reference	
As(V)-reduction	$C_3H_5O_3^- + 2\ H^+ + HAsO_4^{2-} + H_2AsO_4^- \rightarrow 2\ H_3AsO_3 + HCO_3^- + C_2H_3O_2^-$	-172	+135	(Newman *et al.*, 1997)	**(1.1)**
Fe(III)-reduction	$2\ C_3H_5O_3^- + SO_4^{2-} \rightarrow 2\ C_2H_3O_2^- + 2\ HCO_3^- + 0.5\ HS^- + 0.5\ H_2S + 0.5\ H^+$	-81.2 to -158.3	-100 to +100	(Straub et al., 2001)	**(1.2)**
Sulfate-reduction	$0.25\ C_3H_5O_3^- + Fe(OH)_{3(s)} + 1.75\ H^+ \rightarrow Fe^{2+} + 0.25\ C_2H_3O_2^- + 0.25\ HCO_3^- + 2.5\ H_2O$	-34.2	-220	(Newman *et al.*, 1997)	**(1.3)**

The deposition of organic matter in sedimentary environments is highly valued, and organic carbon is quickly depleted by the native microbiota. Consequently, oligotrophic conditions predominate below the subsurface of sedimentary systems where autotrophic microorganisms play a pivotal role in the cycling of reduced species such as H_2, ferrous iron (Fe(II)), reduced sulfur compounds (S(-II), S(0), thiosulfate), iron sulfides, and As(III). These species may act as electron donors during the reduction of an energetically favorable electron acceptor such as nitrate (NO_3^-). The presence of nitrate can trigger the oxidation of As(III), sulfide, and Fe(II) coupled to anoxic nitrate-reduction even, when reducing species (electron donors) are part of minerals as siderite ($FeCO_3$) and pyrrhotite (FeS) (Di Capua et al., 2015). The coexistence of arsenic and nitrate in groundwater has been associated to the excessive use of nitrogen-based compounds (*e.g.* fertilizers) (Shakya and Ghosh, 2018). Simultaneous removal of nitrate and arsenic by microbial processes have been studied, taking into account that some bacterial genera oxidize Fe(II) to Fe(III) or As(III) to As(V), using nitrate as the electron acceptor under anoxic condition (Di Capua et al., 2019).

Many research efforts have focused on understanding the physiological and genetic mechanisms that microorganisms use to metabolize and tolerate the

arsenic, as well as to the isolation and culturing of these microorganisms (Cavalca et al., 2013; Kruger et al., 2013). Recently, several studies showed how the biogeochemical cycle of arsenic is coupled to other biogeochemical cycles of key elements (*i.e.* C, Fe, N, S), these findings provided the more favorable redox reactions in different environments, and their impact on the chemical speciation and fate of arsenic (Moon et al., 2017; Zhang et al., 2018).

Overall, heterotrophic and autotrophic microorganisms interact with the arsenic cycle through multiple bioprocesses including As(V)-, Fe(III), nitrate-, and sulfate-reduction as well as As(III)-, Fe(II)- and sulfide-oxidation. Different mineral phases, immobilizing arsenic, are involved in the arsenic cycle and are susceptible to oxidation or reduction by microbial metabolism as shown in Figure 1.3.

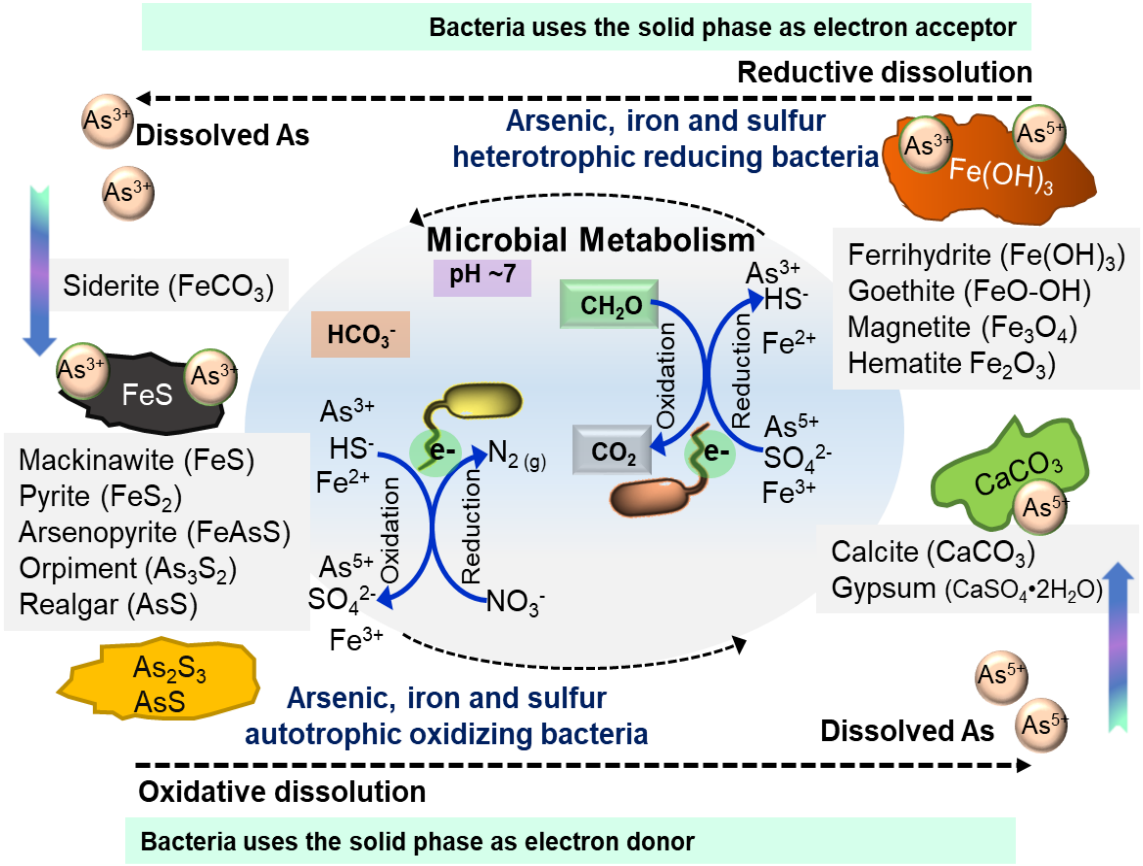


Figure 1.3. Arsenic recycling in terrestrial sediments, considering the interconnection among the biogeochemical cycles of arsenic (As), sulfur (S), iron (Fe), and nitrogen (N) through of common As-bearing minerals.

The coupling of biogeochemical cycles involves numerous redox reactions and multiple microbial genera with different metabolic capabilities, the bacterial genera associated with arsenic-polluted sediments are metabolically very versatile and can use a wide range of electron acceptors and electron donors (Figure 1.4) (Slyemi and Bonnefoy, 2012). The co-occurrence of bioprocesses can be beneficial or detrimental for polluted-sites depending on their biogeochemical features.

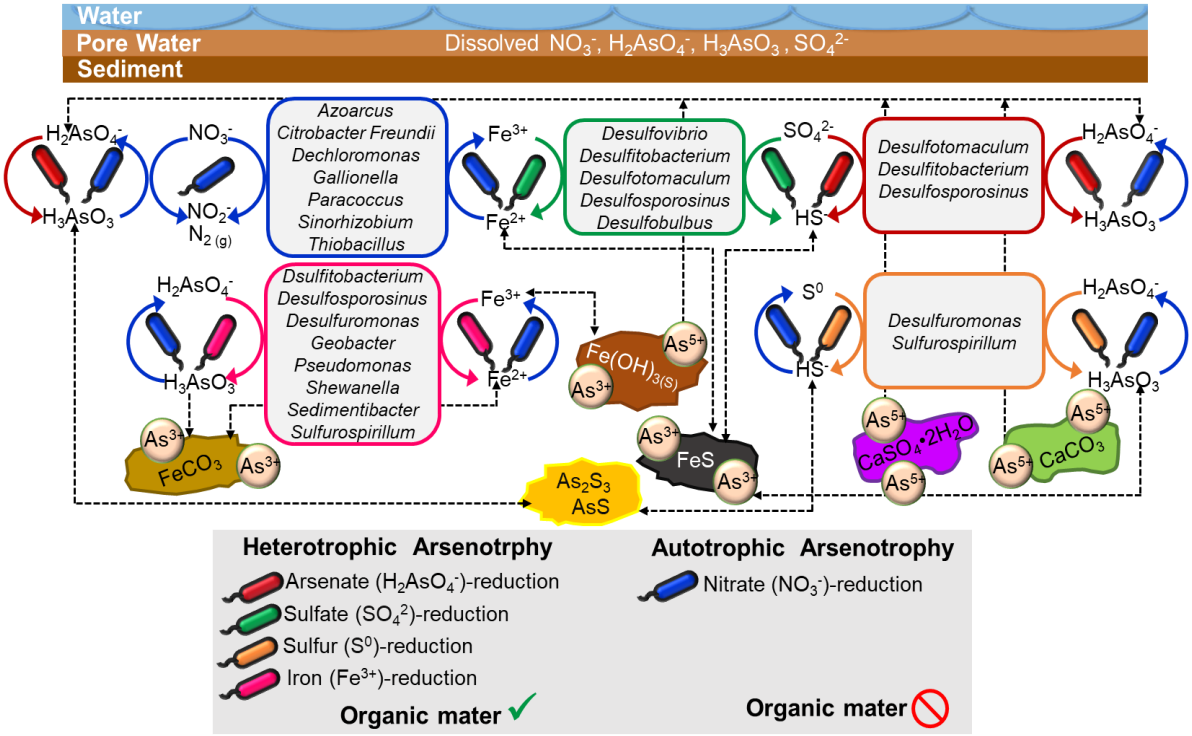


Figure 1.4. Anaerobic arsenotrophy linked with biotransformations of chemical species of sulfur, iron, and nitrogen and the main bacterial genera involved in redox reactions. The color of the arrows denotes the bacterial genera (textbox) that carry out the bioprocess. Calcium carbonates ($CaCO_3$), gypsum ($CaSO_4 \cdot 2H_2O$), ferrihydrite ($Fe(OH)_3$), iron sulfides (FeS), orpiment (As_2S_3) and realgar (AsS), and siderite ($FeCO_3$).

1.7 Arsenic bioremediation in freshwater sediments

Arsenic has ecotoxicological importance, and it is well knowing that the best strategy to reduce the risk and exposure is immobilizing the bioavailable arsenic (*i.e.* dissolved arsenic) as part of minerals. Arsenic sorption onto metal oxide

minerals, especially on iron, manganese, and aluminum (hydr)-oxides, is an important process controlling the dissolved concentration of As in oxidizing environments. As(V) is strongly associated with iron and aluminum (hydr)oxides, whereas As(III) is more mobile than As(V) because, at the typical pH of water bodies, As(III) is an uncharged species (Gorny et al., 2015); hence, As(III)-oxidation is considered an proper pretreatment process. Considering that iron oxides are arsenic sinks in polluted sites, the reductive dissolution of iron (hydr)oxides by dissimilatory iron-reducing bacteria is a relevant mechanism for arsenic release (Mirza et al., 2014). Besides, direct reduction of As(V) adsorbed onto soil minerals by dissimilatory As(V)-reducing bacteria (*arrA*-carriers) is also crucial in arsenic mobilization (Osborne et al., 2015). It is worth to note that the availability of dissolved organic carbon in sediments promotes arsenic destabilization since organic carbon supports reducing microbial processes such as arsenate-, sulfate-, and iron-reduction and consequently, it promotes highly reducing conditions resulting in the instability of amorphous (hydr)oxides (Davranche et al., 2013). As mentioned before As(V) reduction to As(III) is an undesirable process because it increases the toxicity and mobility of arsenic. Nevertheless, to counteract the negative effect of Aa(V)-reduction, this process can be coupled either with sulfate- or iron-reduction under strict reducing conditions (Eh: lower than -100 mV). The presence of reduced species, Fe(II) and S(-II), in combination with As(III), trigger arsenic bio-mineralization removing it from the aqueous phase as part of biogenic arsenic-bearing minerals (Huang, 2014).

For the proper development of bioremediation strategies in arsenic polluted-sites, it is important to consider that the first step is a detailed physicochemical characterization of the site. Secondly, it should be taken into account that the effect of microbial reactions in water bodies depend on hydrology (*i.e.* hydraulic flux), geological composition (major mineral phases), water chemistry (dissolved compounds), available chemical species (oxidation state). In turn, all these are related to the pH, redox potential (Eh), reactions of adsorption/desorption, precipitation/dissolution and ion exchange (Gorny et al., 2015; Huang, 2014). The main bioremediation strategies reported are detailed below.

- The coupling As(V)- and sulfate-reduction can lead to arsenic mineralization as biogenic arsenic sulfides, such as orpiment (As_2S_3) and realgar (AsS). This strategy is recommended for iron-poor sedimentary environments (Battaglia-Brunet et al., 2012; Newman et al., 1997; Rodriguez-Freire et al., 2014).

  $2\ H_3AsO_3 + 3\ HS^- + 3\ H^+ \rightarrow As_2S_{3(s)} + 6\ H_2O$ **(1.4)**

  $H_3AsO_3 + HS^- + 2\ H^+ + e^- \rightarrow AsS_{(s)} + 3\ H_2O$ **(1.5)**

- As(V)-, Fe(III)-, and sulfate-reduction as simultaneous bioprocesses promote the formation of iron sulfides like mackinawite (FeS) and pyrrhotite (FeS). These also promote the formation of secondary minerals such as magnetite (Fe_3O_4) and siderite ($FeCO_3$). These minerals are capable of retaining arsenic by co-precipitation or adsorption (Saalfield and Bostick, 2009).

  $Fe^{2+} + HS^- \rightarrow FeS + H^+$ **(1.6)**

  $Fe^{2+} + 2Fe(OH)_3 \rightarrow Fe_3O_4 + 2H^+ + 2\ H_2O$ **(1.7)**

  $Fe^{2+} + CO_3^{2-} \rightarrow FeCO_3(s)$ **(1.8)**

- Arsenic mineralization was achieved from the precipitation of biogenic calcite by the bacteria *Sporosarcina ginsengisoli* and *Bacillus licheniformis.* Arsenic can be adsorbed in the calcite surface or/and incorporated in the crystal structure. The precipitation of calcium carbonates is stimulated by the enzyme urease and occurs as a consequence of bacterial metabolic activity (Achal et al., 2012). This treatment is feasible in calcareous sediments.

- Microbial stimulation with NO_3^- to support the anoxic oxidation of Fe(II), Mn(II) and As(III). The bacterial Fe(II)- and Mn(II)-oxidation resulted in the precipitation of Fe- and Mn-(hydr)oxides which are effective As(V) adsorbents. Bacteria belonging to the genera *Gallionella* and *Leptothrix* are efficient oxidizing Fe(II) to Fe(III) or/and Mn(II) to Mn(IV) (Lièvremont et al., 2009; Sun et al., 2009). The oxidation of As(III) to As(V), in the range of pH 5 to 9, can only occur chemically (manganese (hydr)-oxides) or biologically (Gorny et al., 2015).

- Some microorganisms have been genetically modified to express arsenic-binding proteins that allow them yielding high rates in As-biotransformation or high capacity of As-bioaccumulation. For example, engineered *Escherichia coli* accumulated 50-60 times more arsenic than wild strain when modified to overexpress *arsR* (Kostal et al., 2004).

1.8 Background and gaps in research related to arsenic biocycle in sediments

Anaerobic bioprocesses strongly contribute to arsenic mobilization/immobilization in contaminated terrestrial sediments, and several investigations have converged on arsenic biotransformations integrating the biogeochemical cycles of key elements (*e.g*. S, Fe, N, and C). These approaches were mainly focused in the following topics: 1) reductive dissolution of arsenic-bearing Mn- and Fe-(hydr)oxides by dissimilatory Fe(III)- Mn(III, IV)- and As(V)-reduction (Das et al., 2016; Neidhardt et al., 2014); 2) arsenic release from sediments as consequence of biostimulation with different electron donors or electron acceptors (Mirza et al., 2014; Pi et al., 2017); 3) oxidative dissolution of iron sulfides by microbial oxidation of S(0), S(-II), As(III) and Fe(II) (Di Capua et al., 2019); 4) arsenic bio-mineralization by coupling dissimilatory Fe(III)-, sulfate- and As(V)-reduction as a bio-remediation strategy (Kirk et al., 2010; Newman et al., 1997).

The development of arsenic-bearing biominerals has been widely studied because they are broadly distributed in the environment and have a fundamental role in arsenic stabilization. Although it has been suggested that the main reservoir of metal sulfide deposits at low temperature (< 100 °C) are of biogenic origin, little is known about the interaction of bacteria and biogenic arsenic sulfide minerals (Picard et al., 2016). Bacteria can change the saturation index of minerals promoting their precipitation, but some questions remain unanswered: Could As-biominerals interfere with the performance and structure of microbial communities? Are there microbial genera closely associated with As-biominerals? What are the

mineralogy and structural morphology of As-biominerals precipitated by indigenous bacterial consortia?

Considering that the reductive dissolution of iron oxides has been identified as a primary cause of hazardous concentrations of arsenic in severely contaminated aquifers (Zhang et al., 2017), the mechanisms of arsenic mobilization in iron-poor environments have received little attention (Meng et al., 2016; Smedley and Kinniburgh, 2002). For instance, in the semi-arid regions in the western USA and northern Mexico, carbonate-rich aquifers, with low iron content and contaminated with arsenic have been identified. These aquifers present large seasonal fluctuations in water recharge, resulting in the dissolution and precipitation of carbonates, which affect the arsenic stabilization (Alarcón-Herrera et al., 2013; Meng et al., 2016; Romero et al., 2004). Furthermore, in these systems, nutrients are continually supplied and oxygen is rapidly depleted, leading to anoxic conditions that promote reducing bioprocesses. Few studies have been conducted focused on arsenic biotransformations in iron-poor environments (Meng et al., 2016).

Arsenic remediation, through coprecipitation with biogenic Fe-(hydr)oxides/oxides or sulfide minerals, represents one of the most attractive approaches for in-situ use because it only requires the biostimulation with the proper electron acceptor or electron donor. Moreover, this approach allows to consider the geochemical characteristics of the polluted-sites and exploit the metabolic capabilities of the native microbiota (Omoregie et al., 2013). Within this framework, it is important to evaluate the stability of the biominerals in carbon-rich/carbon-depleted reducing environments under microbial-reduction and microbial-oxidation, there is a lack of information about microbial-oxidation of biogenic arsenic-sulfides in anoxic sediments. The aforementioned gaps of information regarding arsenic biocycle have inspired the main objectives of this thesis.

1.9 Scope and structure of the thesis

The fate of arsenic in contaminated terrestrial sediments is governed by the interaction of biogeochemical cycles of the most representative elements and microbial processes. Each polluted-site has unique characteristics and requires a suitable design of bioremediation strategies that consider hydrology, mineralogy, redox conditions, organic carbon content, dissolved electron acceptors, and native microbial communities. A wide range of studies has been performed to explain the chemical and biological mechanisms that interfere with arsenic mobilization/immobilization in sediments. Nevertheless, there are some unresolved gaps in knowledge. Accordingly, the global aim of this project was to explore the anaerobic arsenotrophy linked to iron and sulfur biotransformations, with a focus on arsenic remediation in polluted aquifers.

The first approach of this dissertation (Chapter 2) was the characterization of two sediments collected from arsenic-polluted hydraulic systems. The sediments served as inoculum to obtain sulfate- and arsenate-reducing enrichments and promote the biomineralization of arsenic-sulfides. Afterward, bacterial consortia devoid of sediment were obtained from successive transfers of the enrichments. The microbiota associated with the sediments, enrichments, biogenic minerals, and consortia were identified. We focused mainly on the transition of the microbial communities through these microenvironments and the presence of arsenic-reducing bacteria, which was corroborated by the amplification of the functional gene for dissimilative As(V)-reduction (*arrA*).

In Chapter 3, the research was oriented toward the characterization of biogenic minerals formed via As(V)- and sulfate-reduction, without the interference of the sediment composition. For this goal, the biogenic minerals were recuperated from bacterial consortia and two main aspects were studied: physicochemical characteristics of the biomineral (structure, mineralogy, and elemental composition) and interactions biomineral-bacteria.

In Chapter 4, the experimental strategy aimed to find out the fate of arsenic in iron-poor systems (experiments with calcite, gypsum) and iron-rich systems (experiment with ferrihydrite), the latter scenario has been widely investigated. Therefore, we propose mechanisms of arsenic mobilization/immobilization in calcareous and gypsiferous sediments under reducing bioprocesses, as there was no information about it. Three sets of batch experiments were conducted, each one using calcite, gypsum, or ferrihydrite coprecipitated with As(V), the minerals also served as the source of electron acceptors for As(V)-, Fe(III)- and sulfate-reduction.

Finally, the main results obtained in this research are discussed in Chapter 5, the environmental relevance of the project is highlighted, and some recommendations for future research in the field are provided.

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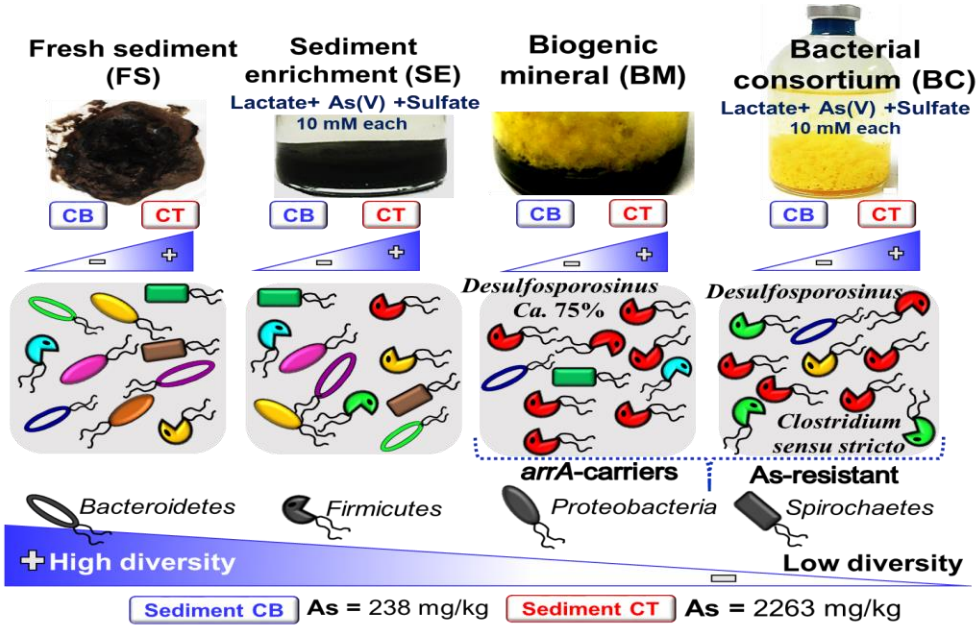
CHAPTER 2

From arsenic rich sediments to sulfate/As(V)-reducing bacterial consortia: Transition of the microbial communities

Highlights

- Arsenic in sediments CB and CT are highly bioavailable since most of the As is part of the soluble, carbonate, and ionic exchangeable fractions.
- The microbial communities of biogenic minerals recovered from sediment enrichments are dominated by the genus *Desulfosporosinus* (up to 79% of abundance).
- Bacterial consortia include *arrA* sequences closely related to recognized arsenate-respirers such is the case *Desulfosporosinus sp Y5* and *Bacterium MPA-C3.*

Graphical Abstract



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ABSTRACT

Investigate different microbial niches as well as the environmental parameters that disturb them, represent an opportunity for a better understanding of the response mechanisms to toxic elements and the strategies of the microbial communities to grow and survive. Which reinforces the available tools for the appropriate design of bioremediation schemes. In this chapter, two different sediments, collected from an arsenic-polluted area in San Luis Potosí, México, were investigated for their arsenic partitioning and culturable microbiota. Dissimilatory arsenate- and sulfate-reducing microbiota were propagated by successive transfers to obtain bacterial consortia (sediment-free). Microbial communities associated with fresh sediments, sediment enrichments, biogenic minerals, and bacterial consortia were explored based on 16S rRNA and *arrA* genes to understand the structure and shifts in the communities through these microenvironments. According to 16S rRNA analysis, microbial diversity decreased as follows: sediments > sediment enrichments > biogenic minerals > bacterial consortia. Removal of the sediment matrix caused substantial shifts in the bacterial communities and promoted the dominance of microorganisms associated to the order Clostridiales. Although *Desulforosporosinus* abundance was very low in the fresh sediments (lower than 0.4%) it increased during the enrichment, being the most representative genus in the biogenic minerals (~75%). This genus was also the most abundant in the microbial consortia (~55%), followed by *Clostridium sensu stricto* 7 (~36%). Results suggested that *Desulforosporosinus and Clostridium sensu stricto* have a pivotal role in arsenic biocycle. The sequencing results of the *arrA* gene revealed the existence of 12 distinct OTUs in all the samples and confirmed the presence of phylotypes closely related to *Desulforosporosinus sp.* Y5 (100% similarity) and *Bacterium MPA-C3* (97% similarity) in the bacterial consortia. Arsenate- and sulfate-reducing bacteria able to resist high arsenic concentrations (~750 mg/L) were linked with the biogenic minerals, which means that arsenite/sulfide biominerals are a good media to disseminate these arsenic-mineralizing bacteria that could be used to implement bioremediation technologies.

Keywords: Sediments, Biogenic minerals, Consortia, Microbiota, *Desulforosporosinus, arsenic*

CHAPTER 2

2.1 INTRODUCTION

Sediments and soils contain complex native microbial communities responsible for key biogeochemical processes that involve changes in the chemical speciation an transport of metals and metalloids, understanding their behavior is essential to monitor the fate of toxic elements in the biosphere (Gillan et al., 2015; Ying et al., 2015; Zhu et al., 2017).

Prokaryotes can respond rapidly to stress conditions, by developing physiological and genetic adaptations. Nevertheless, in some environments, the occurrence of high concentrations of toxic elements, such as arsenic, may act as a selective force allowing the survival and proliferation of microorganisms at extreme conditions. These forces, in turn, can lead to dramatic shifts in the composition, structure, and function of the native microbial communities (Sarkar et al., 2014).

Several members of Bacteria and Archaea have developed resistance strategies to survive in anoxic environments contaminated with arsenic. For instance, there are microorganisms able to use arsenic as energy source and respire this metalloid. Such microorganisms are equipped with a respiratory system known as *arr* system that enables them to use arsenic as an electron acceptor and carry out dissimilative arsenate reduction. Other microorganisms, thriving at high arsenic concentrations, have the machinery to expulse the toxic element from the cell, which is known as *ars* system (arsenic detoxification). Through dissimilative arsenate reduction, some anaerobic microorganisms can obtain energy by coupling the reduction of arsenate (As(V)) to arsenite (As(III)) with the oxidation of some organic compounds (Oremland and Stolz, 2005). The reduction of arsenate is catalyzed by the enzyme arsenate respiratory reductase, encoded by the *arrA* gene (Cai et al., 2009). In sedimentary environments, bacteria that carry the arrA system have received special attention, because they can mediate the reduction of arsenate, that could be sorbed or stabilized in solid phases, and consequently mobilize it as arsenite (Ohtsuka et al., 2013).

Contaminated sites represent ecological niches where historical pollution has resulted in specialized microbial communities that have developed metabolic pathways and adaptive responses to extreme conditions (Atashgahi et al., 2018).

Sediments with long-term arsenic contamination present very diverse arsenic resistant and metabolizing microbiota, such fact is possible because the prokaryotes can acquire the involved genes through horizontal gene transfer (*i.e.* movement of genetic material from one microorganism to another) (Suhadolnik et al., 2017). Recent studies based on the analyses of the functional gene *arrA* have found phylotypes not previously known to be involved in arsenic metabolism manly grouped in *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla (Costa et al., 2014; Lu et al., 2017; Mirza et al., 2014).

According to Carlson et al., (2017) a central goal of environmental microbiology is the understanding of mechanisms that microorganisms use to survive trough geochemical gradients. Within this framework, the study of different microenvironments and the selective pressures that shape them will provide insights into the response mechanisms to toxic elements and strategies that microbial communities use to grow and survive. This information will complement the available tools for the appropriate design of strategies for remediation of contaminated sites either in-situ and ex-situ (Carlson et al., 2017; Sprocati et al., 2006).

Earlier investigations have focused on the microbial communities and genes involved in arsenic speciation in log-term arsenic-polluted sediments; overall, the main findings were the description of highly diverse and unique communities in the polluted-sites, the identification of novel arsenic-transforming genera and the correlation of arsenate-transforming communities with environmental factors (*e.g.* ion concentration, pH, redox potential, temperature) (Costa et al., 2014; Lu et al., 2017; Suhadolnik et al., 2017). However, there are no studies that systematically evaluate the transition and restructure of the microbial communities from sediments to specialized consortia devoid of the original matrix (*i.e.* sediment). Consortia recovered from polluted-sites consist of native microorganisms harboring diversified genes to tolerate and metabolize toxic elements. These mixed cultures also offer the advantage of preserving a diverse metabolic network (*i.e.* fermentation, oxidation of organic carbon using multiple electron acceptors, production of many metabolites), which suits them with greater resilience

compared to pure cultures. Such features make the consortia good candidates for the treatment of highly contaminated effluents and to find out which extreme microorganisms have a key role in biocycles of toxic elements (Sprocati et al., 2006).

The aims of this chapter were: 1) To investigate the stability and bioavailability of arsenic in two sediments with different degree of contamination; 2) To explore the structure and shifts of the microbial communities associated with two arsenic-polluted sediments, sediment enrichments, biogenic minerals and bacterial consortia obtained by successive transfers; 3) To corroborate the presence of arsenic-respiring bacteria, using the *arrA* gene as a proxy, and determine the changes in the function of the community through the distinct microenvironments (sediments, sediment enrichments, biogenic minerals, and bacterial consortia).

2.2 MATERIALS AND METHODS

2.2.1 Sediment collection and procedure for sequential chemical extraction

Surface sediments (5–10 cm depth) were collected from two sites, sediment CB was obtained from an artesian hydraulic complex identified as Cerrito Blanco (100°36´41” W and 23°40´23” N) and sediment CT was obtained from a water spring in the site identified as Club de Tiro (100°38´22” W and 23°38´22” N). The sampling sites are near to the mining district of Santa Maria de La Paz (Matehuala, San Luis Potosi, Mexico), which presents a high degree of contamination by arsenic. The characterization of the sediments, pore- and column-waters was previously reported (Rios-Valenciana et al., 2017). The partitioning of arsenic, sulfate, and iron on the distinct mineral fractions of the sediments were determined by a sequential chemical extraction (SCE) (Keon et al., 2001; Li and Thornton, 2001). Briefly, dry sediment (1 g) was subjected to the sequential extraction procedure to target the following fractions: 1) soluble fraction (deionized water); 2)

ionically bound or bound to surface exchangeable sites (0.5 M $MgCl_2$); 3) bound to carbonate minerals (1 M sodium acetate, pH 5); 4) anionically bound or strongly adsorbed (1 mM NaH_2PO_4); 5) bound to amorphous iron and manganese oxides (0.04 M NH_2OH•HCl at 25% v/v with CH_3COOH); 6) bound to organic matter and sulfides (30% H_2O_2 adjusted at pH 2 with 37% HNO_3 and then 3.2 M CH_3COONH_4 prepared with 20% HNO_3); and 7) residual fraction (HNO_3:HCl, 3:2 v/v, microwave digester, pressure ramp 0-300 psi and temperature set point to 150 ^oC). Continuous agitation was maintained during all extractions. Finally, the elemental composition of each extract was analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian 730-ES, Palo Alto, CA, USA).

2.2.2 Enrichment and cultivation of the As(V)/sulfate-reducing microbiota

We aimed to study the microbial dynamics in four microenvironments from sediment CB or CT. The fresh sediments (FS), the sediment enrichment (SE) obtained under As(V)- and sulfate-reduction, the biogenic minerals formed in this enrichment (BM) and the bacterial consortia (BC) sediment-free. Figure 2.1 illustrates the procedure for the preparation of sediment enrichments, consortia development, and sample collection for the analysis of the microbial communities.

Enriched cultures from sediments CB and CT were prepared in microcosms (125 mL serum bottles) that contained 100 mL of basal medium (1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NH_4Cl, 0.08 mM KH_2PO_4, and 0.1 mL/L of Wolfe's mineral solution) (Burton et al., 2013), amended with lactate, As(V), and sulfate (10 mM each), and 10% (wt./vol.) of sediment. These enrichments still contained sediment, and in the following will be named as sediment enrichments. The pH was adjusted to 7 with $NaHCO_3$ (22 mM), and the headspace of bottles was purged with N_2/CO_2 (80:20, v/v), enrichments were incubated for 30 days. Afterward, the biogenic mineral that was formed above the sediment (Figure 2.1) was separated and used for DNA extraction.

Arsenate- and sulfate-reducing consortia (vigorous shaking) were transferred to 90 mL of fresh medium, this process was repeated at least 6 times to

ensure the complete removal of the sediment. These consortia will be named as bacterial consortia in the following.

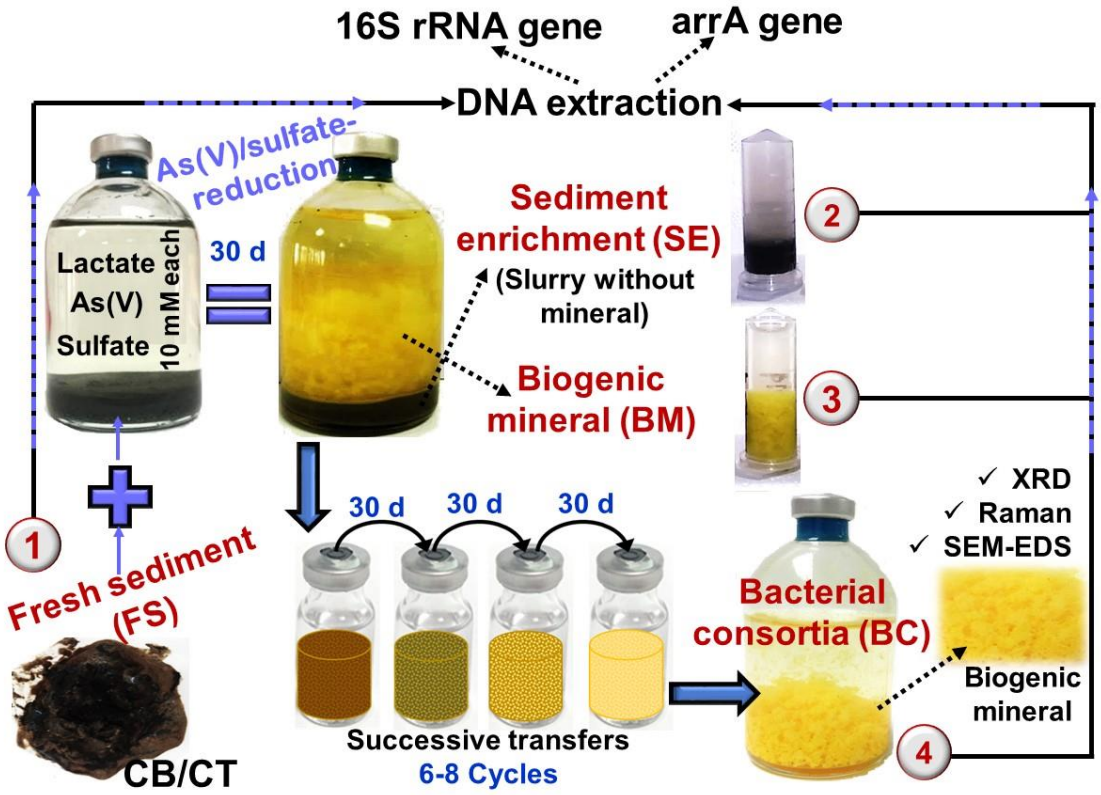

Figure 2.1. Schematic illustration of the experimental procedure to obtain the sediment enrichments, and propagate the bacterial consortia. The different microenvironments (1 to 4) subjected to microbial community analysis are also shown.

2.2.3 Analytic procedures

Arsenic species (V and III) were separated by anion-exchange chromatography (Ficklin, 1983). Total arsenic was analyzed by atomic absorption spectroscopy (Varian Spectra Mod. AA240FS, Santa Clara, CA, USA). Sulfate, lactate and acetate were determined by capillary electrophoresis (Soga, 1999). Dissolved sulfide was determined using the method of Cord-Ruwisch (Cord-Ruwisch, 1985).

The number of indigenous arsenate-reducing bacteria in the consortia was estimated by the most probable number (MPN) technique according to Kuai et al., (2001). Briefly, serial dilutions (10^{-1}–10^{-10}) were inoculated, in triplicate, in serum

vials containing 9 mL of medium amended with 5 mM lactate 5 mM of acetate and 10 mM arsenate. Vials were incubated in the dark for 30 days at 30◦C without agitation. To determine if arsenate reduction took place, we added 100 µL HCl (1 M) and 1.5 mL of sodium sulfide (15 mM) to each vial, the immediate formation (about 30 s) of a yellow precipitate was considered as positive result of arsenate-reduction. The most probable number was estimated from the indexes reported in the appropriate tables of standard methods (Eaton et al., 1998).

2.2.4 DNA extraction and 16S rRNA gene massive sequencing

From each site four samples were processed for DNA extraction, corresponding to the fresh sediment, sediment enrichments, biogenic mineral, and the bacterial consortia (Figure 2.1). All samples were obtained after 30 days of incubation, except in the case of the fresh sediments, the DNA was isolated using the kits ZR Soil Microbe MiniPrep (Zymo Research, Irvine, CA, USA) and DNeasy PowerSoil (Quiagen; Hilden, Nordrhein-Westfalen, Germany) according to specified instructions. The hypervariable regions V3 and V4 of the 16S rRNA gene were amplified with the primers f515 (5´GTGCCAGCMGCCGCGGTAA3´) and r907 (5´CCGTCAATTCMTTTGAGTTT3´), sequencing was performed through the pair-end method (Illumina MiSeq) by RTL Genomics Molecular Sequencing Laboratory (Lubbock, TX, USA).

2.2.5 Bioinformatics treatment

The sequences were analyzed using QIIME2™ software (http://qiime2.org, 2018.8 release). The raw sequencing reads were imported to QIIME 2 artifacts then, were denoised, dereplicated, chimera-filtered, and merged using DADA2 (Divisive Amplicon Denoising Algorithm) (Callahan et al., 2016). A total of 72029 high-quality reads of 370 bp were obtained from the 8 samples, on average 6000 reads per sample, the sequences were gathered in a feature table, 760 features at 99% of similarity were generated. The taxonomic analysis was based on consensus-BLAST classifier trained on 16S rRNA gene and OTUs clustered at 99%

similarities within the Silva 132 database release. Diversity analyses were made through the q2-diversity plugin (QIIME 2's) which computes some alpha (Shannon's diversity index, observed OTUs) and beta (Bray-Curtis distance) diversity metrics, and generates principle coordinates analysis (PCoA) plots using Emperor (interactive visualizations) for each of the beta diversity metrics(Vázquez-Baeza et al., 2013).

The 16S rRNA gene sequences were deposited in NCBI under bioProject ID: PRJNA610123 accession number.

2.2.6 Cloning of the functional gene *arrA* and phylogenetic approach

DNA samples were also used to amplify the functional *arrA* gene by a nested PCR approach (Song et al., 2009). The first PCR was performed with the primers AS1F (5´CGAAGTTCGTCCCGATHACNTGG3´) and AS1R (5´GGGGTGCGGTCYTTNARYTC3´) and the nested PCR was performed with the primers AS2F (5´ GTCCCNATBASNTGGGANRARGCNMT3´) and AS1R (Ohtsuka et al., 2013). The cleaned amplified PCR products (around 630 bp) were used for direct cloning with the kit pGEM®-T Vector. The ligated plasmids were transformed in high transforming efficiency *E. coli* TOP10^{TM} (Invitrogen), following the manufacturer's instructions. Positive clones were amplified by colony PCR, randomly a minimum of 20 *arrA* gene clones were selected per sample for sequencing by the Sanger method (3730x/DNA analyzer, Thermo Fisher Scientific, Waltham, MA, USA) by an external laboratory (University of Arizona Genetics Core, UAGC, Tucson AZ, USA). The sequences were cleaned and the vector removed using VecScreen tool (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Seven libraries were created with a total of 130 *arrA* gene sequences of good quality, fragments of 550 bp were used to create a distance matrix to cluster in operational taxonomic units (OTUs) with a distance cutoff of 90% in Mothur software (Schloss et al., 2009); in the same manner, OTUs were also formed from closely related GenBank reference sequences. The *arrA* nucleotide sequences were translated into amino acid, then aligned with reference sequences using

Clustal W and phylogenies were constructed with Mega 7.0 by the Neighbor-Joining method based on the clustering of 164 amino acid sequences of arsenate respiratory reductase gene (*arrA*) (Kumar et al., 2016).

2.3 RESULTS AND DISCUSSION

2.3.1 Arsenic, sulfate, and iron partitioning in the sediments

The partitioning of metal and metalloids species associated with different sediment fractions is important for assessing the potential mobility and bioavailability of toxic elements in contaminated sediments, furthermore, geochemical features of the sediments influence their microbial phylogeny. The sediments CB and CT were submitted to chemical sequential extraction to assess the stability and bioavailability of arsenic, sulfate, and iron (Figure 2.2).

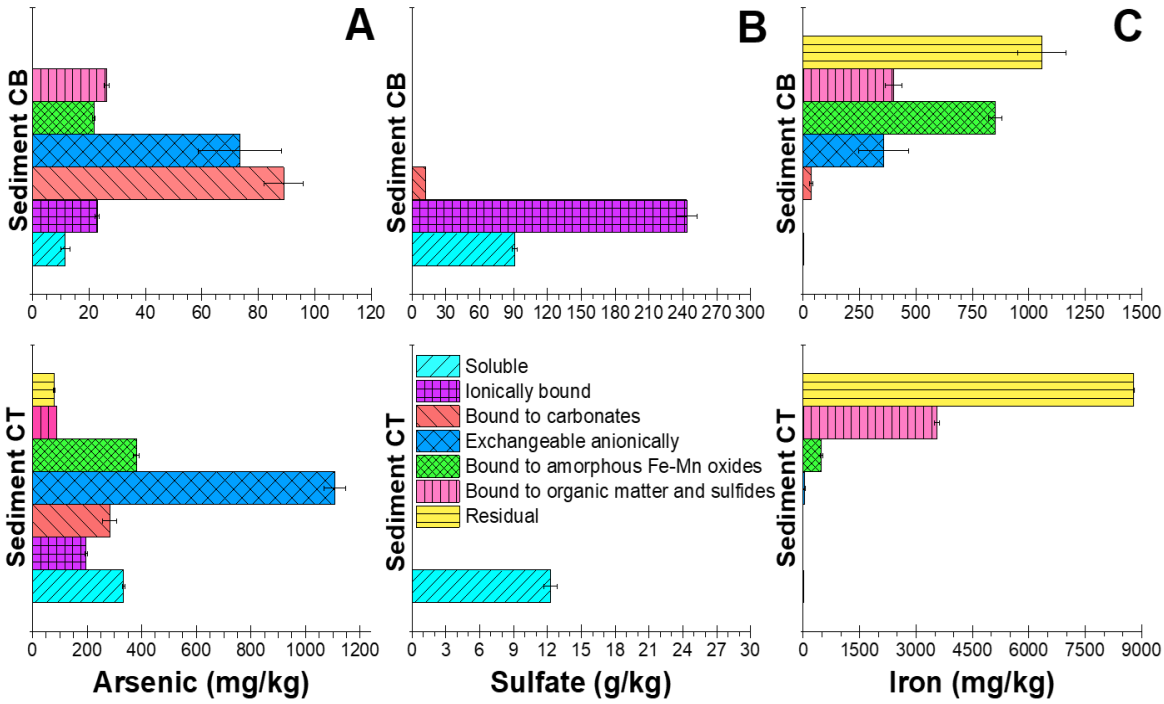

Figure 2.2. Sequential chemical extraction (SCE) procedure from sediments of the sites Cerrito Blanco (CB) and Club de Tiro (CT). A) arsenic partitioning; B) sulfate partitioning and C) iron partitioning.

The arsenic concentration in sediment CT (2263.1 ± 167.7 mg/kg) was 10 times higher than in sediment CB (238 ± 4.1 mg/kg). The extraction procedure revealed that the arsenic distribution among the two sediments differed significantly, in the sediment CB, the highest portion of arsenic was associated with carbonates (36.3%, or 89 ± 7 mg/kg), followed by the exchangeable anionic fraction (30.1%, or 745 ± 15 mg/kg). While most of the arsenic in sediment CT was found in the exchangeable anionic fraction (44.9%, or 1107 ± 39 mg/kg) followed by arsenic associated to amorphous iron and manganese oxides (15.4%, or 380 ± 10 mg/kg) and then the soluble fraction (13.5%, or 334 ± 6 mg/kg). The substantial arsenic concentration solubilized by deionized water may explain the high concentration of arsenic previously found in the column water of this site (48.3 ± 0.5 mg/L) (Rios-Valenciana et al., 2017).

In sediment CB, the arsenic associated with carbonates was higher compared with arsenic bound to amorphous iron oxides, despite carbonate minerals retain less arsenic than Fe(III)-oxides (Smedley and Kinniburgh, 2002). This result is supported by the low iron content in this sediment (~3000 mg of Fe/kg) equivalent to 0.3% (*wt.%*) of iron (Figure 2.2B), sediments that have less than 6% of iron are classified as iron-poor (Boggs, 2009). It is worth to note that arsenic bound to carbonates is untestable in semi-arid regions, such is the case of the sampling sites. Temporal changes in groundwater elevation influence redox conditions and cause dissolution and precipitation of carbonate minerals, triggering arsenic mobilization (Meng et al., 2016).

In both sediments, more than 30% of arsenic was mobilized by anionic exchange, which means that arsenate ($HAsO_4^{2-}$) was exchanged by phosphate (HPO_4^{2-}), the release of arsenic by the phosphate extracting agent occurs by competitive displacement, mobilizing the arsenic complexed with crystalline iron oxides or humic acids (Keon *et al*., 2001). Only in sediment CT, arsenic was quantified in the residual fraction (3.2%), which is the most stable fraction composed by arsenic coprecipitated with primary minerals (*i.e.* pyritic minerals) and other recalcitrant solids (*i.e.* crystalline silicates) (Das et al., 2016; Meng et al., 2016). Accordingly, the majority of iron was also determined in the residual fraction

(Figure 2.2C), most probably iron was present as pyrite. Comparing the two sediments, the content of sulfate was at least 19 times higher in sediment CB than in sediment CT, in the later sulfate was only in the soluble fraction, possibly of anthropogenic origin (Figure 2.2B). The mineralogy of sediment CB consisted mainly of gypsum and quartz whereas sediment CT was composed mainly by calcite and quartz (Rios-Valenciana et al., 2017).

According to the Risk Assessment Code, a guideline proposed to assessing the risk of toxic elements in sediments, if a toxic element, as arsenic, is associated to the exchangeable and carbonates fractions in percentages between 31% and 50%, the toxic element presents a high risk of mobilization (Baran and Tarnawski, 2015; Hahladakis et al., 2016). Hence, the sediments from both sites belong to this category, with 50% and 33% of arsenic distributed in ionically bound (exchangeable cationic) and carbonate fractions, respectively. High risk of arsenic release was determined in both sediments and therefore it could be subject to microbial action. These results further emphasize the importance of understanding the functional dynamics and metabolic capabilities of native microbiota.

2.3.2 Development of the arsenate/sulfate-reducing consortia

Each sediment microbiota was enriched in microcosms and used for successive transfers to obtain sediment-free cultures. The aim of this step was to establish cultivable communities of arsenate/sulfate-reducing microorganisms, in the following identified as Consortium CB and Consortium CT. Figure 2.3 demonstrates that both consortia were able to reach near-stoichiometric reduction of As(V) to As(III) at day 6. Sulfate-reduction was also achieved in both consortia, and it was visually supported by the formation of a yellowish precipitate responsible for the removal of the aqueous arsenic by its mineralization.

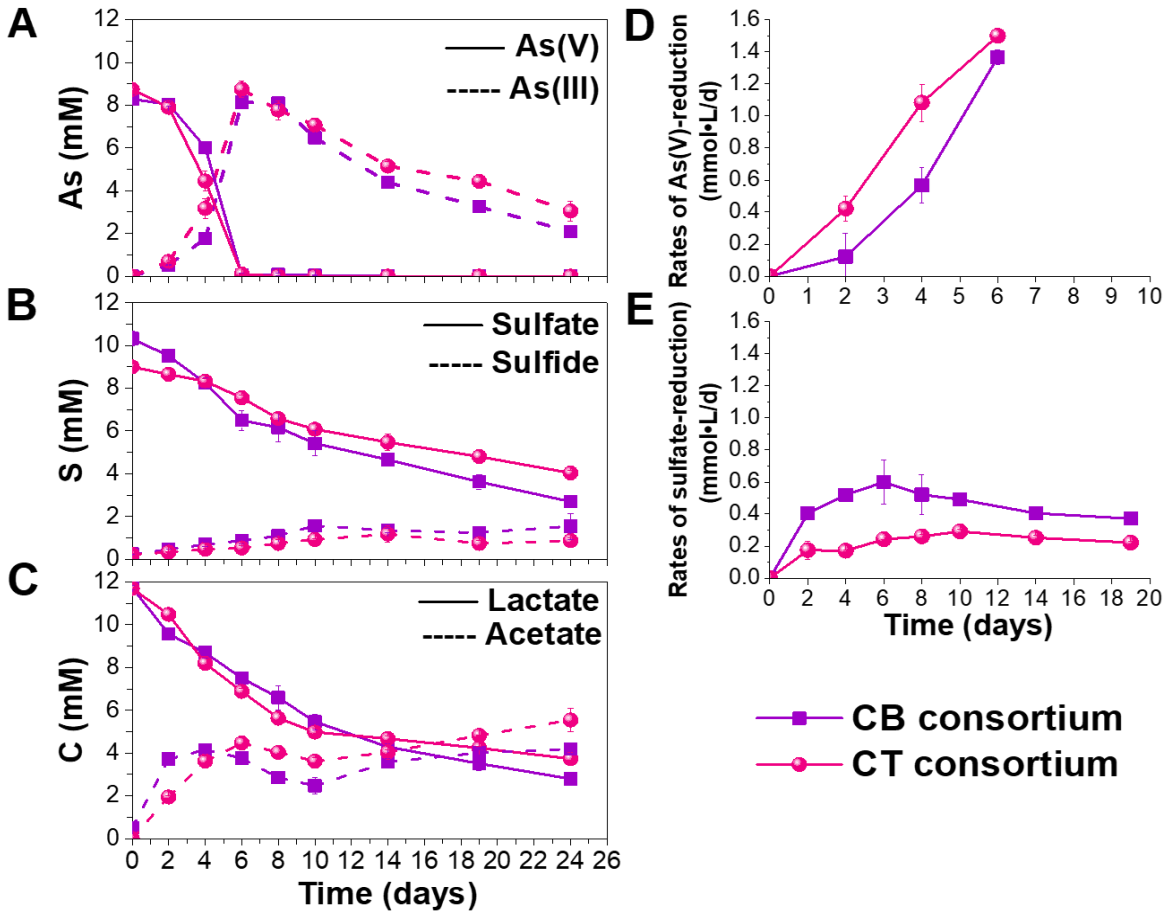

Figure 2.3. Arsenate- and sulfate-reducing activities of the microbial consortia obtained from sediments CB and sediment CT through successive transfers. A) arsenate (As(V)) reduction and arsenite (As(III)) production, B) sulfate reduction and sulfide production, C) lactate consumption and acetate production, D) rates of As(V)-reduction, E) rates of sulfate-reduction.

The rates of arsenate-reduction were higher for Consortium CT (1.10 mmol/L•d at day 4) compared to those of consortium CB (0.60 mmol/L•d at day 4) (Figure 2.3D); in agreement, the most probable number of As(V)-reducing bacteria was about 15 times higher for the consortium CT (15 × 10^8 MPN/mL) than for consortium CB, which could be related with the high degree of arsenic contamination found in sediment CT (2263.1 ± 167.7 mg/kg) (Figure 2.2A). The rates of sulfate-reduction were low for both consortia (Figure 2.3E), the consortium CB showed the higher sulfate-reduction rate (0.50 mmol/L•d at day 4), which was expected because sediment CB is a gypsiferous sediment promoting a mayor sulfate-reducing activity. Consistently with this results, in an earlier study we

determined that sediment CT has a rate of As(V)-reduction about three times higher than sediment CB, whereas the latter has a higher rate of sulfate-reduction (under the same culture conditions than the present study). Besides, the MPN of arsenate-reducing bacteria in the fresh sediment CT was 6.2 times higher (4.7×10^8 MPN/g sediment dry weight) than in sediment CB (7.6×10^7 MPN/g sediment dry weight) (Rios-Valenciana et al., 2017).

2.3.3 Microbial community transition through the distinct microenvironments

Based on the 16S rRNA analysis, we explored the bacterial diversity of the distinct microenvironments, two arsenic-contaminated fresh sediments, sediment enrichments obtained under As(V)- and sulfate-reduction, the biogenic minerals formed in this enrichments, as well as the bacterial consortia (see Figure 2.1). Comparing the samples, the microbial communities of the fresh sediments presented the highest richness and diversity according to the Shannon index (higher than 6) and the amount of accumulated OTUs (up to 305) (Table 2.1), The rarefaction curves approach an asymptote, indicating that enough sample coverage was obtained (Figure A1, Appendix).

Table 2.1 Alpha diversity based on 16S rRNA Illumina MiSeq analysis of the different microenvironments from CB and CT sediments. Operational taxonomic units (OTUs) at 99% of similarity and Shannon index.

Sample	# OTUs	Shannon Index
CB Fresh sediment (CB-FS)	238 ± 0.52	6.5 ± 0.07
CT Fresh sediment (CT-FS)	305 ± 1.23	7.4 ± 0.01
CB Sediment enrichment (CB-SE)	93 ± 0.33	4.9 ± 0.01
CT Sediment enrichment (CT-SE)	213 ± 1.20	6.3 ± 0.01
CB Biogenic mineral (CB-BM)	58 ± 0.42	3.5 ± 0.06
CT Biogenic mineral (CT-BM)	69 ± 0.32	3.8 ± 0.03
CB Bacterial consortia (CB-BC)	20 ± 0.32	1.6 ± 0.02
CT Bacterial consortia (CT-BC)	27 ± 0.92	2.1 ± 0.07

As the sediments were further cultivated, the diversity of the communities decreased as follows: fresh sediments > sediment enrichments > biogenic minerals

> bacterial consortia (Table 2.1). Interestingly, the diversity in the biogenic minerals is greater than in consortia, this can be explained because the biominerals were recovered from sediment enrichments, whereas the consortia community suffered a strict selection thought the removal of the sediment by the successive transfers.

There was a radical shift in the dominance of bacterial groups between the samples, even at phylum level. In the fresh sediments, the community was integrated by 22 phyla, the most representative (>2.5%) were *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Proteobacteria* and *Spirochaetes* (Figure 2.4). The dominant phylum in both sediments was *Proteobacteria,* representing 59% and 55% of the sequences in sediment CB and CT, respectively (Figure 2.4). In the sediment enrichments, *Proteobacteria* was also the most abundant phylum (more than 40% of the sequences), but the phylum *Firmicutes* increased significantly reaching abundances higher than 24% in both samples. Conversely, in the biogenic minerals, the phylum *Firmicutes* represented more than 88% of the communities, and the abundance of *Proteobacteria* decreased up to 2.8% and 4.8% in the biominerals CB and CT, respectively. In the bacterial consortia, more than 97% of the sequences belonged to *Firmicutes* (Figure 2.4).

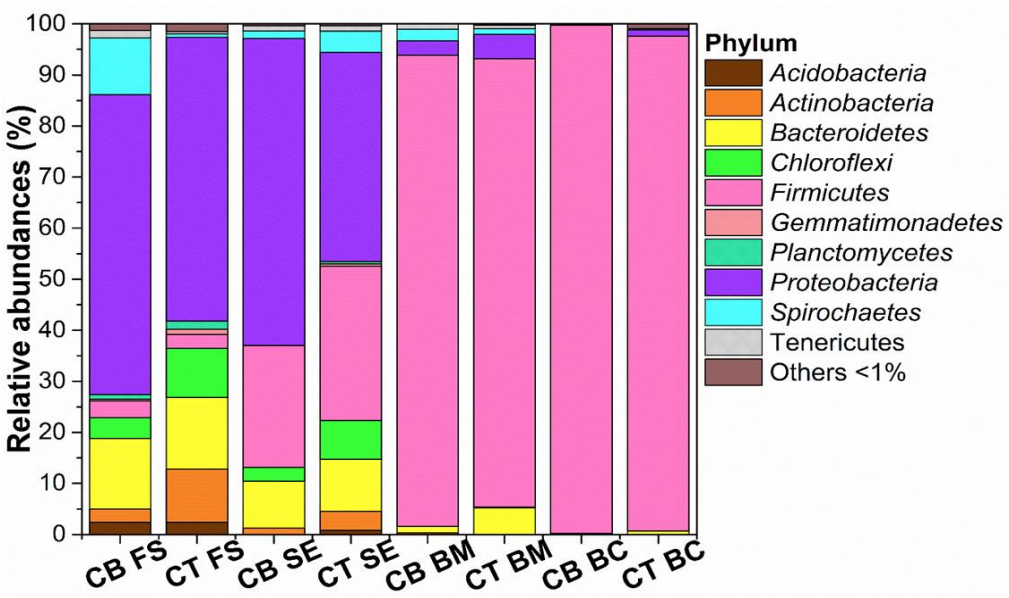

Figure 2.4 Bacterial community composition based on 16S rRNA gene analysis at phylum level of the distinct microenvironments of the sites CB and CT: fresh sediment (FS); sediment enrichment (SE); biogenic mineral (BM) and bacterial consortium (BC).

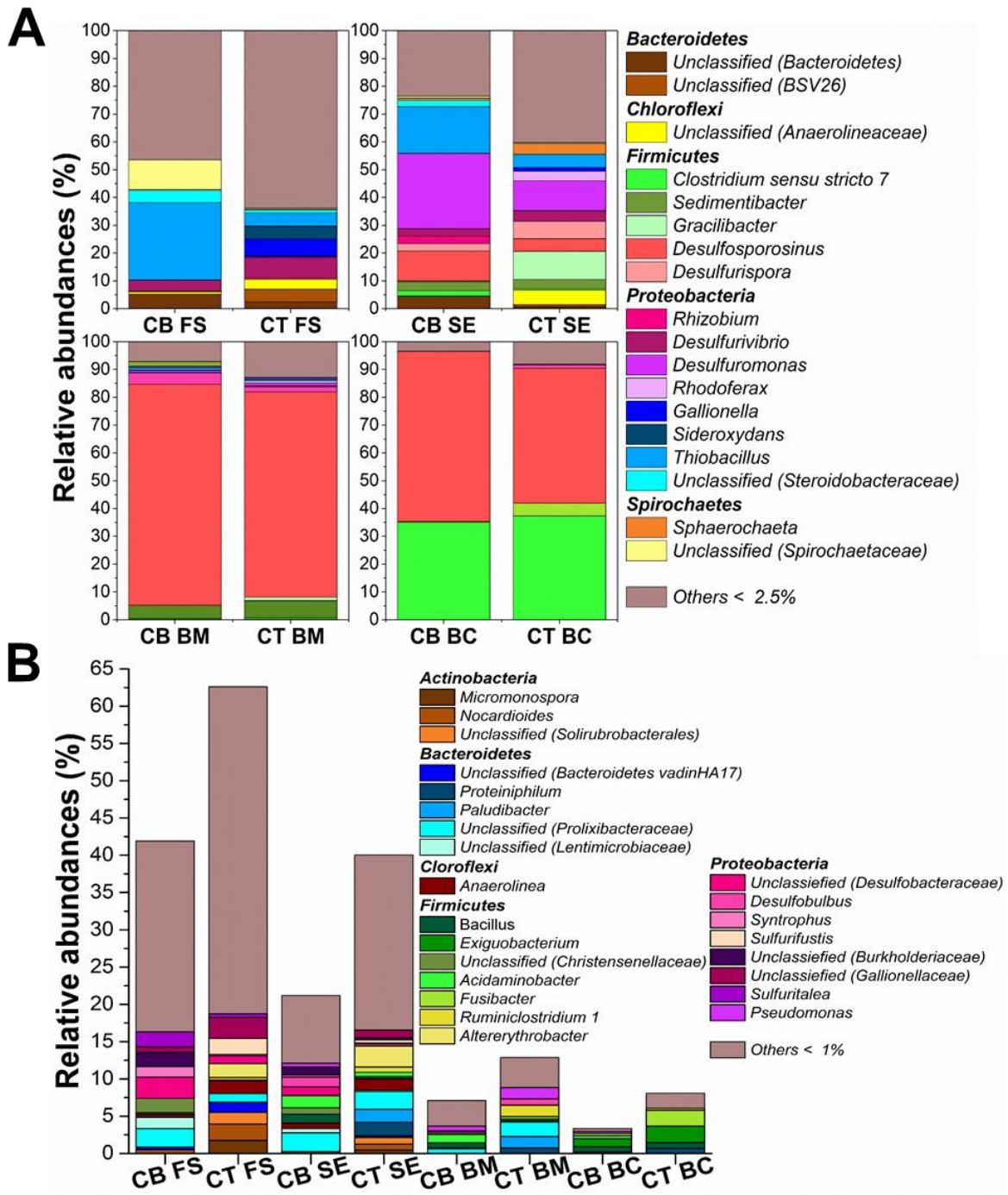

Figure 2.5. Bacterial community composition based on 16S rRNA gene analysis at genus level of the distinct microenvironments of the sites CB and CT: fresh sediment (FS); sediment enrichment (SE); biogenic mineral (BM) and bacterial consortium (BC). A) genera with abundances higher than 2.5%; B) genera with abundances lower than 2.5% and higher than 1%.

A total of 262 genera were detected considering all the samples, but only 18 of them were present in abundances higher than 2.5% (Figure 2.5A). As mentioned

before the fresh sediments and sediment enrichments showed the highest richness, therefore up to 63% of the genera were present in abundances lower than 2.5% (Figure 2.5B).

It is remarkable the abundance of *Desulfosporosinus* in the biogenic minerals, which represented on average 75% of the sequences in both biominerals. Regarding the bacterial consortia, these were composed by the same genera but in different abundances, *Proteiniphilum*, *Bacillus, Exiguobacterium, Fusibacter, Clostridium sensu stricto 7, Desulfosporosinus* and *Sedimentibacter* (Figure 2.5). Six of these genera belong to the phylum *Firmicutes* and are widely known for including arsenate-respiring species (Costa et al., 2014; Lu et al., 2017). The bacterial consortia were dominated by *Desulfosporosinus* and *Clostridium sensu stricto 7,* both genera comprised 96% of the sequences in consortium CB and 86% in consortium CT (Figure 2.5A).

Bacterial consortia were the least diverse among the samples, consortium CB was integrated only by 13 genera and consortium CT was integrated by 16 genera. When the sediment was removed, the planktonic biomass did not serve as inoculum and the biominerals were the main source to propagate the consortia. Possibly, in both consortia the high concentrations of As(III) (*ca.* 10 mM) and the complete removal of the sediment acted as selective forces, because the sediment receives the deposition of organic matter from the water column and provides a matrix of complex nutrients and solid surfaces for microbial growth (Huang et al., 2017). In great extent, the applied selective forces are responsible for the significant differences in the microbiota associated among the different microenvironments (Figure 2.5).

Figure 2.6 shows the bacterial genera enriched in the different microenvironments, taking as reference the microbial composition of the fresh sediments. The adverse conditions in biogenic minerals restricted the enrichment of certain genera, this microenvironment consists mainly of arsenite and sulfide and the cells can be mineralized gradually. According to some authors, mineralized cells are those that are embedded in mineral aggregates (Le Pape et al., 2017;

Stanley et al., 2018). In both biogenic minerals only three genera were enriched, *Desulfosporosinus, Sedimentibacter,* and *Desulfurispora* (Figure 2.6), which belong to the *Firmicutes* phylum and *Clostridiales* order, All of them are able to form spores, and have been implicated in arsenic metabolism (Kaksonen et al., 2007; Suhadolnik et al., 2017). Under extreme conditions the capacity to produce spores is very helpful, although mineralized cells are unable to metabolize, the generation and release of spores serve as a growth strategy to maintain the genome, allowing the microorganisms to survive (Stanley et al., 2018). In contrast with the biogenic minerals, besides *Desulfosporosinus, Clostridium sensu stricto* 7 (up to 37%) was also enriched in the consortia (Figure 2.6), this genus is cataloged as a fermentative, but has also been related with arsenic respiration (Mirza *et al*., 2014). We hypothesized that two selective forces operated in favor of arsenate-respiring bacteria in the bacterial consortia: the high arsenic concentrations and the presence of biogenic arsenic minerals without the protection of the sediment matrix.

Sediment CT has 10 times more arsenic than sediment CB, in spite of this, sediment CT presented a greater diversity, which agreed with previous reports that found a great diversity in arsenic polluted sediments (Costa et al., 2014; Suhadolnik et al., 2017). Cai et al. (2009) reported that more bacterial species were isolated from a site with long-term and high-level of arsenic contamination than from sites with intermediate and low levels of contamination. According to the authors, that fact was attributable to an evolutionary adaptation of specific bacterial species to arsenic stress.

It is worth to note that although *Desulfosporosinus* was almost absent in the fresh sediments (less than 0.4% of the OTUs), in the biogenic minerals and bacterial consortia, it was the dominant genus (reaching about 75% in biominerals). *Desulfosporosinus* is highly versatile genera which is capable of using As(V), Fe(III), sulfate, thiosulfate, and nitrate as respiratory electron acceptors (Liu et al., 2004; Pérez-Jiménez et al., 2005). Members of this genus are efficient in promoting saturating geochemical conditions for arsenic sulfide

precipitation (Stanley et al., 2018; Suhadolnik et al., 2017; Zhang et al., 2018). Hausmann et al. (2016) reported that *Desulfosporosinus* possesses a high number of ribosomal operons that enables the microorganism to respond quickly, by increasing their ribosome content and metabolic activity, to favorable growth conditions. Frequently, *Desulfosporosinus spp.* and *Clostridium spp.* have been related with exceptional resistance to toxic elements, attributed to their physiologic and metabolic capabilities, since they are Gram +, able to form spores, and they use a wide range of electron donors and electron acceptors (Hwang et al., 2009).

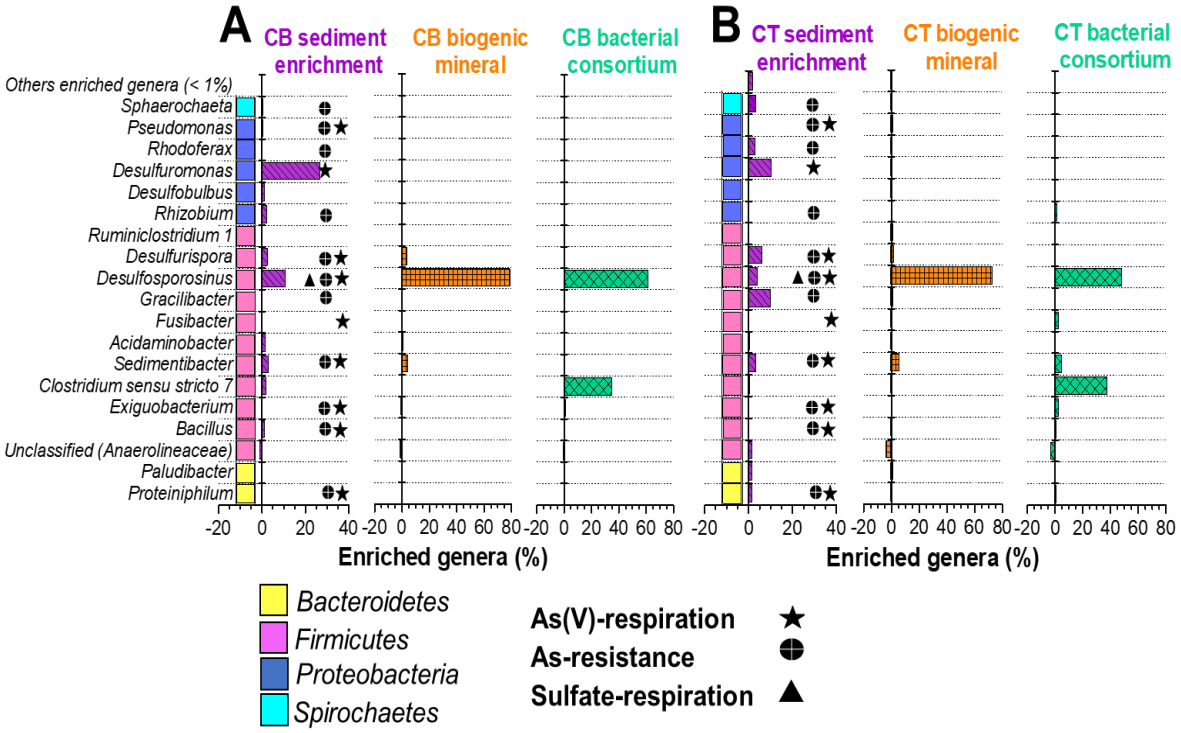

Figure 2.6. Bacterial genera that increased their relative abundance taking as reference the fresh sediments. A) Microenvironments from site CB; B) Microenvironments from site CT. The genera whose enrichment was less than 1% in all the samples were grouped in others.

Based on the 16S rRNA analysis and Bray-Curtis dissimilarity, the communities in the bacterial consortia and the biogenic minerals are quite similar irrespectively of the sediment of origin (Figures 2.5 and 2.7).

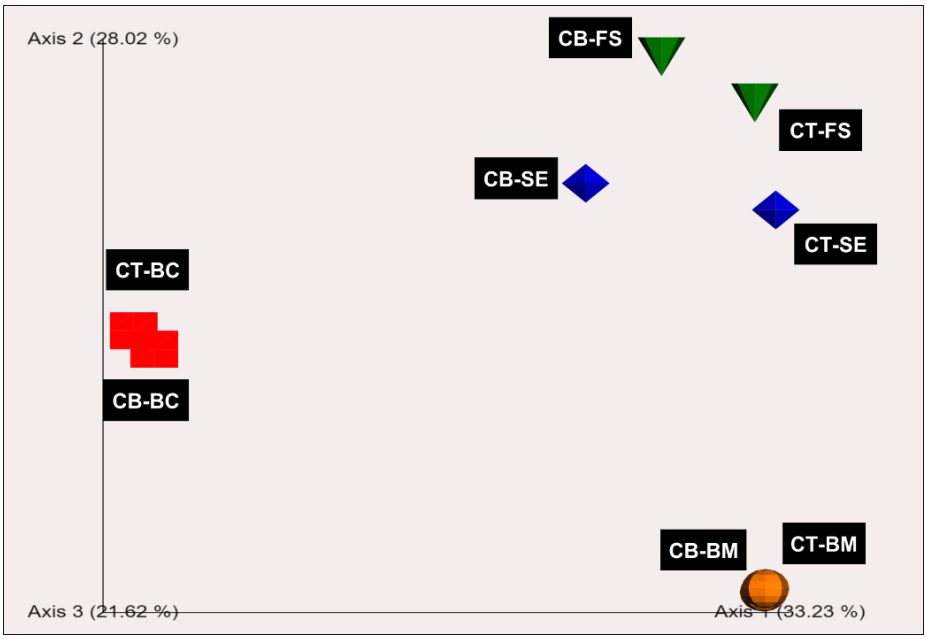

Figure 2.7. Beta diversity based on Bray-Curtis distance, a quantitative measure of community dissimilarity considering the16S rRNA Illumina MiSeq analysis. Fresh sediment (FS, ▼), sediment enrichment (SE, ♦), and biogenic mineral (BM, ●) and bacterial consortium (BC, ■).

In summary, the microbial microbiota of the biogenic minerals was represented mainly by the genus *Desulfosporosinus* that reached abundances about 75%, whereas the microbiota of the microbial consortia was represented by *Desulfosporosinus* (*ca.* 55%) and *Clostridium sensu stricto 7* (*ca*. 36%). On a global scale, many studies of arsenic-contaminated sites have found *Desulfosporosinus* as one of the principal members of the indigenous communities (Moon et al., 2017; Yamamura et al., 2018; Zhang et al., 2018). Evidently, this genus has an important role in the arsenic biogeochemical cycle possibly due to its high adaptability to extreme environments (Hausmann et al., 2016). In anoxic microcosms with As-polluted sediments amended with glucose (28 mM) it was detected a significant increment of *Clostridium sensu stricto 7,* which was also correlated with *arrA* positive clones (Mirza *et al*., 2014).

2.3.4 Phylogenetic analysis of functional *ArrA* gene

To confirm the presence of dissimilative arsenate-reducing bacteria, the *arrA* functional gene was amplified and cloned, *arrA* phylotypes were identified in fresh sediment CT, sediment enrichments, biogenic minerals, and bacterial consortia. Regrettably, after a series of attempts, the *arrA* gene was not amplified from the sample of fresh sediment CB, possibly because this sediment presents a low degree of arsenic-contamination (Figure 2.2A). Similarly, in a previous report, the amplification of the *arrA* gene was not successful from fresh sediments with low arsenic concentration (lower than 10 mg/kg); the authors attributed this to the low abundance of the *arrA* gene in the sample (Mirza et al., 2014). The occurrence of the *arrA* gene has been preferentially associated with highly contaminated sites (Desoeuvre et al., 2016).

In the current study, the *arrA* sequences were clustering into OTUs at 97% and 90% cutoff, obtaining 28 and 12 OTUs respectively, considering the BLAST analysis and for easy representation, we have reported only the results at 90% cutoff. The phylogenetic tree of the *arrA* gene was constructed with 130 *arrA* gene sequences clustering in 12 distinct OTUs and 34 closely related a*rrA* sequences from GenBack from these, 26 sequences corresponded to uncultured bacteria grouped in 11 OTUs (at 90% cutoff) and 8 sequences of pure cultures (Figure 2.8). In our study the *arrA* diversity was relatively low, this was expected for biogenic precipitates and microbial consortia because the diversity-based in the 16S rRNA gene was low (less than 69 OTUs).

BLASTp analysis of the clone sequences revealed similarities between 72 and 100% with *arrA* gene sequences from the GeneBank database. In the bacterial consortia of both sediments it was detected the OTU ER9, which is identical to the sequence of the *arrA* gene of *Desulfosporosinus sp. Y5* (ABB02056.1, 100% of similarity at amino acid level). In Consortium CT, only two *arrA* OTUs were amplified: 32% of the sequences corresponded to the OTU ER9 and the rest of the sequences were gathered in OTU ER3 (Figure 2.8), which has high similarity to the *arrA* gene of *Bacterium MPA-C3* (AGE47823.1, 97% of similarity at amino acid level). *Bacterium MPA-C3* was isolated from arsenic-bearing sediments, it can

respire As(V), Se(VI), Fe(III), NO_3^-, S^0, but is unable to reduce sulfate, moreover it can precipitate alacranite an arsenic sulfide (Mumford et al., 2013). OTU ER3 also showed with the *arrA* gene of *Desulfitobacterium hafniense Y51* (BAE85920.1, 96% of similarity at amino acid level). The OTU ER12, retrieved from sediment CT, was linked to *Desulfuromonas sp. WB3* (AKM12623.1, 82% of similarity at amino acid level).

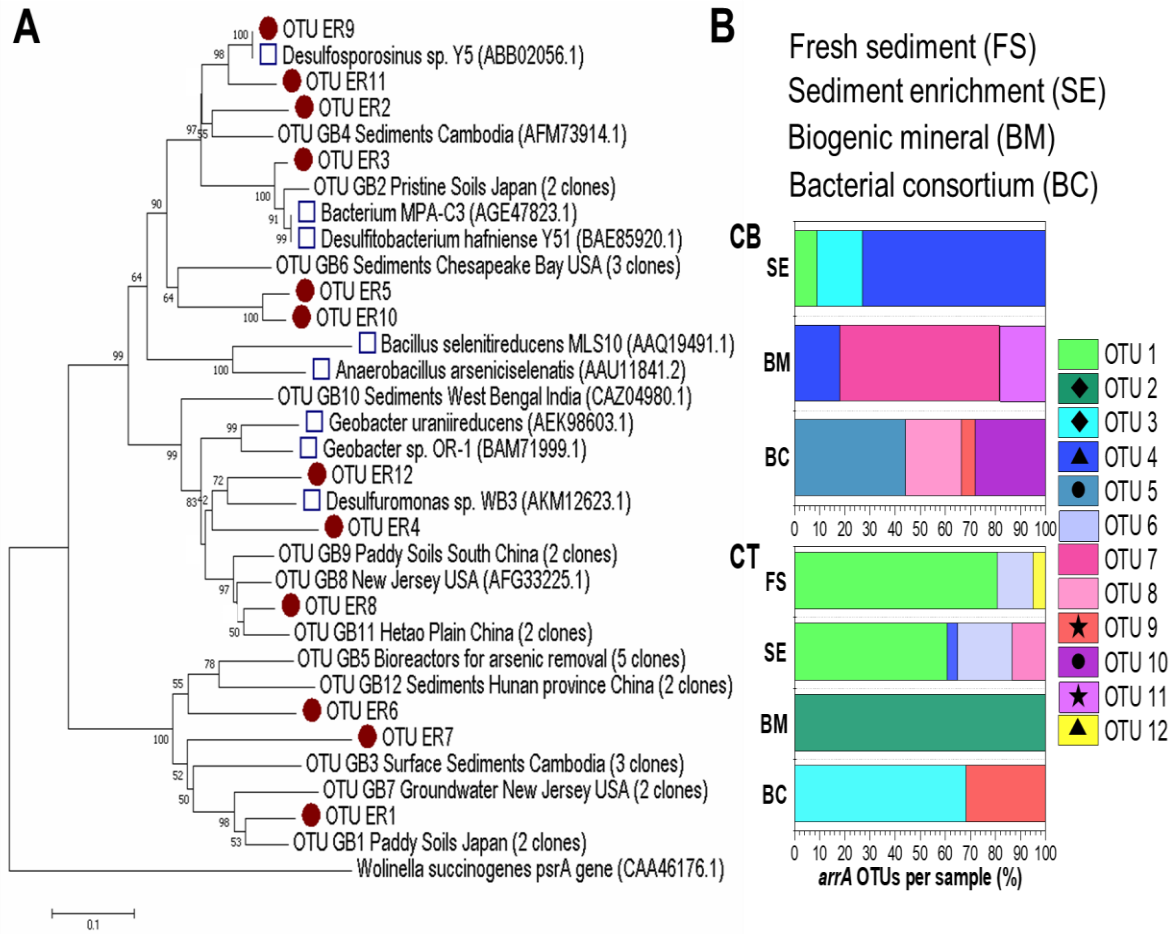

Figure 2.8. Phylogeny of arsenate respiratory reductase gene (*arrA*). A) Neighbor-Joining phylogenetic tree based on the clustering of 168 amino acid sequences of *arrA* gene. Seven libraries were generaed with a total of 130 *arrA* genes. The tree was constructed considering 26 arrA gene sequences from uncultured microorganisms and 8 pure cultures (blue squares). The *arrA* sequences were clustered in OTUs (at 90% cutoff) in Mothur, and then representative sequences were translated into amino acid. OTUs marked with a circle represent the sequences obtained in the present study. Numbers at nodes show bootstrap values obtained from 1000 replicate bootstraps. B) Distribution and diversity of *arrA* OTUs in the microenvironments CB and CT. The same black figure indicates close phylogeny between the OTUs.

The *arrA* phylotypes distribution was different between each sample (Figure 2.8B). As presented before (Figure 2.8A), only the OTUs ER9, ER3, ER12 were closely related with pure cultures, the OTUs ER5, ER6, ER7, and ER10 are significantly different from the *arrA* gene sequences obtained from GenBank (similarity lower than 80%) highlighting their novelty. The rest of the OTUS exhibited similarity (> 80%) with uncultured bacterial clones recovered from arsenic-contaminated sediments, soils, and groundwater from different sites Cambodia, Japan, China and the USA (Figures 2.8). The arsenate-respiring prokaryotes are highly diverse and are widely distributed in the environment, the first arsenate-respiring was described in 1994 and since then only about 40 cultivable arsenate-respiring bacteria have been identified so far, and the *arrA* gene has been identified only in 32 pure strains (Andres and Bertin, 2016; Costa et al., 2014; Lu et al., 2017). Therefore, more efforts are necessary to identify and cultivate novel arsenate-respiring microorganisms.

Through the 16S rRNA analysis, we found that communities in biogenic minerals and bacterial consortia were quite similar irrespectively of the sediment origin (Figures 2.5 and 2.6). However, according to the *arrA* gene analysis, noticeable differences were detected (Figure 2.8), the original sediment could have affected the function of arsenate-respiring bacteria given that consortium CT, obtained from the most contaminated sediment, only presented *arrA* sequences linked with highly specialized microorganisms (*i.e. Desulfosporosinus Y5* and *Bacterium MPA-C3*), recognized for their important role in the arsenic biogeochemical cycle of hydrogeological systems (Garcia-Dominguez et al., 2008; Liu et al., 2004; Pérez-Jiménez et al., 2005).

2.4 CONCLUDING REMARKS

The sequential fractionation of the sediments showed that more than 77% of the arsenic was highly bioavailable (*i.e.* associated with soluble, carbonate and

ionically bound fractions), this fact did not negatively impact the native microbiota. Sediment CT presented the highest concentration of arsenic (~2263 mg/kg) and was the most diverse (Shannon index of 7.4 and 305 OTUs). It is probable that the long-term exposure to the high arsenic concentration caused the adaptation of its native microbiota, by the acquisition of genes related to detoxification and arsenic respiration probably by horizontal gene transfer events. In contrast with the fresh sediments, the sediment enrichments showed a transition in their communities, genera gathered in *Proteobacteria* and *Firmicutes* phylum increased notably such as *Desulfuromonas* and *Desulfosporosinus* despite this, sediment enrichments maintained a high diversity. However, when the sediment matrix was removed completely, the assembly of the communities was independent of the origin sites and the differences were observed when the sample belonged to either biogenic minerals or bacterial consortia.

In these microenvironments, the structure and function of the microbial community changed with respect to the fresh sediments and sediment enrichments. As it was promoting the growth of specialized groups with the ability to resist and metabolize arsenic (*Desulfosporosinus*, *Clostridium sensu stricto 7 and Sedimentibacter).* This was supported by the *arrA* results since in the consortia were determined *arrA* sequences with high similarity to *Desulfosporosinus sp. Y5* and *Bacterium MPA-C3* which have been recognized by their pivotal role in As(V)-respiration and precipitation of arsenic sulfides.

To the best of our knowledge, this is the first report that explores the arsenate reducing community in biogenic minerals formed by As(V)/sulfate-reducing activity. The biogenic arsenic sulfide mineral is a reservoir of arsenic resistant and arsenic-respiring microorganisms.

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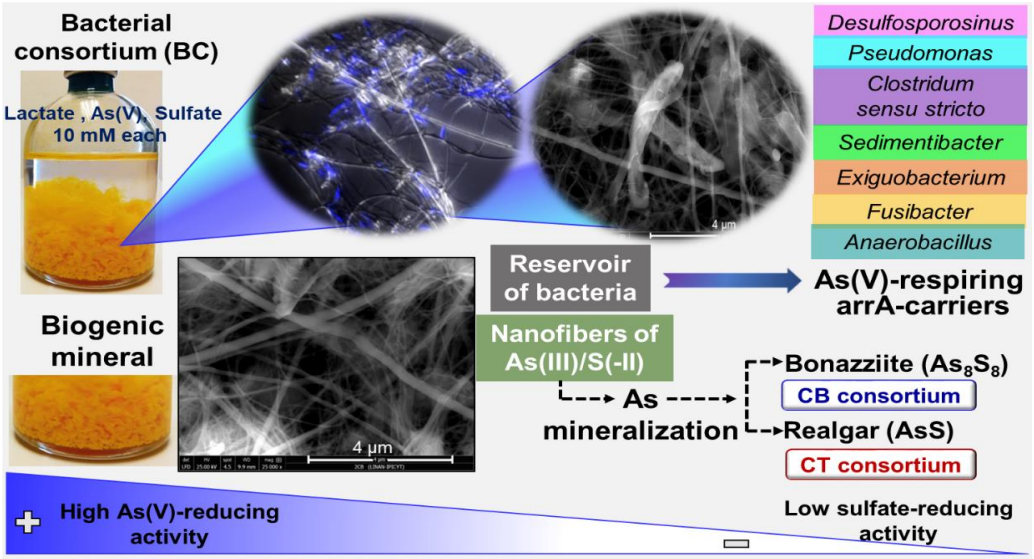
CHAPTER 3

Biogenic minerals and extreme bacteria a team in arsenic-polluted environments

Highlights

- Biogenic minerals formed via sulfate- and arsenate-reduction in cultures from consortia CB and CT corresponded to As(III)/S(-II) nanofibers (< 250 nm of diameter).
- Each bacterial consortium developed arsenic-sulfides with different mineralogy, the biomineral CB was identified as bonazziite and the biomineral CT was identified as crystalline realgar.
- As(III)/S(-II) biogenic mineral is a reservoir of arsenic-respiring bacteria and it represents a good media for bioprospection of arsenic mineralizing bacteria.

Graphical abstract



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ABSTRACT

The formation of arsenic-bearing minerals by microbial anaerobic respiration is crucial in the natural arsenic cycle and the bioremediation processes. The interaction between microorganisms and biogenic arsenic-sulfides, and the role of these biominerals in shaping the structure of microbial communities in polluted environments has remained elusive. To address this issue, microcosms experiments inoculated with arsenate/sulfate-reducing bacterial consortia devoid of sediments were used to recover the biogenic minerals and investigate their morphology, composition, and mineralogy. Biominerals formed via sulfate- and arsenate-reduction in cultures from consortia CB and CT corresponded to As(III)/S(-II) nanofibers (< 250 nm of diameter), which had different mineralogy. The biomineral CB was identified as bonazziite (As_8S_8), while the biomineral CT, highly crystalline, was identified as realgar (AsS). The development of the different mineral phases was related to the composition of the microbial communities and therefore, with the rates of arsenate- and sulfate-reduction. *Desulfosporosinus*, *Clostridium sensu stricto*, *Pseudomonas*, *Exiguobacterium*, *Sedimentibacter*, *Fusibacter,* and *Anaerobacillus* represented 90% of the genera linked to both biominerals*,* all these genera include dissimilative arsenate-reducing species (*arrA*-carriers). *Desulfosporosinus*, a sulfate-reducer, dominated in biomineral CB and *Pseudomonas*, an arsenate-reducer, was the dominant genus in biomineral CT. Consequently, the biogenic sulfide supply was low in the biomineral CT allowing the maturation of the amorphous arsenic-sulfide phase into crystalline realgar. Until recently it was believed that crystalline realgar and their polymorphous as is the case of bonazziite could only be formed at high temperatures and under hydrothermal conditions. We found that the bacterial consortia were able to overcome the thermodynamic barriers and catalyze the formation of these mineral phases under mesophilic conditions and short periods (< 40 days). The key was to have high arsenate reduction rates and low sulfate-reduction rates that were possible to achieve thanks to the microbial community that we selected.

Keywords: Biominerals, microbial community, arsenate-reducing bacteria, biogenic arsenic-sulfide, realgar, bonazziite

CHAPTER 3

3.1 INTRODUCTION

Biomineralization refers to the processes by which the organisms catalyze the formation of mineral phases, such processes include redox reactions for energy conservation, detoxification, and production of extracellular metabolites (Gadd, 2010). A wide range of phylogenetic groups of prokaryotes can induce the precipitation of biogenic mineral phases with different morphologies and mineralogy such as calcium carbonates, phosphates, silicates, Fe and Mn-oxides, iron secondary minerals (*e.g.* siderite, magnetite) and sulfides, these biominerals can adsorb or coprecipitate metals and metalloids (Gadd, 2010; Picard et al., 2016). In natural environments, biomineralization is crucial to control the fate of hazardous inorganic elements; specifically, in reducing environments containing sulfate and metals, the mineralization of metals as part of sulfides is a pivotal process (O'Day et al., 2004). In low-temperature environments (< 100 °C, *e.g.* lakes, rivers aquifers) about 97% of the sulfide produced globally is attributable to the activity of sulfate-reducing prokaryotes, the remaining 3% are produced at volcanoes and deep-sea hydrothermal vents (Picard et al., 2016). In this framework some reports have been emphasized the significant role of bacteria in the formation sedimentary metal sulfide deposits (Demergasso et al., 2007; Picard et al., 2016). However, the relationship between metal sulfides and bacteria is often overlooked in the low temperature environments. It has been demonstrated that, besides creating saturating geochemical conditions for mineral precipitation, microorganisms promote mineral nucleation and grow of the mineral phases using different mechanisms. For instance, at circumneutral pH, microbial cell walls have a net negative charge, due to deprotonation of organic ligands contained in various polymers, such as peptidoglycan and lipopolysaccharides. Thus, cell walls can provide scaffolds for metal binding and microenvironments for super-saturation of chemical species, resulting in the fast precipitation of minerals (Picard et al., 2016; Stanley et al., 2018). In addition, the role of exopolymeric substances, consisting of organic ligands that bind metals, has also been highlighted to play an important role. Sulfate-reducing bacteria (SRB) can produce large amounts of exopolymeric

substances, which have high affinity for cationic elements (Ca^{2+} and Mg^{2+}) (Braissant et al., 2007).

As with all toxic metals and metalloids, the safest way to avoid arsenic toxicity is to maintain the element in its non-bioavailable form, ideally as part of minerals (Gadd, 2010). From a kinetics point of view, the precipitation of biogenic minerals that immobilize arsenic is advantageous over abiotic precipitation; consequently, biominerals have a primary role in the arsenic cycle and they also represent an effective alternative for arsenic attenuation (Picard et al., 2016).

One interesting aspect of the formation of biological arsenic-sulfide minerals is that it occurs when the respiratory processes of arsenate- and sulfate-reduction occur simultaneously (Altun et al., 2014; Rodriguez-Freire et al., 2014). Arsenic sulfides such orpiment (As_2S_3) and realgar (AsS), are highly insoluble, stable in reducing environments, and they may contain up to 60-70% wt. of arsenic (Kirk et al., 2010). Under mesophilic conditions, the formation of orpiment and realgar in sediments has been reported to be possible only catalyzed by microbial activity (Demergasso et al., 2007; O'Day et al., 2004). Some research has focused on the precipitation of biogenic arsenic sulfides, highlighting their importance in the arsenic biogeochemical cycle and their application in the bioremediation processes (Altun et al., 2014; Demergasso et al., 2007). Additional information is required to better understand the interaction biomineral-microorganism and the role of these minerals in shaping the structure of the microbial communities in contaminated sites. There are few studies with this approach and focusing on iron sulfides (Picard et al., 2018; Stanley et al., 2018).

Chalcogenide minerals result from the reaction of group VI elements (mainly S, Se, Te) with more electropositive elements (As, Sb, Si, Cd) (McFarlane et al., 2015). Depending on their composition and synthesis, these compounds can be formed as nanostructures and be crystalline, glassy, semiconductive, and ionic conductors. Such properties give them functionality to be used in sensors and photoactive devices (Lee et al., 2007; McFarlane et al., 2015). The physicochemical synthesis of chalcogenide nanostructures has been investigated, but frequently it requires templates, precursors, and extreme conditions (high

pressures and temperatures) (Rao and Govindaraj, 2009), hence microbial routs (green synthesis) have gained attention.

Arsenic sulfides (*e.g*. orpiment, realgar, alacranite) belong to chalcogenides; in the pioneer studies of arsenate reduction, biogenic sulfides were considered to have amorphous nature and low crystallinity (Newman et al., 1997). Later reports demonstrated the synthesis of extracellular arsenic-sulfide nanostructures by anaerobic bacterial respiration (Jiang et al., 2009; Lee et al., 2007). The precipitation of arsenic-sulfide nanostructures has been described in pure cultures of *Shewanella* during the reduction thiosulfate to sulfide, and As(V) to As(III), different strains of this genus can produce a network of arsenic-sulfide nanofibers/nanotubes in anoxic cultures. The efficient formation of the arsenic-sulfide nanotubes by *Shewanella* strains was linked to active arsenate-reductase systems and the presence of the *arrA* and *arsC* genes (Jiang et al., 2009; McFarlane et al., 2015). There is only one report of the production of arsenic sulfide nanomaterials by bacterial consortia performing As(V)- and sulfate-reduction under acidic conditions (pH 4.5) (Le Pape et al., 2017); the acid pH favors the precipitation of arsenic sulfides (Battaglia-Brunet et al., 2012; Eary, 1992). A major challenge of biogenic synthesis is the control of proprieties as size, shape, and structure of biominerals. A better understanding of the mechanisms of formation, parameter handling in cultures (proper concentrations of electron donor and electron acceptors), as well as finding out what other bacteria are involved in the production of As-S nanofibers, will help us to design adequate biological systems for the production of nanostructures.

The aims of this chapter were: 1) To investigate the mineralogy and structural morphology of biogenic minerals recovered from cultures of two bacterial consortia under As(V)- and sulfate- reduction; 2) to elucidate novel bacterial genera involved in the development of As(III)-S(-II) nanofibers and discuss their physiological and genetic features.

3.2 MATERIALS AND METHODS

3.2.1 Microcosms for recuperation of biogenic minerals

Bacterial consortia from the sediments CB and CT were grown under arsenate- and sulfate-reducing conditions. Roughly, 90 mL of basal medium (Chapter 2), amended with lactate, arsenate, sulfate (10 mM each), and pH adjusted to 6.8 with $NaHCO_3$ were placed in 125 mL serum bottles. The bottles were sealed with rubber stoppers and aluminum rings, purged with N_2/CO_2 (80:20, v/v), and sterilized (autoclaving 20 minutes, 121 °C and 103 kPa). The microcosms were started from sterile media inoculated with 10 mL of either of consortium CT or consortium CB. Cultures were incubated during 25 days in the dark, without stirring.

3.2.2 Recover and characterization of the biogenic minerals

From each consortium (CB and CT) two samples of biogenic minerals were analyzed, one of them to observe the intact cells and the structure of the biominerals after 25 days of culturing the microcosms, it was only analyzed by SEM-EDS. The other sample was obtained after 40 days of incubation to make the physicochemical characterization by XRD, Raman, and SEM-EDS. Details are explained below.

The samples to observe intact cells were prepared inside of the anaerobic chamber (N_2/H_2, 95:5 % v/v) (COY 14500, Grass Lake, MI, USA), a fraction (*ca.* 5 µL) of yellow wet precipitate recently withdrawn from a culture was deposited directly on the mounting pin, coated with double-sided carbon tape, and once the sample dried up (*ca.* 1 hour) it was analyzed by scanning electron microscopy (SEM, FEI-QUANTA 250 FEG, Hillsboro, Or, USA) combined with energy dispersive spectroscopy (EDS, EDAX, Mod. DX4). Also, an elemental analysis mapping was performed at 14 kV with a working distance of 8.5 mm using a silicon drift detector (EDAX).

For physicochemical characterization, the microcosms were also open inside of the anaerobic chamber, then biogenic minerals were recovered using a

Pasteur pipette and they were deposited in Eppendorf tubes (2 mL), pelleted by centrifugation (4000 *g*) and decanted. The biominerals were washed three times with O_2 free deionized water to remove the salts finally, biominerals were vacuum dried (2 hours) and pulverized in an agate mortar. The powdered samples were analyzed by X-ray diffraction (Diffractometer SmartLab, Rigaku, Tokyo, Japan), the patterns obtained (2θ =10° to 90°, step = 10 s, step size = 0.02°) were compared against the database of the International Center of Diffraction Data (ICDD). Ramman spectroscopy (Micro-Raman Renishaw, Barcelona, Spain) was conducted at 633 nm of laser excitation and a SEM-EDS analysis (as described above).

3.2.3 DAPI stain and optical microscopy

The biogenic mineral was prepared for visualization of the total cells using 4´,6´-diamidino-2-phenylindole (DAPI) a DNA-binding staining. Briefly, samples of biominerals recuperated from the microcosms were fixed as follows: per one volume of sample (0.5 mL) three volumes of paraformaldehyde (4%) were added and the mix was stored at 4 °C for 3 hours, then the pellet was recovered by centrifugation (5000 *g*) and washed two times with PBS buffer. Finally, cold ethanol (100%) was added, to maintain the sample. 6 µL of the fixed sample were deposited into wells of a Teflon-coated slide (Thermo Scientific™ PTFE Diagnostic Slides), and 10 µL Vectashield® (antifade Mounting Medium)/DAPI were distributed over the slide, covered with a thin film and sealed. DAPI stained cells were observed using Axio Scope A1 epifluorescence microscope using a wavelength of 358 nm and a blue filter (Carl Zeiss, Jena, Germany).

3.2.4 DNA extraction and 16S rRNA gene analysis from the biogenic minerals

DNA was extracted from the biogenic minerals using the commercial kit ZR Soil Microbe MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The composition of the bacterial community was determined targeting the V4 hypervariable region of the prokaryotic 16S rRNA

gene with the primers 515 F (5´GTGCCAGCMGCCGCGGTAA3´) and 806 R (5´GGACTACHVGGGTWTCTAAT3´) (Walters *et al*., 2015). Pair-end sequencing method (Illumina MiSeq) was performed by an external laboratory (RTL Genomics Molecular Sequencing Laboratory, Lubbock, TX, USA).

3.2.5 Bioinformatics treatment and taxonomic assignment

Quantitative Insights into Microbial Ecology (QIIME2, 2018.8 release) software was used for the analysis of 16S rRNA libraries. The analysis of raw sequences included denoising, dereplicating, chimera-filtering and merging using Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2016). A total of 28284 good-quality sequences of 260 bp were obtained from the two samples (average 14142 reads per sample), the sequences were gathered in a feature table, generating 24 features. Taxonomic analysis was based on consensus-BLAST classifier trained on 16S rRNA gene clustered at 99% sequence identity within the SILVA database (132 release).

3.3 RESULTS AND DISCUSSION

3.3.1 Interaction mineral-microorganisms

The production of arsenic sulfides was visually detected as yellow precipitates in cultures inoculated with the consortia after 6 days of incubation, the proportion of precipitates increased through the incubation period. Precipitates taken directly from the cultures (without washing or drying) were analyzed by SEM-EDS after 25 days of incubation (Figure 3.1) to observe the morphology of the cells and analyze their interaction with the biogenic minerals.

The biominerals obtained from both consortia consisted of a network of filamentous nanofibers with different diameters (lower than 250 nm) that were closely associated with the microorganisms. Apparently, the minerals were formed in an extracellular way and mineralized cells were observed (*i.e.* cells embedded in

mineral aggregates, whose surface was covered by filamentous mineral structures) (Le Pape et al., 2017; Stanley et al., 2018) (Figures 3.1E and F).

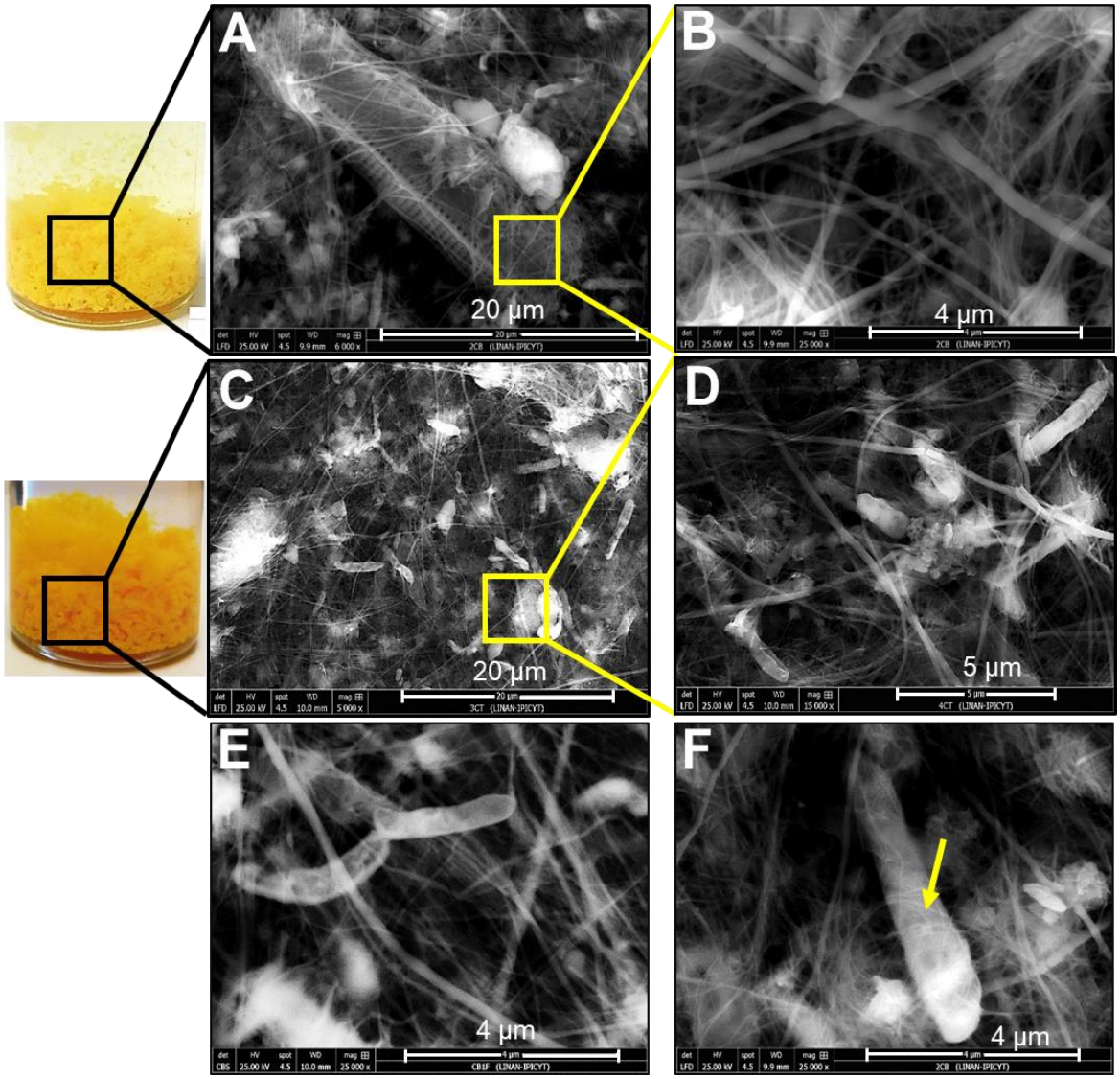


Figure 3.1. Macroscopic and microscopic appearance of biogenic minerals obtained after culturing the consortia during 25 days. A) and B) nanostructures of biomineral CB; C) and D) nanostructures of biomineral CT, and E) and F) Mineralized cells, yellow arrow points out the mineral nanofibers covering the cell surface.

The EDS of the filamentous nanostructures mainly revealed the presence of arsenic, sulfur, and calcium (Figure 3.2). Lee et al. (2007) observed similar filamentous structures in a precipitate formed by the dissimilative metal-reducing strain *Shewanella sp. HN-41* in presence of arsenate and thiosulfate as electron

acceptors. The authors concluded that the structures corresponded to As-S nanotubes (20-100 nm in diameter) that initially precipitated as amorphous orpiment (As_2S_3), and then were gradually transformed into realgar (AsS) and duranusite (As_4S) due to the increase of the As/S ratio over the incubation time (after 50 days).

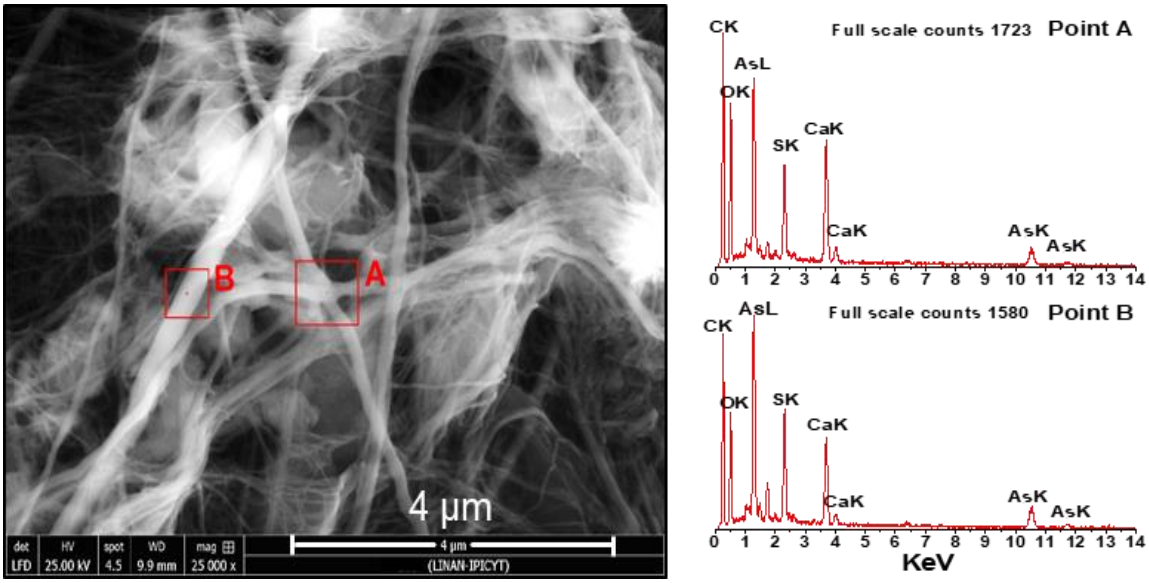
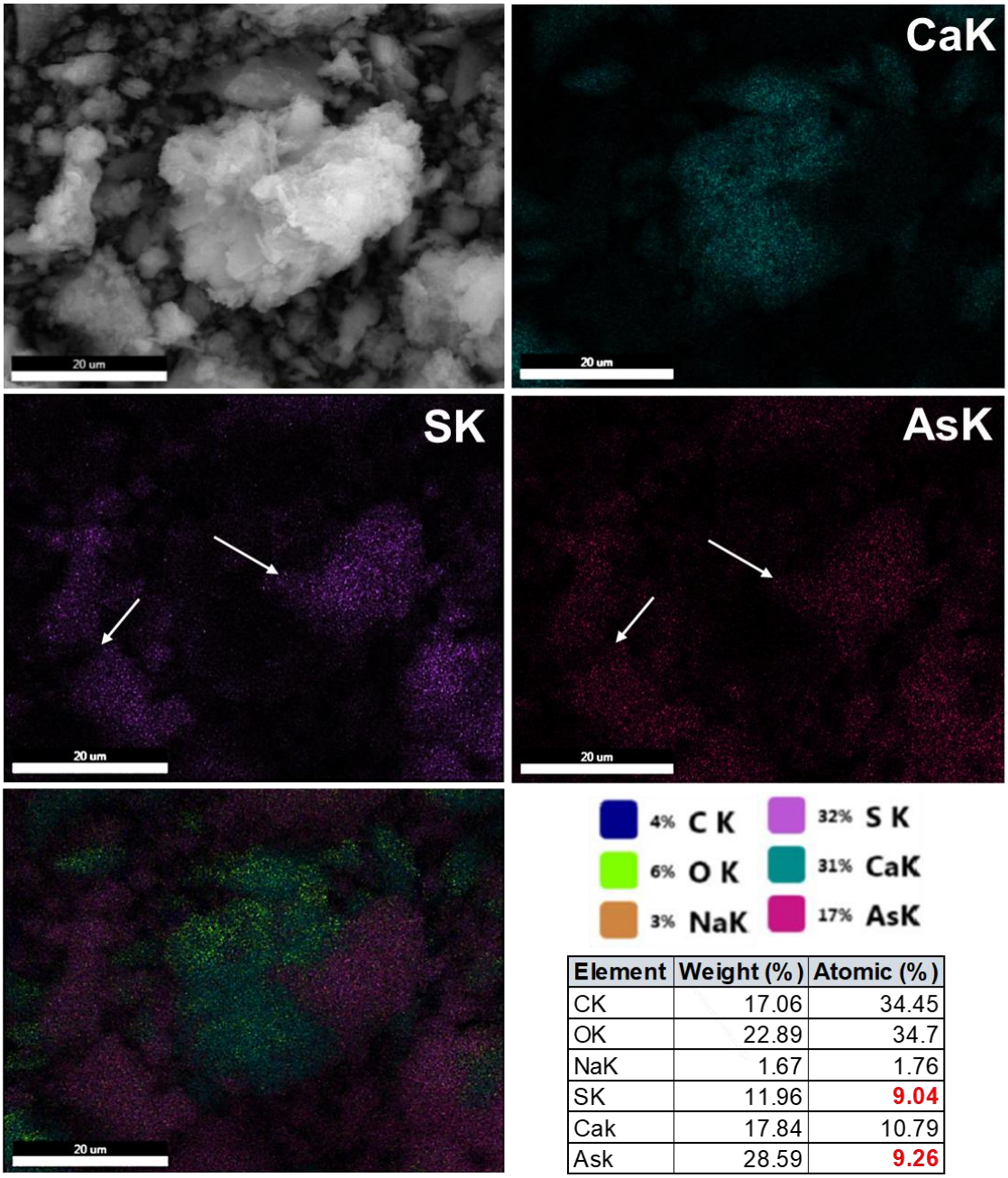


Figure 3.2. Elemental composition of biogenic mineral from consortium CB based on SEM-EDS analysis.

The precipitation of arsenic-sulfide nanofibers has been well described in pure cultures of *Shewanella* strains, this genus produces arsenic-sulfide nanomaterials by respiration routes of arsenate and thiosulfate (Jiang et al., 2009; Lee et al., 2007; McFarlane et al., 2015). There is only one report of the production of arsenic sulfide nanostructures by an indigenous SRB consortium under As(V)- and sulfate-reduction in acidic conditions (pH 4.5), this consortium was obtained after one month of sub-culturing of sediment of the Carnoulès acid mine drainage (Le Pape et al., 2017). However, the role of specific bacterial genera in the formation of nanostructures has not been discussed in deep. The formation of arsenic-sulfide nanotubes is expected only at low sulfate-reducing rates equivalent to a slow dosage of biogenic sulfide (µM order), this condition prevailed in the consortia CB (0.50 mmol of sulfate/L•d) and CT (0.17 mmol of sulfate/L•d).

An exploration of the biomineral CT through an elemental mapping (ESEM-EDS) confirmed the predominance of sulfur, arsenic, and calcium, the correspondence in the spatial distribution was evident between arsenic and sulfur (Figure 3.3). According to the EDS analysis, the relative As/S molar ratio was 1.02, which corresponds to the realgar stoichiometry (As/S=1).



Element	Weight (%)	Atomic (%)
CK	17.06	34.45
OK	22.89	34.7
NaK	1.67	1.76
SK	11.96	**9.04**
Cak	17.84	10.79
Ask	28.59	**9.26**

Figure 3.3. ESEM-EDS map showing spatial distribution of the most abundant elements in the biogenic mineral recuperated from a culture of CT consortium. The bars in all the images represent 20 µm. The arrows point out the same distribution of arsenic and sulfur.

Despite that calcium was abundant in the biomineral, the results showed that calcium had a different spatial distribution than arsenic and sulfide (Figure 3.3). It was reported that exopolymeric substances of sulfate-reducing bacterial communities have a strong calcium-binding capacity (Braissant et al., 2007), which could explain the presence of calcium in the biomineral. Possibly, calcium favored the nucleation of the biominerals by forming complexes with the exopolymeric substances that served as scaffolds for As(III)/S(-II) mineralization. A similar mechanism was described for other minerals such as carbonates and phosphates (Braissant et al., 2007; Gadd, 2010).

Biogenic minerals were also observed by optical microscopy, using DAPI staining. Figure 3.4 shows a filamentous network closely associated with the microorganisms of consortium CB and CT, because of DAPI staining binds to DNA, in blue fluorescence, it can appreciate the great abundance of microorganisms associated with the As(III)/S(-II) nanofibers.

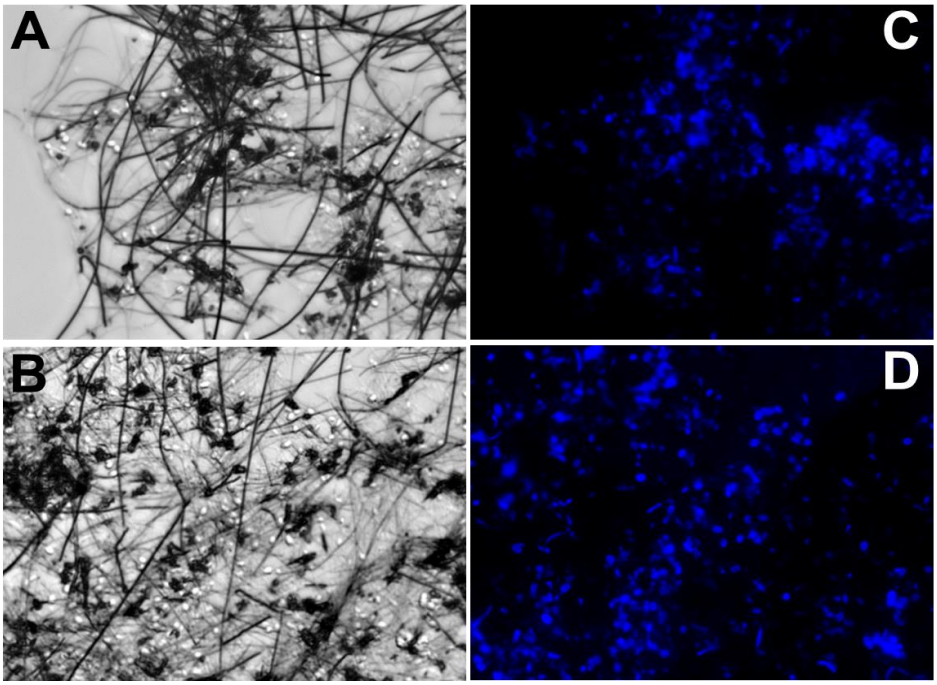


Figure 3.4. Mineral fibers (A, B) observed by differential interference contrast (DIC) microscopy and cells (C, D) stained with 4´,6´-diamidino-2-phenylindole (DAPI). DAPI binds to DNA, blue fluorescence shows the cells. Upper panels: biomineral from consortium CB and bottom panels: biomineral from consortium CT.

As previously discussed, the formation of nanostructures of arsenic sulfides depends on the right proportions of arsenite and sulfide, we propose that the arsenate-reduction first occurred since it is thermodynamically more favorable (+135 mV) than sulfate-reduction (Newman *et al*., 1997), thus enough As(III) was available in solution. Subsequently, the sulfate-reduction process started to dose biogenic sulfide promoting that arsenic-sulfides were oversaturated and their precipitation was feasible. However, during the process of mineralization some active cells were mineralized and inactivated, this fact strongly diminished the sulfate-reducing activity, this was supported by lactate and sulfate remnants after 24 days of incubation (Figure 2.3, Chapter 2), suggesting a partial inhibition of the sulfate-reducing activity and as consequence, the supply of biogenic sulfide was slow allowing the development of As(III)/S(-II) nanostructures.

3.3.2 Physicochemical features of the biogenic minerals

The consortia used to inoculate the microcosms was devoid of sediments, this was an advantage because the sediment composition could no longer interfere with the characterization of biogenic minerals as observed previously (Rios-Valenciana et al., 2017). Figure 3.5 shows the results, in the case of the biogenic mineral obtained from a culture of consortium CT, the XRD patterns displayed 17 well-defined diffraction peaks that correspond to realgar (AsS, ICDD card 09-0441). The best match with the biogenic mineral from consortium CB was consistent with the reference pattern of bonazziite (As_8S_8, ICDD card 00-4220) a realgar polymorph (*i.e.* with the same composition than realgar but different crystal structure).

Considering that arsenic-sulfide has a good Raman scattering proprieties the biogenic mineral phases were also analyzed by this technique (Figure 3.5B) (Vermeulen et al., 2018). Raman spectral analysis of the biomineral CT show six distinct band positions at 143,182, 193, 220, 342, and 353 cm^{-1}, these bands are expected for the mineral realgar and corroborate the XRD findings (Figure 3.5B) (Marucci et al., 2018). In the case of biomineral CB not was obtained a good Raman scattering signal, highlighting that this phase has amorphous nature (Vermeulen et al., 2018).

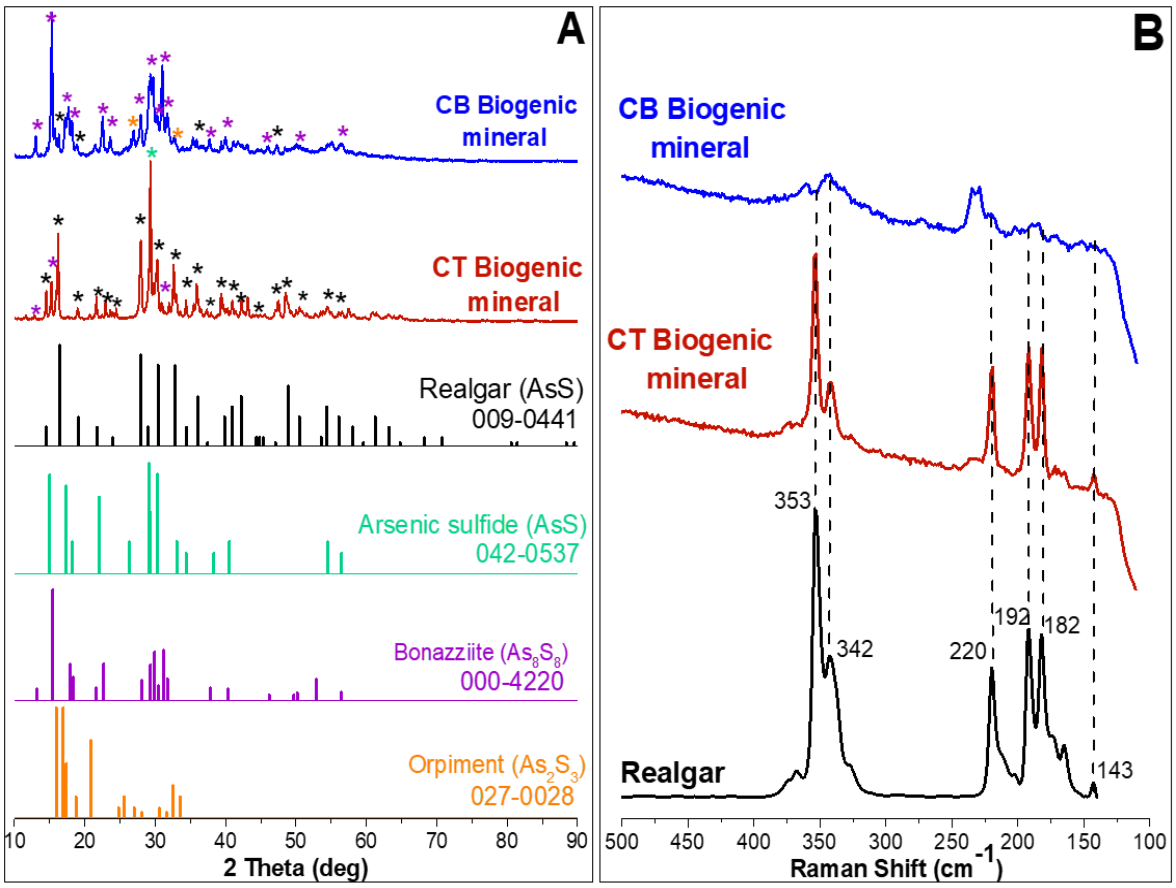


Figure 3.5. Characterization of As(III)/S(-II) biogenic minerals recovered of the bacterial consortia obtained from sediments CB and CT. A) XRD patterns; B) Raman spectra, with 633 nm of laser excitation. To perform the analysis, the biominerals were extracted after 40 days of incubation, washed with O_2 free water to remove salts, dry at vacuum, and pulverized.

Moreover, the EDS analysis confirmed that sulfur and arsenic were the dominant elements in both biominerals (Figure 3.6). Comparing the biogenic arsenic-sulfides recovered from each bacterial consortium, we observed that their mineralogy and crystallinity were different. The main mineral phase identified in the biomineral CB was bonazziite whereas in the biomineral CT was realgar. Unlike the biomineral from consortium CB, the biomineral CT showed defined peaks in the Raman spectra (Figures 3.5A and B). Consortium CB has higher sulfate-reduction rate than consortium CT (Figure 2.3, Chapter 2), impacting the development of different mineral sulfides since their formation is highly dependent on the sulfate-reduction rates (O'Day et al., 2004). Clearly, the metabolic capabilities of the

microbial communities in each consortium was a decisive factor in the formation of specific phases of biogenic arsenic-sulfides. The ability to precipitate arsenic sulfides is widespread among various phylogenetic groups of prokaryotes. According to previous works, some pure cultures were able to develop different realgar metastable polymorphs as duranusite (As_4S), bonazziite (As_8S_8), and alacranite (As_8S_9) (Falteisek et al., 2019; Lee et al., 2007; Mumford et al., 2013). For instance, *Bacterium MPA-C3 (Deferribacteres* phylum) precipitated alacranite during the reduction of S^0 and As(V) (Mumford et al., 2013).

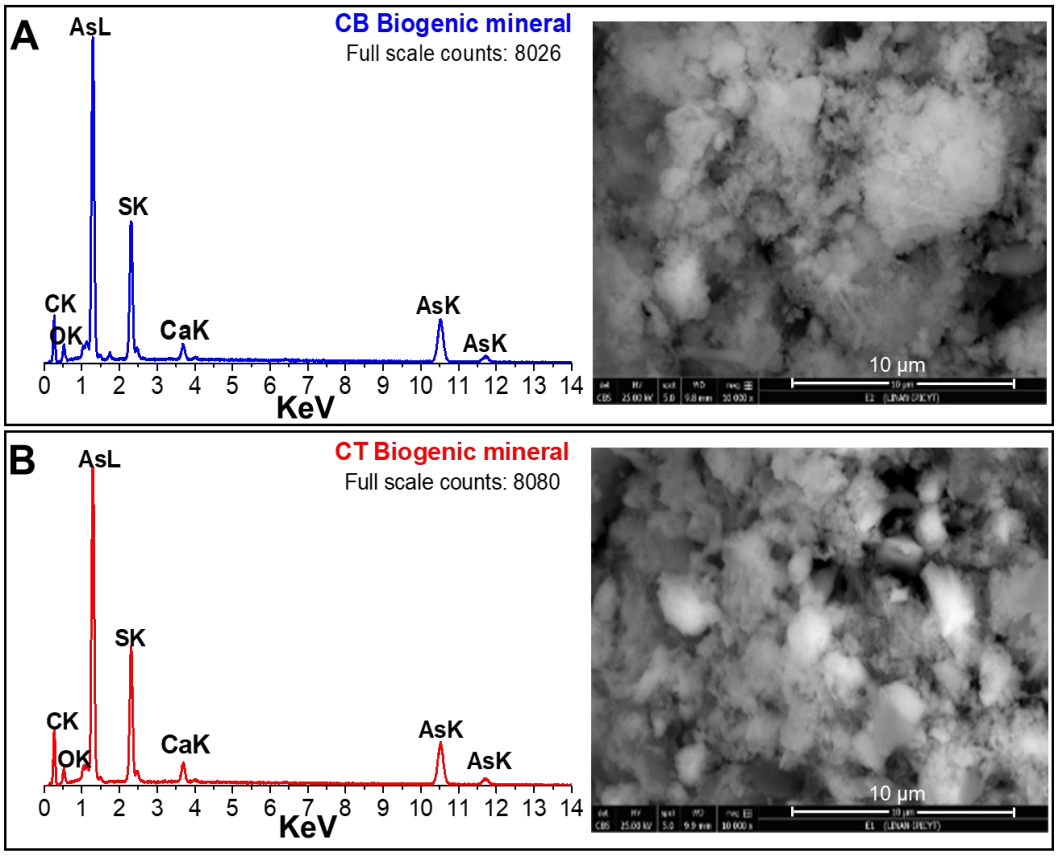


Figure 3.6. Analysis SEM-EDS of As(III)/S(-II) biogenic minerals recovered of the bacterial consortia: A) biomineral CB, B) biomineral CT. To perform the analysis, the biominerals were extracted after 40 days of incubation, washed with O_2 free water to remove salts, dry at vacuum, and pulverized.

Microbial groups such as sulfate-reducing bacteria can accelerate the transformation of amorphous sulfides to metastable phases (Lee *et al*., 2007; Picard *et al*., 2018); possibly in our consortia, initially amorphous orpiment

precipitated and crystalized gradually, later transformed into bonazziite an realgar due to the constant supply of biogenic sulfide. In previous studies using anaerobic consortia, the biogenic arsenic sulfides formed by dissimilatory arsenate reduction consisted of a mix of amorphous orpiment and realgar (Le Pape et al., 2017; Rodriguez-Freire et al., 2014). Le Pape et al., (2017) postulated that orpiment can be reduced into realgar by biogenic sulfide since the transformation of amorphous orpiment to crystalline realgar implies the reduction of As(III) to As(II) (Reaction 3.1). However, the increase of the S/As molar ratio may yield to the formation of thioarsenites (Reaction 3.2), due to the excess of biogenic sulfide (experiments performed in pH 4.5-5). Thioarsenites inhibit the precipitation of arsenic sulfides and even could dissolve the existing mineral phases such as amorphous orpiment (Altun et al., 2014).

$As_2^{(III)}S_3^{(-II)} + H_2S \rightarrow 2\ As^{(II)}S^{(-II)} + H_2S_2$ **(3.1)**

$As_2^{(III)}S_3^{(-II)} + 3\ H_2S \rightarrow 2\ As^{(III)}(SH)_3$ **(3.2)**

Other authors have proposed that microbial communities associated with realgar precipitation usually show low sulfate-reducing activity (Falteisek et al., 2019). In our case, the reducing equivalents from lactate (*ca.*10 mM) were enough to support As(V)-reduction and sulfate-reduction. All the As(V) (*ca.*10 mM) was reduced in less than 6 days but less than 50% of sulfate (5 mM) was consumed after 10 days of incubation (Figure 2.3, Chapter 2). Perhaps there was a certain degree of inhibition of sulfate-reducing activity by the precipitation of As(III)/S(-II). We also observed lactate remnants in microcosms assays despite the availability of sulfate (Rios-Valenciana et al., 2020, Chapter 4 of this thesis). It is possible that our system was self-regulated ensuring the precipitation of arsenic sulfides, the high concentrations of sulfate in the culture was not a limiting factor. A consortium capable of transforming amorphous orpiment into crystalline realgar is desirable because realgar contains up to 70% of arsenic (Kirk et al., 2010), it has a lower solubility than orpiment (K_{sp}= -11.9) and it is stable under reducing conditions, crystalline realgar is less sensitive to dissolution even it is stable under leaching conditions (Eary, 1992; Shakya and Ghosh, 2019).

3.3.3 Microbial communities associated to the biominerals

Through the taxonomic characterization based on the 16S rRNA gene sequencing, 100% of sequences corresponded to the bacteria domain. The community of the biomineral retrieved from Consortium CB was composed of the phylum *Firmicutes (*96%*)*, whereas the biomineral from Consortium CT was composed by *Firmicutes* (61%) and Proteobacteria (33%) (Figure 3.7A). In agreement with the conclusion in Chapter 2, the biogenic minerals are reservoirs of arsenate-respiring bacteria, regardless if the biomineral was obtained from sediment enrichments or bacterial consortia. The most representative genera (abundance > 2.5%) were *Desulfosporosinus*, *Clostridium sensu stricto*, *Pseudomonas*, *Exiguobacterium*, *Sedimentibacter*, *Fusibacter* and *Anaerobacillus* (Figure 3.7B)*,* all these genera include non-assimilative arsenate-reducing species (Cai et al., 2016; Serrano et al., 2017; Zhang et al., 2018). *Desulfosporosinus* was the dominant genus in biomineral CB reaching relative abundance of 34%, and biomineral CT was composed mainly by *Pseudomonas* (36%). In Chapter 2, it was discussed the primary role of *Desulfosporosinus* in the arsenic biocycle. Concerning *Pseudomonas*, this was not an abundant genus in the biominerals from sediment enrichments (less than 1.5% of abundance, Figure 2.5B of Chapter 2) but became more abundant in the biomineral from CT consortia. *Pseudomonas* is a prominent member (reaching relative abundances of up to *ca.*15%) of the microbial communities of arsenic-contaminated groundwater (Ghosh and Sar, 2013; Zhang et al., 2018).

In the biomineral CB, 93% of the bacterial genera are recognized by comprise arsenic-respirers, spore-forming and gram (+) species; meanwhile in the biomineral CT, only 56% of the genera have these features and 36% correspond to *Pseudomonas.* Some *Pseudomonas* species contain the *arsC* and *aio*A genes that confer arsenic resistance; this genus has also been related to arsenic respiration by energy-conserving pathways (*arrA* gene). The simultaneous presence of these genes can give to *Pseudomonas* extreme resistance to arsenic, and therefore the advantage to survive and proliferate under high arsenic stress (Cai et al., 2009; Freikowski et al., 2010). Interestingly, *Pseudomonas* is a non-spore forming genus.

We suggest that, during mineral precipitation, the viable cells could be inactivated and mineralized (see Figure 3.1); as a consequence, spore-forming bacteria preferably were preserved through successive transfers of the consortia (Figure 3.7). From all the retrieved genera, only *Pseudomonas* is Gram (−). Some authors have discussed that Gram (+) bacteria are more effective in forming biominerals than Gram (−) bacteria because peptidoglycan layers create a consistent anionic surface charge that improves metal binding (Picard et al., 2016; Stanley et al., 2018).

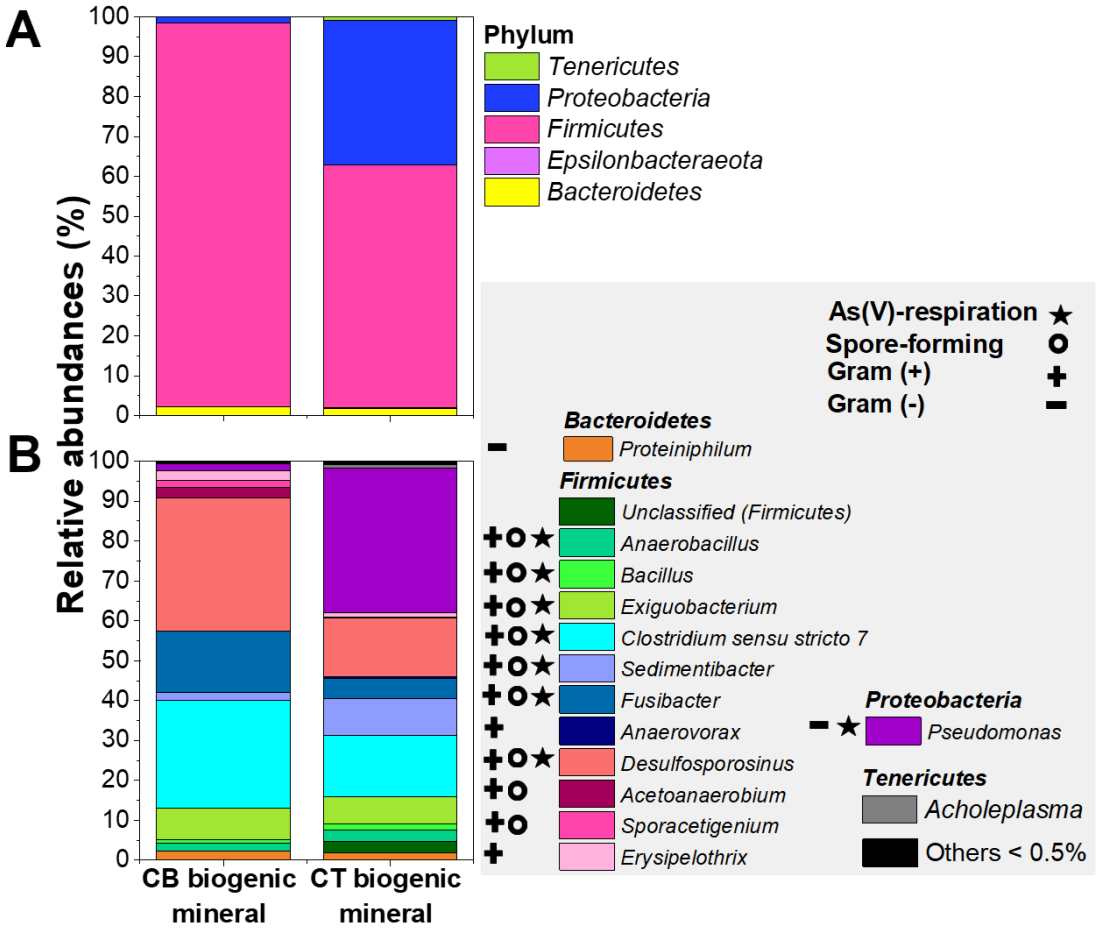


Figure 3.7. Microbial communities linked with biogenic minerals recovered from cultures of the bacterial consortia CB and CT. A) Phylum level, B) Genus level.

The microbial communities associated with realgar formation usually show low sulfate-reducing activity and fermentative metabolism (Falteisek et al., 2019). Fermentative bacteria increase the reducing conditions promoting the precipitation

of sulfide minerals in sulfide deficient (µM order) solutions. A suite of fermentative bacteria such as *Clostridium sensu stricto*, *Sedimentibacter* and *Exiguobacterium* predominated in the biominerals. Realgar formation has not been reported before in communities dominated by sulfate-reducing bacteria, in this study *Desulfosporosinus* (*Firmicutes)* was the only sulfate-reducer associated with the microbiota from the consortia, whose abundance in biomineral CT (14%) was lower than in biomineral CB (34%). In agreement, biomineral CT was identified as crystalline realgar, the lower crystallinity of bonazziite in biomineral CB could be related with the high abundance of *Desulfosporosinus* and consequently a greater sulfate-reducing activity.

3.4 CONCLUDING REMARKS

The bacterial consortia (CB and CT) were able to precipitate extracellular As(III)/S(-II) filamentous nanofibers via arsenate- and sulfate-reduction. The biomineral CB was identified as bonazziite, and the biomineral CT was identified as realgar, which was highly crystalline. The consortia reported here could be applied in arsenic stabilization and remediation.

Moreover, the biosynthesis of arsenic sulfides using the sulfate/arsenate-reducing consortia could be exploited for the production of nanomaterials with potential application in sensors and photoactive devices. Taking into account that for their biosynthesis, the crystalline nanofibers of realgar only required a short period (less than 40 days), simple operation conditions (mesophilic temperature, neutral pH) and a resilient bacterial consortium.

The most noticeable difference in the communities was the relative abundance of *Desulfosporosinus* (in biomineral CB) or *Pseudomonas* (in biomineral CT). Interestingly, the composition of the bacterial community strongly impacts the precipitation and mineralogy of specific phases of arsenic-sulfides.

For both consortia, the rates of arsenate-reduction were higher than the rates of sulfate-reduction but this was not the only condition to obtain biominerals with high crystallinity. It was noticed that to obtain biominerals with high crystallinity

as realgar (biomineral CT) the sulfate reducing rate was about 5 times lower than the arsenate-reducing rate. This research demonstrates that indigenous bacterial consortia from arsenic-polluted sediments are efficient in the precipitation As(III)/S(-II) filamentous nanofibers, using arsenate and sulfate as electron acceptors.

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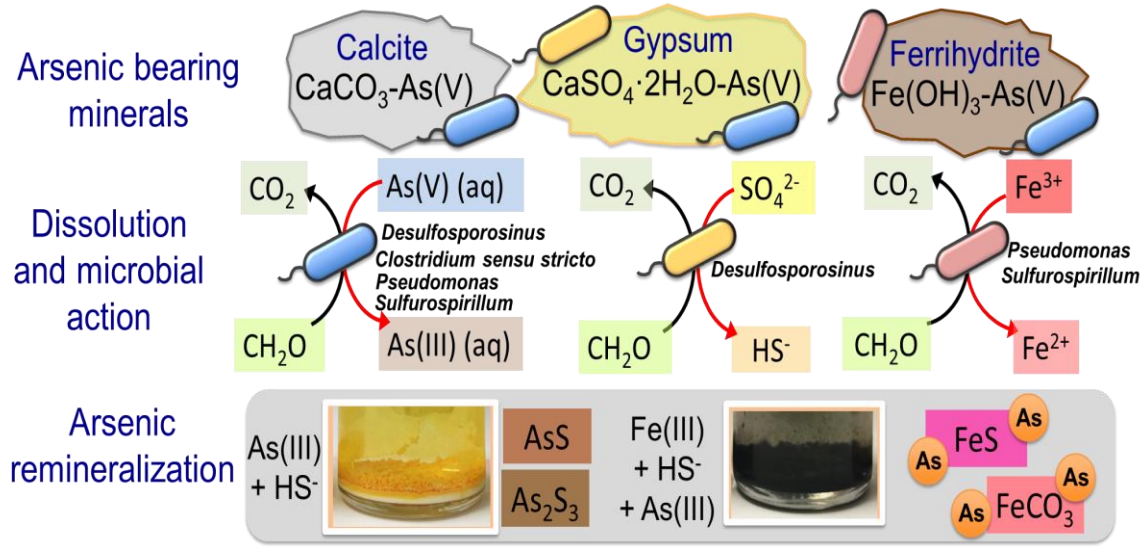
CHAPTER 4

Dissolution and final fate of arsenic associated with gypsum, calcite, and ferrihydrite: influence of microbial reduction of As(V), sulfate, and Fe(III)

Highlights

- Microbial reduction caused dissolution of As bound to calcite, gypsum or ferrihydrite.
- Arsenate reduction resulted in release of As(III) from calcite-As(V).
- Reducing bioprocesses with gypsum-As(V) led to As(III) remineralization
- Mineral composition and sulfate availability led to microbial community shifts.

Graphical Abstract



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CHAPTER 4

ABSTRACT

Several studies have demonstrated that gypsum ($CaSO_4 \cdot 2H_2O$) and calcite ($CaCO_3$) can be important hosts of arsenic in contaminated hydrogeological systems. However, the extent to which microbial reducing processes contribute to the dissolution and transformation of carbonate and sulfate minerals and, thereby, to arsenic mobilization is poorly understood. These processes are likely to have a strong impact on arsenic mobility in iron-poor environments and in reducing aquifers where iron oxyhydroxides become unstable. Anoxic batch bioassays with arsenate (As(V)) coprecipitated with calcite, gypsum, or ferrihydrite ($Fe(OH)_3$) were conducted in the presence of sulfate or molybdate to examine the impact of bioprocesses (*i.e.* As(V), sulfate, and Fe(III)-reduction) on arsenic dissolution, speciation, and eventual remineralization. Microbial reduction of As(V)-bearing calcite caused an important dissolution of arsenite, As(III), which remained in solution up to the end of the experiment (30 days). The reduction of As(V) from gypsum-As(V) also led to the release of As(III), which was subsequently remineralized, possibly as arsenic sulfides. The presence of sulfate triggered arsenic dissolution in the bioassays with ferrihydrite-As(V). This study showed that although gypsum and calcite have a lower capacity to bind arsenic, compared to iron oxides, they can play a critical role in the biogeochemical cycle of arsenic in natural calcareous and gypsiferous systems depleted of iron since they can be a source of electron acceptors for reducing bioprocesses.

Keywords: Arsenate, Biogeochemistry, Bioprocesses, Mobilization, Remineralization, Sulfide

CHAPTER 4

4.1 INTRODUCTION

The release of arsenic from sediments into groundwater represents a major hazard to public health since this metalloid is a priority pollutant due to its ecotoxicological nature (Moon et al., 2017; O'Day et al., 2004). Depending on the geological composition of hydraulic systems, arsenic is associated with different minerals. The association of arsenic with iron and sulfur minerals plays a leading role in the distribution of arsenic in polluted sedimentary environments (Zhang et al., 2018), and the occurrence of arsenic in the water column is mainly related to the dissolution of arsenic-bearing minerals.

On a global scale, most of the aquifers with severe arsenic contamination that have been studied are iron-rich, and the mobilization of arsenic in these systems has been attributed to microbial reductive dissolution of iron oxides (Deng et al., 2018). The problem is maximized because Fe(III)-(hydr)oxides, such as ferrihydrite, have a high capacity to adsorb arsenic, but are susceptible to microbial attack, because they are the source of electron acceptor for Fe(III)-reduction (Meng et al., 2016; Muehe et al., 2013; Zobrist et al., 2000). Nevertheless, due to the biogeochemical heterogeneity (different geological, mineralogical and microbial composition) of the sites affected by arsenic contamination, it is important to consider other scenarios that can potentially impact the fate and bioavailability of arsenic.

Minerals such as gypsum and calcite have recently attracted the interest of research due to their affinity to incorporate arsenic, either through adsorption or co-precipitation, in addition to their abundance in the Earth's crust, specifically in hydrogeological systems with high arsenic concentrations (Winkel et al., 2013; Bardelli et al., 2011; Smedley and Kinniburgh, 2002). In sedimentary environments, where iron oxides are not sufficiently abundant to act as major scavengers of arsenic, calcite and gypsum can play an important role in the fate of arsenic. However, the role of these minerals on the mobilization of arsenic in hydrogeological systems has received little attention (Meng et al., 2016). It has been estimated that sorbed arsenic loadings in gypsum and calcite are much smaller than those determined in iron oxides, but gypsiferous and calcareous sites

contaminated with arsenic have not been studied in deep (Smedley and Kinniburgh, 2002). Recently, other authors reported that the role of calcium carbonates in controlling arsenic distribution could have been underestimated because they are important components in semi-arid subsurface systems that can immobilize high levels of arsenic. For instance, up to 913 mg of As/kg were quantified in carbonate deposits (Meng et al., 2016; Renard et al., 2015; Winkel et al., 2013).

Studies that involve microbial processes and calcite have mainly focused on the precipitation of calcium carbonates by bacteria, which is very relevant because it could contribute to arsenic immobilization in the environment (Winkel et al., 2013). In addition, the mechanisms for arsenate (As(V)) and arsenite (As(III)) uptake in biogenic calcite have been studied (Catelani *et al*., 2018). Overall, the mechanisms of arsenic uptake in calcite have demonstrated that at circumneutral pH the As(V) interacts more strongly than As(III) with the calcite surface, since As(V) forms inner-sphere surface complexes (Alexandratos et al., 2007; Catelani *et al*., 2018). Gypsum and other sulfate minerals are potential electron acceptors for sulfate-reducing bacteria, which can be harnessed to remediate industrial wastes containing toxic elements (*i.e*. phosphogypsum, mining wastes) (Karnachuk et al., 2002). However, the role of microbial reduction on the solubilization and remineralization of arsenic coprecipitated with calcite and gypsum has not been investigated to date.

Native microbiota enriched from arsenic-rich environments is characterized by high metabolic versatility and it is usually able to perform the dissimilatory reduction of As(V), sulfate, and Fe(III) simultaneously, even when these electron acceptors are bound to minerals (Lu et al., 2017; Suhadolnik et al., 2017). Therefore, it is essential to understand the relationship between bioprocesses and geochemical factors in calcareous and gypsiferous contaminated sites. To the best of our knowledge, this is the first report that investigates systematically the role of microbial reducing process in arsenic mobilization and transformation in systems with gypsum and calcite.

The aims of this work were: 1) to investigate the role of a bacterial consortium, obtained from As-polluted sediments, on the release of arsenic from arsenic-bearing minerals (*i.e.* gypsum, calcite, or ferrihydrite); 2) to establish whether the solubilized arsenic is remineralized under reducing conditions or it remains in solution and 3) to explore how the microbial structure of the consortium changes depending on the availability of different minerals coprecipitated with arsenic (calcite, gypsum, or ferrihydrite) that could be used as electron acceptors.

4.2 MATERIALS AND METHODS

4.2.1 Synthesis of calcite, gypsum, and ferrihydrite coprecipitated with As(V)

Calcite ($CaCO_3$), gypsum ($CaSO_4 \cdot 2H_2O$), and ferrihydrite ($Fe(OH)_3$) were synthesized in presence of As(V). Briefly, calcite with As(V) was prepared by mixing 250 mL of $CaCl_2 \cdot 2H_2O$ (0.5 M) and 250 mL of $NaHCO_3$ (0.5 M); the sodium bicarbonate solution (pH 7.5) contained 0.05 M of $Na_2HAsO_4 \bullet 7H_2O$ (Román-Ross et al., 2006; Yokoyama et al., 2012). Gypsum with As(V) was obtained by mixing 168 mL of $CaCl_2 \cdot 2H_2O$ (0.5 M) with 64 mL $Na_2HAsO_4 \cdot 7H_2O$ (0.16 M). The pH was adjusted to 7.2 by adding NaOH (1 M), and subsequently 168 mL of Na_2SO_4 (0.5 M) were added (Zhang et al., 2015). Ferrihydrite with As(V) was prepared by simultaneous addition of 100 mL of $FeCl_3 \cdot 6\ H_2O$ (0.5 M) and 100 mL of a stock solution of $Na_2HAsO_4 \cdot 7H_2O$ (0.1 M) in 200 mL of deionized water at a constant flow rate (2.5 mL/min); the pH was adjusted to 7.5-7.8 with NaOH (1 M) by titration (Zobrist et al., 2000). The arsenate concentration in the final mixtures was the same for the synthesis of each mineral (0.025 M). All the precipitates were recovered by centrifugation (4000 g, 15 min), washed with deionized water, and finally dried at room temperature (25 °C ± 2). Calcite, gypsum, and ferrihydrite were selected as solid electron acceptors due to their high affinity for arsenic (Yokoyama et al., 2012; Zhang et al., 2015).

4.2.2 Inoculum and batch microcosms assays

The inoculum was the consortium free of sediment obtained through eight successive transfers from primary enriched cultures of the sediment CT. Each transfer consisted in inoculating 10 mL from the primary enrichment or the previous transfer to 90 mL of sterile basal medium (see below) amended with lactate, arsenate, and sulfate (10 mM each); the process was repeated after 30 days of incubation. We pursued to work with a consortium free of sediment to avoid the interference of the sediment mineralogy in the analysis of the solid fractions.

Lactate (20 mM) was added as electron donor to the microcosms with the arsenic-bearing minerals. The arsenic-bearing minerals (solid electron acceptors), were added at the following concentrations: gypsum-As(V) or calcite-As(V) 20 g/L and ferrihydrite-As(V) 10 g/L. Each assay was inoculated with 10 mL of the consortium culture (0.007 g of volatile suspended solids).

All microcosms assays were performed in serum bottles (125 mL) with100 mL of sterile basal medium (1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NH_4Cl, 0.08 mM KH_2PO_4, 0.1 mL/L of Wolfe's mineral solution) (Burton et al., 2013). The pH was adjusted to 7.0 ±2 with $NaHCO_3$ (ca. 22 mM). The headspace of the serum bottles (~10 mL) was purged with N_2/CO_2 (80:20, v/v). Assays were incubated (30 ºC) in the dark without agitation.

Three different biotic assays were carried out by triplicate with each one of the solid electron acceptors, calcite-As(V), gypsum-As(V), or ferrihydrite-As(V), as follows: A1, amended with lactate; A2, with lactate and 15 mM of sulfate; A3, with lactate and 25 mM Na_2MoO_4 as inhibitor of sulfate-reduction. Corresponding abiotic controls (without inoculum) were set up (C1, C2, and C3).

4.2.3 Sample collection and chemical analysis

Microcosms were sampled periodically, every 2 or 4 days during 30 days, to follow the concentrations of lactate, acetate, propionate, sulfate and sulfide, total aqueous arsenic and arsenic species (III and V), aqueous Fe(II) and total Fe(II). Species of As(III) and As(V) were determined by anion-exchange chromatography (Ficklin,

1983). Total arsenic was analyzed by atomic absorption spectroscopy (Varian Spectra Mod. AA240FS, Santa Clara, CA, USA). Sulfate, lactate, acetate, and propionate were analyzed by capillary electrophoresis (Soga and Ross, 1999). Dissolved sulfide was determined using the Cord-Ruwisch method (Cord-Ruwisch, 1985). The reduction of Fe(III) was followed by the concentration of total Fe(II), determined as HCl-extracted Fe(II), which considers ferrous iron incorporated into amorphous precipitates using the ferrozine assay (Stookey, 1970). The pH, redox potential (Eh), and volatile suspended solids concentration were determined according to standard methods (Eaton et al., 1998).

4.2.4 Recovery of the solid fraction and characterization

At the end of the experiments (30 days), the solid fraction from the biotic and abiotic assays was recovered. The bottles were opened inside an anaerobic chamber and the solid phase was collected with a Pasteur pipette; the pellet was recovered by centrifugation (14,000 g, 10 min), decanted and vacuum dried. Composed samples from the solid fraction of the replicas of each assay were characterized by X-ray diffraction (Diffractometer SmartLab, Rigaku®). In addition, the solid fraction was subjected to acid digestion in a microwave equipment using a mixture of HNO_3:HCl (3:2 v/v), a pressure ramp 0-300 psi during 10 minutes and temperature set point to 150 ^oC. Further elemental analysis was achieved by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Varian 730-ES, Palo Alto, CA, USA) after 20-fold dilution of the digested sample with deionized water.

4.2.5 DNA extraction and 16S rRNA bacterial next generation sequencing

Bacterial DNA was extracted from the inoculum (day 0) and from a composite sample of the three replicates at the end (30 days) of each biotic assay (A1, A2, or A3), using the commercial kit ZR Soil Microbe MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The composition of the bacterial community was determined targeting the V4 hypervariable region of the

prokaryotic 16S rRNA gene with the primers 515 F (5´GTGCCAGCMGCCGCGGTAA3´) and 806 R (5´GGACTACHVGGGTWTCTAAT3´) (Walters et al., 2015). Pair-end sequencing method (Illumina MiSeq) was performed by an external laboratory (RTL Genomics Molecular Sequencing Laboratory, Lubbock, TX, USA).

4.2.6 16S rRNA gene analysis and taxonomic assignment

The raw next generation sequencing reads were analyzed using QIIME2 software 2018.8 release, sequences were dereplicated and chimera-filtered using DADA2 (Divisive Amplicon Denoising Algorithm) (Callahan et al., 2016). A total of 227545 good-quality sequences of 260 bp were obtained from 10 samples (average 22000 reads per sample), the sequences were gathered in a feature table, 45 features were generated. Taxonomic analysis was based on consensus-BLAST classifier trained on 16S rRNA gene clustered at 99% sequence identity within the SILVA 132 database release. Sequence data were deposited under BioProject ID: PRJNA528962 and accession number: SRP189375 (NCBI).

4.3. RESULTS

4.3.1 Arsenic reduction and mobilization from solid electron acceptors

The time profiles of arsenic concentration showed that at day 0, in the experiments with calcite-As(V) and gypsum-As(V), around 0.5 mM of As(V) was dissolved into the aqueous phase, including the abiotic controls (Figure 4.1). In the abiotic controls, the concentration of dissolved As(V) increased with time but in all the biotic assays, with calcite or gypsum, the aqueous As(V) decreased due to microbial reduction to As(III) (Figure 4.1). In the biotic assays with gypsum-As(V), arsenic was solubilized because both As(V) and sulfate were used as electron acceptors (Figure 4.1). On the contrary, there was significant less dissolution of

arsenic (about 10 fold) in the experiments with ferrihydrite, even in the bioassays where As(V) was also reduced to As(III) (Figure 4.1).

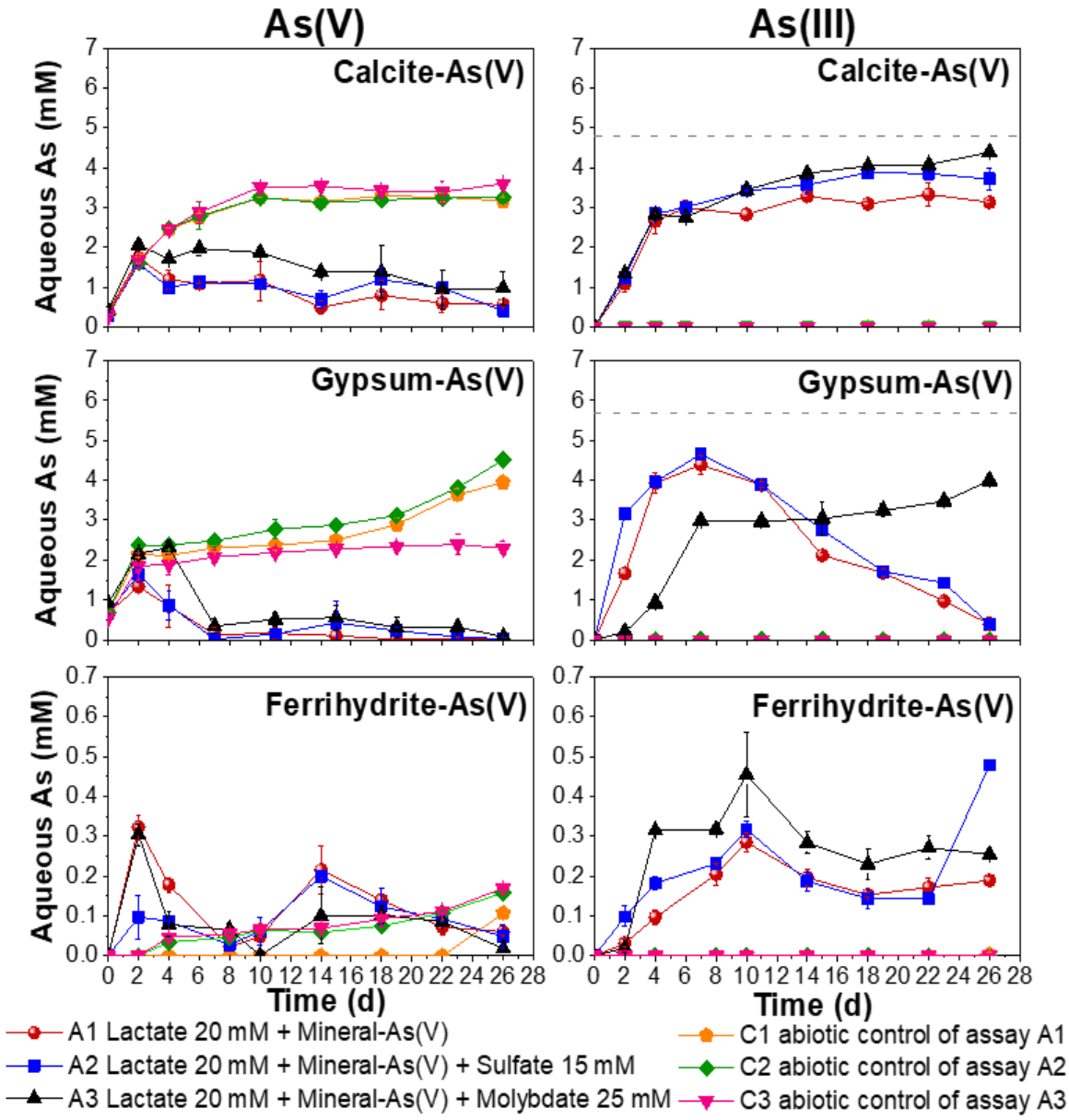

Figure 4.1. Time profiles of the concentration of arsenate, As(V), and arsenite, As(III), in batch experiments with As(V)-bearing minerals: calcite-As(V); gypsum-As(V); and ferrihydrite-As(V). The dotted lines in D) and E) indicate the theoretical concentration of As(III) that would be formed from the reduction of all the As(V) present in the mineral.

In the experiments with calcite (Figure 4.1), about 96% of As(III) remained in solution irrespective of the presence of sulfate (assay A2) or molybdate (assay A3). Conversely, in the assays with gypsum-As(V), the concentration of As(III) in the aqueous phase decreased due to the formation of yellowish precipitates with the sulfide produced by sulfate-reduction (Figure 4.1). Around 99% of the dissolved As(III) was remineralized from the aqueous phase in bioassays A1 and A2 at day 30, where As(III) was almost completely removed from the liquid phase when sulfate reduction occurred without restrictions (A1 and A2, Figure 4.1). However, when sulfate-reduction was inhibited with molybdate (A3 assay), the concentration of As(III) continued to increase reaching 4 mM at day 26.

With ferrihydrite-As(V), the maximum concentrations of As(III) in solution were found at day 10, being *ca.* 0.45 mM when molybdate was present (A3, Figure 4.1). Afterward, the concentration of As(III) in solution declined. Close to the end of the experiment (day 26), As(III) increased in the assay with sulfate (A2) reaching concentrations of 0.6 mM, most probably as a result of sulfate-reduction, that produced sulfide able to stimulate the quick transformation of ferrihydrite and consequently the dissolution of arsenic.

In addition to As(V)-reduction, we also monitored the reduction of the other potential electron acceptors, such as sulfate in gypsum assays and Fe(III) in ferrihydrite assays, or the supplemented sulfate in assays A2 (Figure 4.2). Without exception, in all the biotic assays with sulfate, the concentration of aqueous sulfide was less than 0.8 mM (Figure 4.2). Microbial Fe(III)-reduction occurred with ferrihydrite, as shown by the constant increase of total Fe(II), reaching concentrations between 3.3 and 4.5 mM (Figure 4.2).

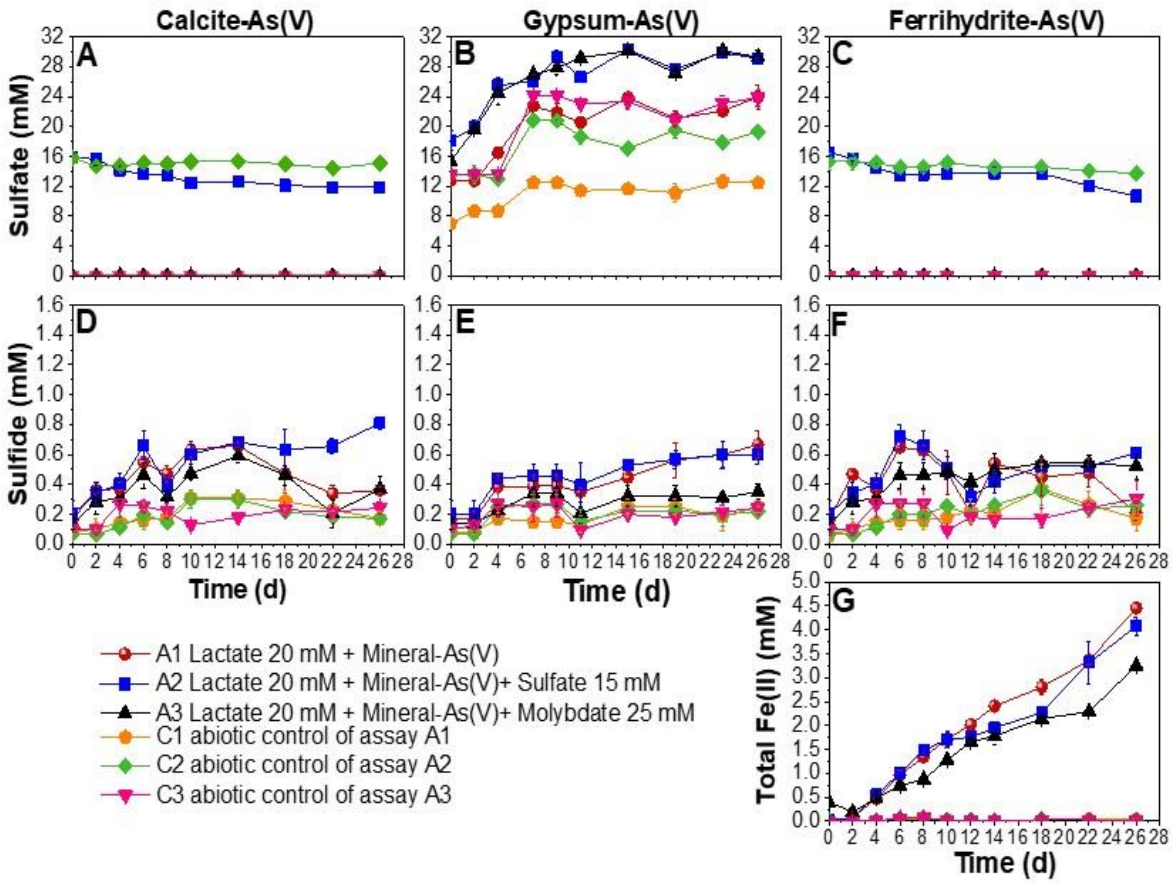

Figure 4.2. Kinetics of sulfate and iron reducing activities in batch systems with arsenic bearing minerals. Sulfate reduction in assays with calcite-As(V) (A), gypsum-As(V) (B) and ferrihydrite-As(V) (C); sulfide production in assays with Calcite-As(V) (D), gypsum-As(V) (E) and ferrihydrite-As(V) (F); total Fe(II) (HCl-extracted Fe(II)) in assays with ferrihydrite-As(V) (G).

The rates of As(V)-, Fe(III)- and sulfate-reduction in the batch biossays were calculated to further investigate the magnitude of the bioprocesses with each mineral, the magnitude of the different respiration processes changed depending on the mineral (Table 4.1). Overall, As(V)-reduction was the most favorable process in the assays with calcite and gypsum (0.66-0.99 mmol/L·d), whereas it was the less favored process in assay with ferrihydrite (0.03-0.08 mmol/L·d). In the latter case, however, the rates of As(V) and Fe(III)-reduction could have been underestimated due to the immediate precipitation of iron and arsenic species.

Table 4.1 Rates of reduction of As(V), sulfate and Fe(III) in the batch bioassays performed with synthetic calcite, gypsum, and ferrihydrite co-precipitated with arsenate.

Reduction rate (mmol·L/d)	Lactate + mineral-As(V) A1	Lactate + mineral-As(V) + sulfate A2	Lactate + mineral-As(V) + molybdate A3
As(V)-reduction			
Calcite-As(V)	0.66 ± 0.08	0.72 ± 0.05	0.71 ± 0.02
Gypsum-As(V)	0.98 ± 0.06	0.99 ± 0.05	0.43 ± 0.03
Ferrihydrite-As(V)	0.03 ± 0.003	0.05 ± 0.003	0.08 ± 0.001
Sulfate-reduction			
Calcite-As(V)	NA	0.40 ±0.07	NA
Gypsum-As(V)	-	-	-
Ferrihydrite-As(V)	NA	0.52 ± 0.15	NA
Fe(III)-reduction			
Ferrihydrite-As(V)	0.17 ± 0.01	0.19 ± 0.02	0.10 ± 0.01

NA, not applicable; **-**, Determination was not possible

4.3.2 Fate of the electron donor (lactate)

Lactate was consumed in all the bioassays yielding acetate and propionate as byproducts (Figure 4.3). In addition to the incomplete oxidation of lactate to acetate, *via* respiration of As(V), sulfate, or Fe(III) (Table 1.1 Chapter 1), the presence of propionate suggests that lactate was also fermented as shown in the Reaction 4.1 (Kwon et al., 2014).

$$3\ C_3H_5O_3^- \rightarrow 2\ C_3H_5O_2^- + C_2H_3O_2^- + HCO_3^- + H^+ \quad \textbf{(4.1)}$$

The experiments with gypsum-As(V) showed that around 70% of lactate was consumed in assays A1 and A2, whereas only 35% of lactate was consumed when sulfate-reduction was inhibited (A3). In these experiments, lactate was oxidized incompletely to acetate coupled with the reduction of the two electron acceptors present in the mineral, As(V) and sulfate, and it was also fermented to propionate reaching 6 mM in assays A1 and A2 at day 15 (Figure 4.3H). Afterward, propionate was consumed by sulfate-reduction contributing to the accumulation of acetate (around 10 mM, Figure 4.3E). In assay A3, acetate concentration was

approximately 3 times lower comparing with A1 and A2, due to the inhibition of sulfate-reduction.

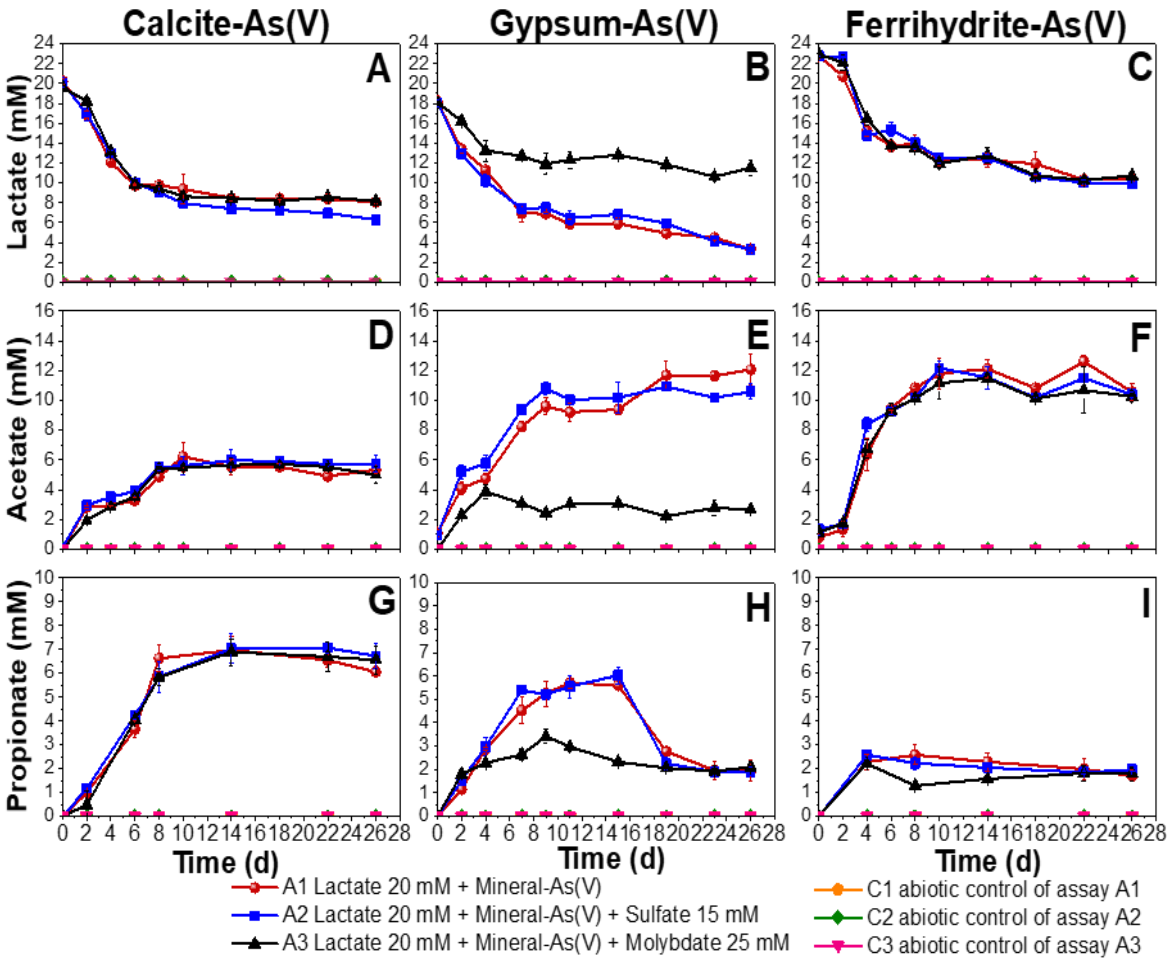

Figure. 4.3. Lactate, acetate, and propionate time profiles in batch bioassays: A), D) and G) with calcite-As(V); B), E) and H) with gypsum-As(V) and C), F) and I) with ferrihydrite-As(V).

Concerning the experiments with ferrihydrite-As(V), lactate consumption was very similar (53 - 56%) because Fe(III) was always available as electron acceptor. Acetate reached almost the stoichiometric concentration (11 mM) corresponding to the incomplete oxidation of lactate (12.2-12.8 mM) to acetate with Fe(III) or As(V) as electron acceptors (Table 1.1, Chapter 1), this result pointed out that iron- and arsenic-reduction were preferred over sulfate-reduction, even when sulfate was available (assay A2).

The accumulated propionate with ferrihydrite-As(V) was very low (1.6-1.8 mM) (Figure 4.3I), suggesting that fermentation was limited, in contrast to the assays with calcite/gypsum-As(V) (Figures. 4.3G and H).

4.3.3 Biotransformation of the solid fraction

To gain additional information about the role of microbial activity on arsenic mobilization, the composition of the solid fraction was determined at the beginning and at the end of the experiments.

The initial concentration of arsenic in calcite was 20 ± 0.52 mg of As(V)/g or 2 wt.% (Figure 4.4A). The arsenic associated to the solid and liquid fractions at the end of the bioassays, showed that 73−87% of the total arsenic was solubilized, compared to only 50−56% in the abiotic controls (Figures 4.5A and 4.5B). In the case of gypsum, the synthesized mineral incorporated 28.4 ± 0.81 mg As(V)/g or 2.8 wt.% (Figure 4.4B). After 30 days of incubation, more than 98% of arsenic was present in the solid phase of bioassays A1 (with gypsum only) and A2 (with gypsum and sulfate), which is in agreement with the low dissolved arsenic levels observed in these assays (0.3 mM) at day 26 (Figure 4.1). When sulfate-reduction was inhibited with molybdate (assay A3) only 50% of arsenic was in the solid phase (Figures 4.4B and 4.5C).

The synthesized ferrihydrite contained 79.2 ± 0.49 mg As(V)/g corresponding to 8 wt.% (Figure 4.4C). Less than 1% of the arsenic was dissolved in the abiotic controls (Figure 4.5F) and the liquid phase of the biotic assays, A1 and A3 (Figure 4.5E), contained less than 5% of the total arsenic after As(V)- and Fe(III)-reduction took place. Conversely, in the assay with sulfate (A2), arsenic in solution was 31%; iron dissolution (11-19%) was also detected due to Fe(III)-reduction to soluble Fe(II) (Figure 4.4C).

Yellowish precipitates resembling arsenic sulfides were observed in assays A1 and A2 with gypsum and in A2 with calcite (in a lesser extent). Meanwhile, black precipitates were observed with ferrihydrite, possibly a mixture of iron carbonates and iron sulfides (Figure 4.4).

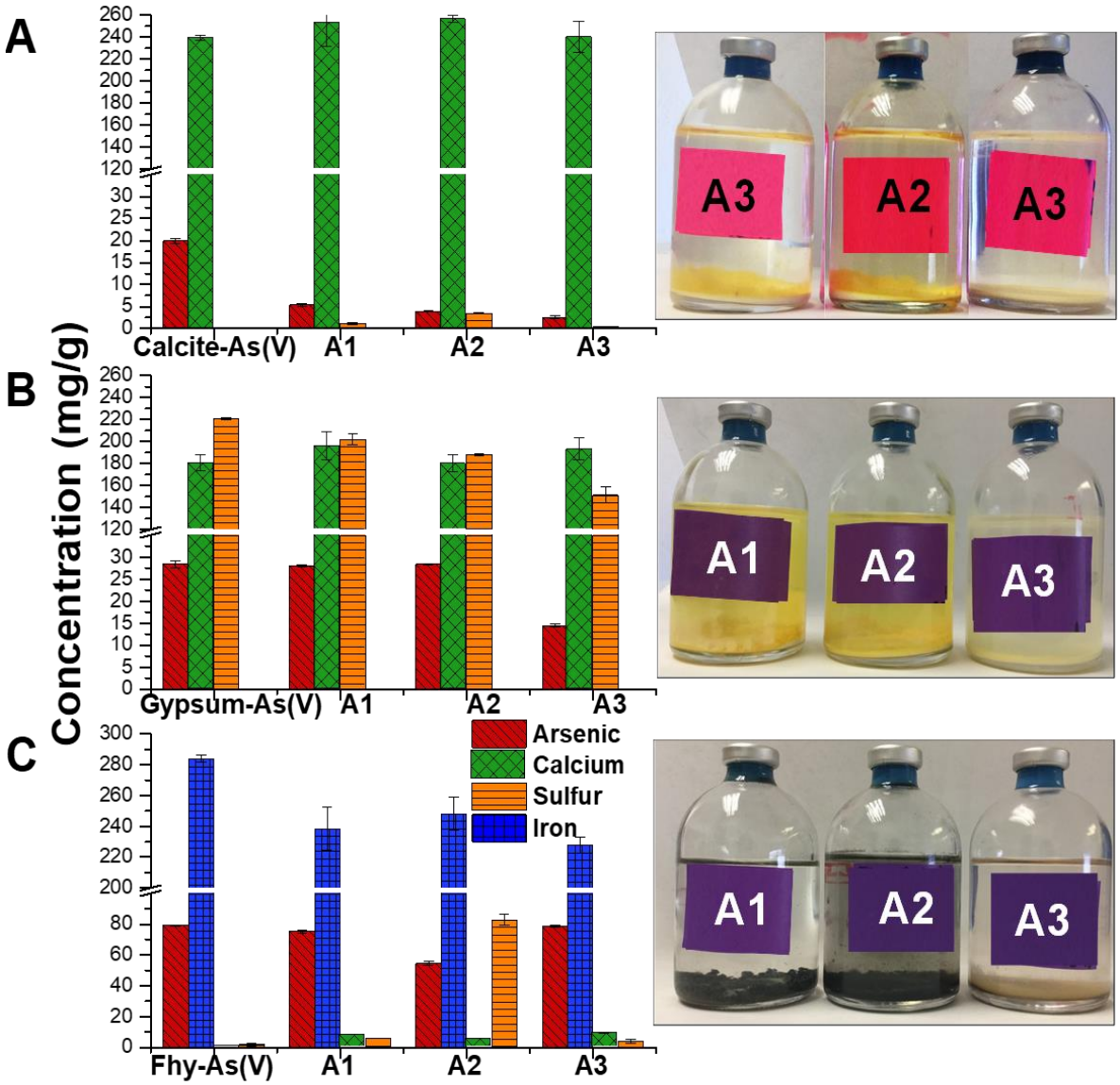

Figure 4.4. Composition and visual appearance of the solid fraction of the biotic assays (A1, A2, and A3) after 30 days of incubation with A) calcite-As(V); B) gypsum-As(V); and C) Fhy-As(V): ferrihydrite-As(V). The initial composition (day 0) is also provided under the name of each mineral.

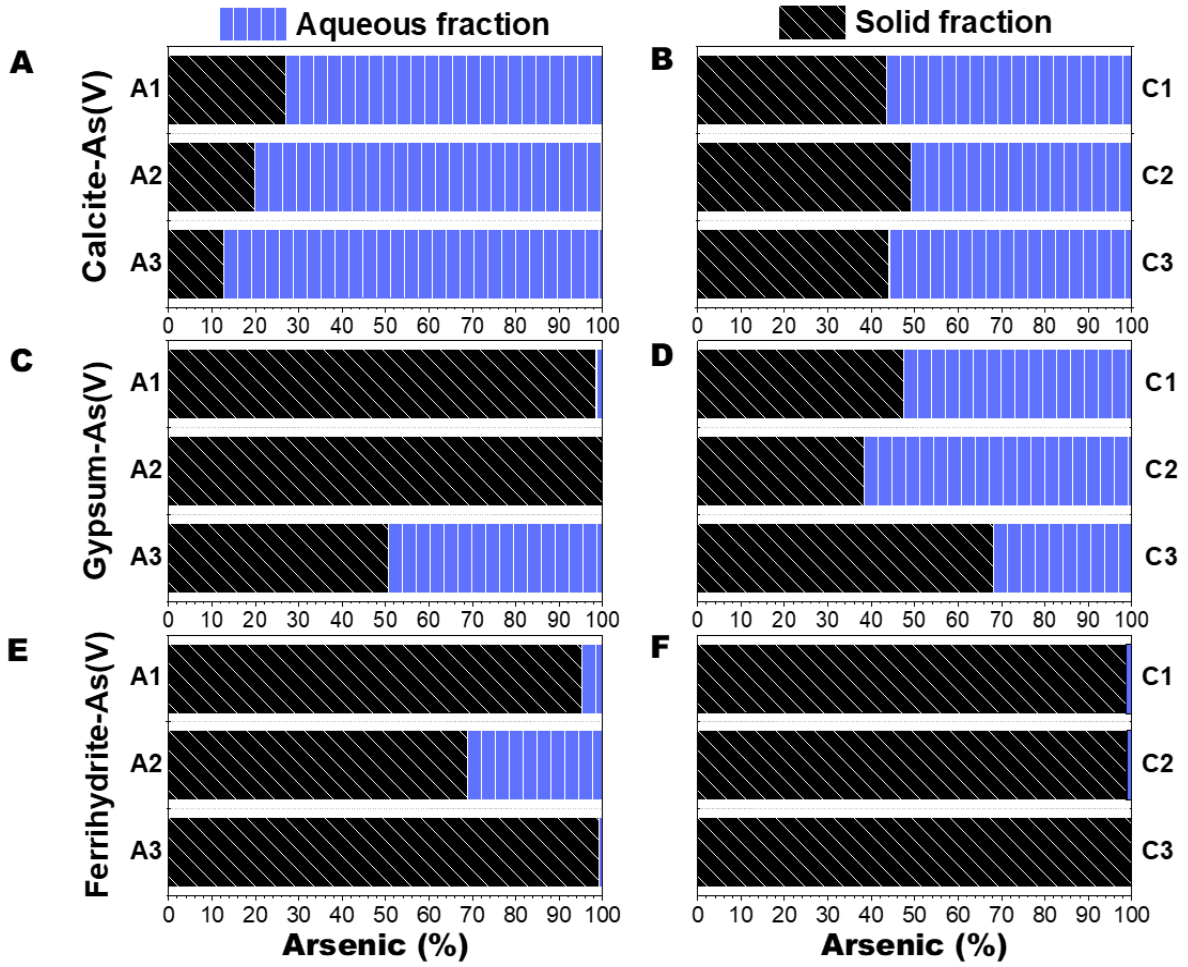

Figure 4.5. Arsenic distribution in the solid and aqueous fraction of batch assays after 30 days of incubation. Calcite-As(V): A) biotic and B) abiotic. Gypsum-As(V): C) biotic and D) abiotic. Ferrihydrite-As(V): E) biotic and F) abiotic.

Furthermore, XRD analysis allowed to corroborate the mineralogy of the synthesized minerals and to assess the mineralogy of the solid fraction in the biotic assays after 30 days of incubation (Figure 4.6). The initial calcite-As(V) consisted of a mixture of calcite and vaterite, which is a precursor of calcite during synthesis and in the early stage of precipitation (Yokoyama et al., 2012). At the end of the experiments with calcite-As(V), the vaterite initially detected in the mineral phase was transformed completely into calcite, as corroborated by the XRD patterns (Figure 4.6A), and most of the arsenic initially incorporated in the synthesized calcite-As(V) was liberated to the aqueous phase (73-87%, in biotic assays Figure 4.6A). In the case of gypsum and ferrihydrite co-precipitated with As(V), the corresponding footprints were confirmed by XRD patterns (Figures 4.6B and C).

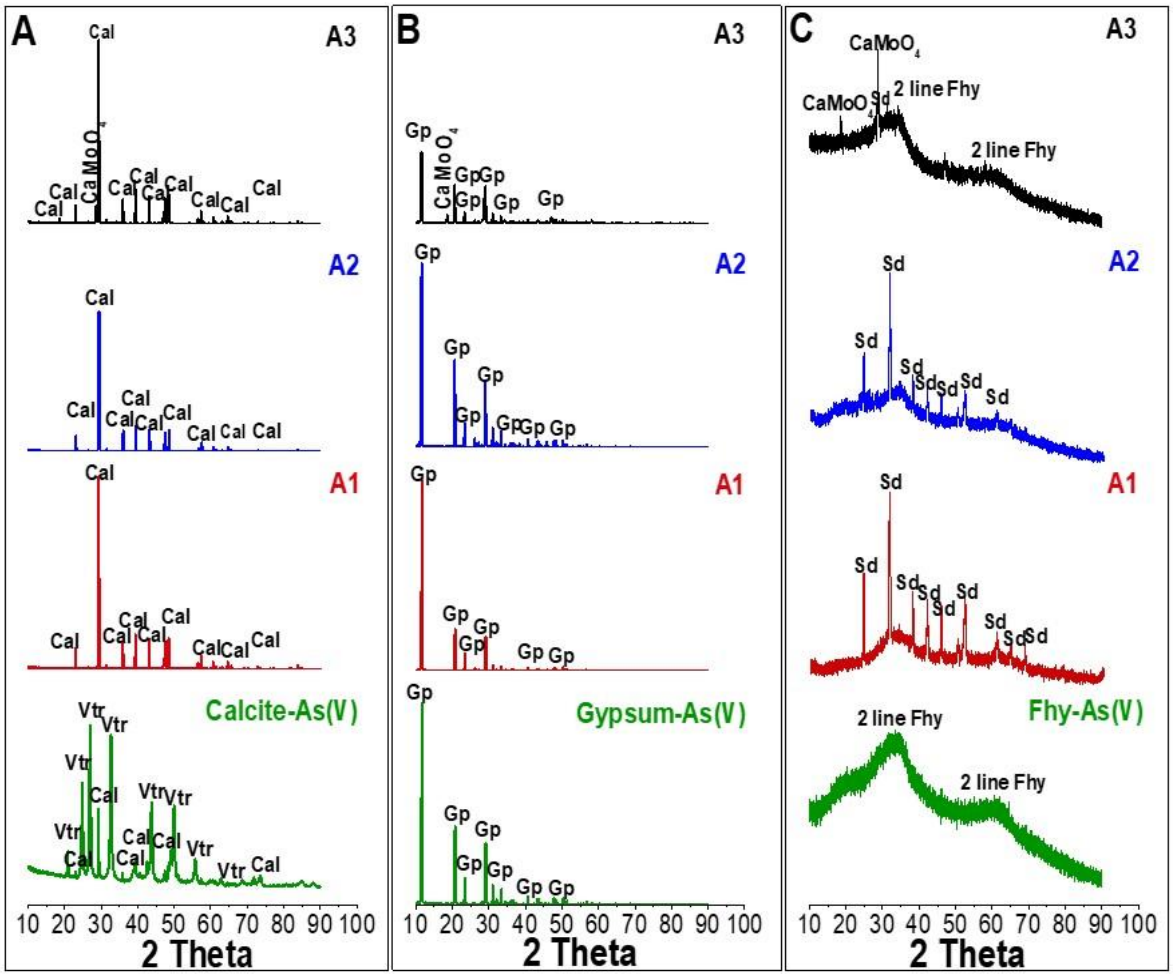

Figure 4.6. XRD diffractograms of the solid fraction obtained after 30 days of incubation from batch systems to evaluate the stability of three minerals co-precipitated with arsenic under reducing bioprocesses. A) Calcite-As(V); B) Gypsum-As(V) and C) Ferrihydrite-As(V). Cal: calcite ($CaCO_3$), Vtr: vaterite ($CaCO_3$), Gp: gypsum ($CaSO_4 \cdot 2H_2O$), $CaMoO_4$: calcium molybdate, Fhy: ferrihydrite ($Fe(OH)_3$), and Sd: siderite ($FeCO_3$).

After 30 days of incubation, the solid fractions of the bioassays with calcite-As(V) or gypsum-As(V) correspond to calcite and gypsum (Figure 4.6A and B). In comparison, the solid phase of the experiments with ferrihydrite in bioassays A1 and A2 showed the presence of siderite ($FeCO_3$), whereas ferrihydrite was still detected in the assays with molybdate (A3, Figure 4.7C). Although the solid fraction in the experiments amended with sulfate, A2 (Figure 4.4C), showed an important incorporation of sulfur (82.7 ± 3.7 mg of S/g of ferrihydrite), it was not possible to detect iron sulfides through XRD analysis most probably because sulfides were present below the detection limit (<5%). However, when calculating

the saturation indexes considering the species in solution, the iron sulfides resulted to be oversaturated, and their precipitation was expected (Figure 4.7).

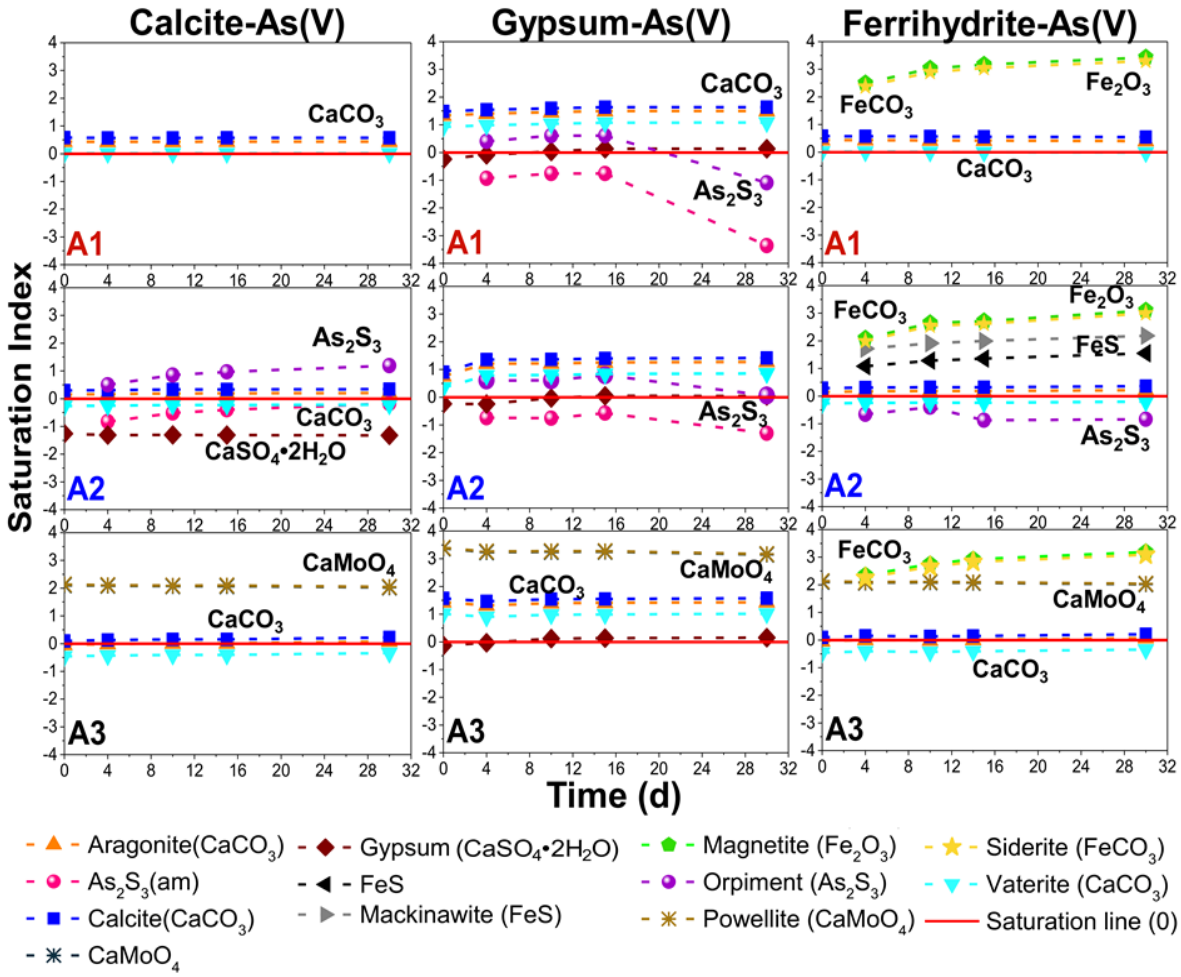

Figure 4.7. Values of the saturation indexes calculated using Visual MINTEQ® 3.1, considering the chemical species identified in the aqueous phase as product of the microbial action. The indexes allowed evaluating the mineral phases possibly formed in the biotic assays with the arsenic-bearing minerals (*i.e.* phases above saturation line, red). Based on our experimental conditions, the temperature was fixed to 30 °C and the pH to 7.5.

4.3.4 Bacterial community linked to arsenic-bearing minerals

The inoculum was not very diverse as the community was composed of 24 OTUs; similarly, at the end of the experiments, the microbiota of the batch assays contained 18 to 24 OTUs (Figure A2, Appendix). The original inoculum was

composed by 6 phyla, *Actinobacteria, Bacteroidetes*, *Tenericutes*, *Epsilonbacteraeota*, *Firmicutes* and *Proteobacteria* the last three were the most represented (> 97% in all assays). Figure 4.8 illustrates that the main genera identified in the inoculum were *Pseudomonas* (36%), *Clostridium sensu stricto 7* (16%), *Desulfosporosinus* (15%), and in a lesser extent but higher than 5%: *Exiguobacterium* (7%), *Sedimentibacter* (9%), and *Fusibacter* (5%).

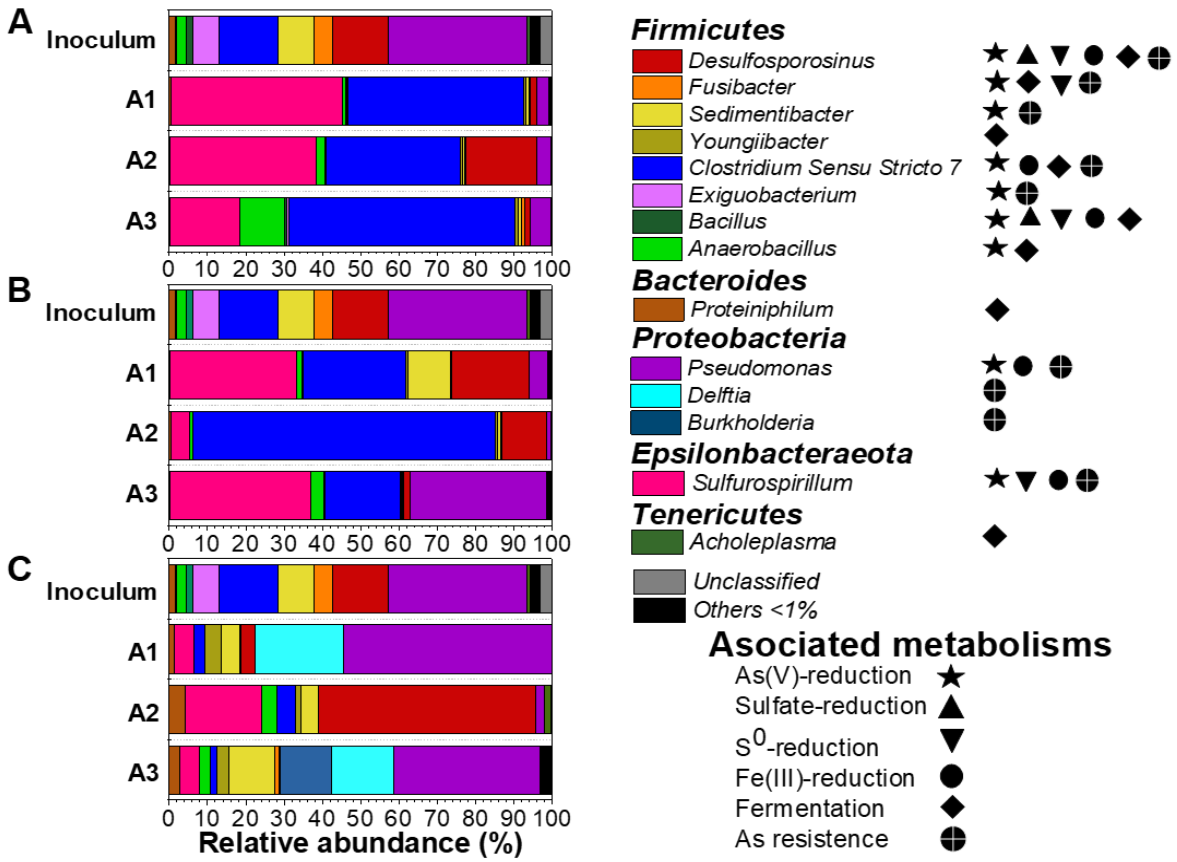

Figure 4.8. Taxonomic affiliations of bacteria domain at the genus level of the inoculum and the microbiota associated to batch systems after 30 days of incubation. A) calcite-As(V); B) gypsum-As(V); C) ferrihydrite-As(V).

Figure 4.9 presents a global perspective of the microbial compositions obtained in the different assays with each bearing mineral. The enriched genera, *i.e.* the genera whose relative abundance increased compared to the inoculum, are shown as bars to the right. The solid electron acceptor (As-bearing mineral) and the addition of sulfate or molybdate defined the communities obtained at the end of

each bioassay. *Sulforuspirillum* was enriched in all the experiments, and *Clostridium* was mostly enriched in the assays with calcite and gypsum. D*esulfosporosinus* remained almost the same as in the inoculum in the experiments with calcite and gypsum (when sulfate was present). *Pseudomonas* was only enriched in the absence of sulfate in assays A1 and A3 with ferrihydrite (up to 19%). These four genera were involved in the transformation of arsenic, all of them can use As(V) as electron acceptor (Figure 4.9).

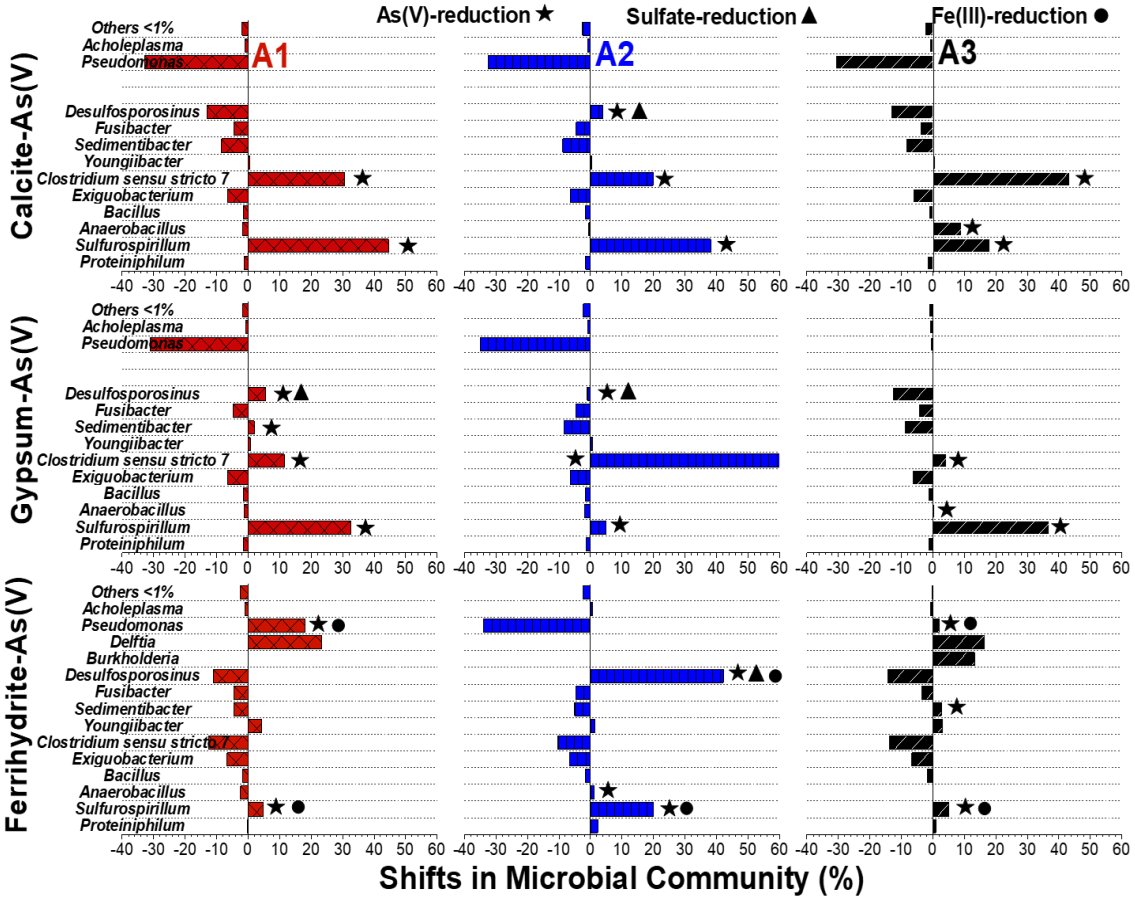

Figure 4.9 Changes in the community profiles at genus level of the different assays (A1, A2, A3) with the three different arsenic-bearing minerals at the end of the experiments (after 30 days) compared with the microbial composition of the inoculum (day 0). Enriched genera are represented as bars to the right; bars to the left represent genera that decreased their relative abundance.

It is remarkable the enrichment of *Desulfosporosinus* in assay A2 with ferrihydrite, 42% more abundant than in the inoculum (Figure 4.9). Conversely, in

all the assays where sulfate-reducing activity was inhibited (A3) the abundance of *Desulfosporosinus* decreased dramatically around 13% (Figure 4.9). Similarly, in assays with calcite or ferrihydrite without sulfate (A1), the presence of *Desulfosporosinus* also diminished (around 12%) in spite that *Desulfosporosinus* was among the dominant populations in the inoculum with a relative abundance of ca. 15% (Figure 4.8).

As presented before (in section 3.2) lactate was fermented to propionate and possibly this fermentation was carried out by members of the genus *Clostridium sensu stricto 7*, which was highly favored in the experiments with calcite (enriched up to 43%) and gypsum (enriched up to 60%) as shown in Figure 4.9. The contrary was found in the communities developed with ferrihydrite, the abundance of *Clostridium sensu stricto 7* decreased 12% (Figure 4.9), which is consistent with the low propionate production in the ferrihydrite-As(V) assays (Figure 4.3).

4.4. DISCUSSION

4.4.1 Dynamics of reducing bioprocesses in the aqueous fraction

This work demonstrates that microbial activity favored the release of the arsenic co-precipitated in the three minerals as a result of using them as solid electron acceptors. It is worth noting that the dissolution of carbonate and sulfate minerals, unlike iron oxides, is not an exclusive microbially-driven process. The dissolution of arsenic was anticipated since calcite and gypsum will eventually reach chemical equilibrium with the liquid medium, leading to the release of labile sorbed As(V) (Alexandratos et al., 2007; Zhang et al., 2015). Nonetheless, the reduction of As(V) to As(III) was only driven by microbial activity.

Calcite is a stable mineral, even when the redox potential changes, it has a limited affinity for As(III) at circumneutral pH because arsenite is an uncharged molecule (Renard et al., 2015). In the experiments with calcite and sulfate (A2) the concentration of sulfide was negligible, limiting the formation of arsenic sulfides. In

the event that arsenic sulfides were formed these would remain under-saturated; this situation, in addition to the low capacity of calcite to incorporate As(III), would explain why arsenite remained in solution. Possibly, sulfate-reduction was limited because lactate fermentation was the dominant bioprocess as shown by the lactate/propionate ratio (between 1.7 and 1.9), very close to the theoretical ratio (1.5) of lactate fermentation (Reaction 4.1). It has been reported that lactate fermenting microbes can outcompete lactate oxidizers (Kwon et al., 2014), when sulfate is the available electron acceptor at high lactate concentrations (> 10 mM), such as in the present study. The prevalence of fermentation in the bioassays with calcite is also supported by the fact that sequences resembling *Clostridium sensu stricto 7* (20-43% of enrichment) dominated the community (Figure 4.9).

In the experiments with gypsum, the decrease of dissolved As(III) was caused by its remineralization as part of the yellowish precipitates, *i.e.* arsenic mineral sulfides. In a similar experiment, anglesite ($PbSO_4$), served as electron acceptor for sulfate-reducing bacteria and lead (Pb) was remineralized as lead sulfide (galena, PbS) (Karnachuk et al., 2002). Related to fermentation of lactate to propionate, probably propionate was the electron donor for sulfate-reduction (see Reactions 4.2 and 4.3) (Kwon et al., 2016, 2014). Overall, our results demonstrate the beneficial effect of the sulfate-reducing bioprocess in the immobilization of arsenic in gypsiferous environments.

$$4\ C_3H_5O_2^- + 7\ SO_4^{2-} \rightarrow 12\ HCO_3^- + 7HS^- + H^+ \quad \textbf{(4.2)}$$

$$4\ C_3H_5O_2^- + 3\ SO_4^{2-} \rightarrow 4\ C_2H_3O_2^- + 4\ HCO_3^- + 3\ HS^- + H^+ \quad \textbf{(4.3)}$$

In contrast, sulfate-reduction had a negative effect on the experiments with ferrihydrite-As(V). Biogenic sulfide could mobilize arsenic through the sulfidization of ferrihydrite (Reaction 4.4), either by promoting the formation of iron sulfides (*e.g.*, mackinawite) (Reaction 4.5), which can sequester arsenic but in minor proportion, or by accelerating the reduction of Fe(III) to Fe(II), leading to the formation of secondary iron minerals (siderite, magnetite) (Burton et al., 2011).

$$2\ Fe(OH)_{3(s)} + HS^- + 5H^+ \rightarrow 2\ Fe^{2+} + S^0 + 6\ H_2O \quad \textbf{(4.4)}$$

$$Fe(OH)_{3(s)} + 2HS^- + H^+ \rightarrow FeS_{(s)} + S^0 + 3\ H_2O \quad \textbf{(4.5)}$$

4.4.2 Arsenic dissolution and remineralization

From a real situation perspective, when arsenic is mobilized from minerals, either by abiotic or biotic routes, it is desirable that remineralization occurs. Depending on the redox conditions and on the lithology of the site, microbial reducing processes may promote arsenic dissolution, but simultaneously they could be the main actors in arsenic attenuation promoting new mineral phases that serve as arsenic sinks. From this point of view, it was essential to determine the fate of arsenic.

Some authors have discussed the stable association between arsenic and calcite under reducing conditions since As(III) could be incorporated to calcite by substitution of CO_3^{2-} in the crystal lattice ($CO_3^{2-} \leftrightarrow AsO_3^{3-}$) (Bardelli et al., 2011; Catelani et al., 2018). However, if As(V) is reduced to As(III), as observed in this study, the mobility of arsenic will greatly increase because the distribution coefficient (K_d) of As(III) on calcite is around 2000 times smaller than that of As(V), therefore calcite only retains low quantities of As(III) (Winkel et al., 2013; Yokoyama et al., 2012). Our study shows that it is important to consider that microbial As(V)-reduction has a detrimental effect in calcareous systems because it increases the arsenic dissolution and the reduction of As(V) to As(III) promotes its permanence in the aqueous phase. These observations are consistent with the hydrogeochemical model proposed by Sø et al. (2008) who concluded that As(III) is very mobile in calcite bearing sediments.

Conversely, arsenic remineralization was observed in the biotic systems with gypsum in the assays where sulfate-reduction took place (A1 and A2, Figures 4.4B and 4.5C). Gypsum is highly soluble, thus sulfate and calcium ions (Ca^{2+} and SO_4^{2-}) and even As(V) could be dissolved until equilibrium with the solution is reached. Consequently, arsenate and sulfate were bioavailable for reducing bacteria and the products, As(III) and sulfide, precipitated as arsenic sulfides, most probably as amorphous orpiment (Saturation Indexs Figure 4.7). Sulfate consumption will in turn break the equilibrium and promote the dissolution of more gypsum until the electron donor (lactate) was depleted. The development of arsenic sulfides is desirable because they can contain between 60% to 70%

arsenic by weight and are poorly soluble and stable phases under anoxic conditions (O'Day et al., 2004).

According to the XRD analysis of the solid fractions of biotic assays with calcite or gypsum, the signals of these minerals were still detected after 30 days (Figure 4.7). The abundance of both crystalline phases could have masked the existence of arsenic sulfides. Additionally, sulfide minerals may have poor crystallinity, making difficult their detection through XRD, as observed previously (Doerfelt et al., 2016).

Regarding ferrihydrite-As(V), our results are in agreement with previous reports that found arsenic mobilization from ferrihydrite under sulfate-reduction (Burton et al., 2011; Kocar et al., 2010). Ferrihydrite is highly reactive under microbial activity and reducing conditions, therefore it is also possible that sulfidization of ferrihydrite occurred. In this process, biogenic sulfide reacts with iron oxides promoting the formation of iron secondary minerals, elemental sulfur, and traces of iron sulfides (Saalfield and Bostick, 2009). The formation of iron secondary minerals is desirable because they can coprecipitate and adsorb arsenic (Guo et al., 2010). Siderite was found in two assays (A1 and A2) with ferrihydrite-As(V), the fast precipitation of siderite is common in reduced systems with Fe(II) and bicarbonate (in the order of mmol/L); the bicarbonate used to buffer the basal medium and the reducing bioprocesses were sources of bicarbonate in the experiments. Furthermore, the formation of siderite is related to high rates of Fe(III)-reduction (Zachara et al., 2002) and the adsorption capacity of natural siderite has been estimated in 1040 mg of As(III)/kg at 1 mg/L of As(III) in solution (Guo et al., 2007). Siderite has a log k_{sp} of -10, although it is more soluble than ferrihydrite, it is more stable under reducing conditions (Muehe et al., 2013).

Hydrous ferric oxides, such as ferrihydrite, have the highest capacity to retain As(III) as indicated by their high K_d values (from 7340 to 670,000 L/kg) (Smedley and Kinniburgh, 2002). Compared with calcite (log K_{sp} -8.4) or gypsum (log K_{sp} -4.6), ferrihydrite (log K_{sp} -39) is much less soluble (Hansel et al., 2004; Renard et al., 2015; Rodríguez-Blanco et al., 2007). Thus, the partial dissolution/desorption of As(V) from gypsum was expected given the high solubility

of this mineral. Moreover, during the precipitation of calcite or gypsum, highly soluble calcium arsenates can precipitate over the mineral surfaces. Calcium arsenates have been associated with arsenic mobilization in calcareous and gypsiferous soils (Martínez-Villegas et al., 2013; Rodríguez-Blanco et al., 2007).

4.4.3 The role of the microbial community

All the batch assays were inoculated with a consortium that initially contained bacterial genera that include species identified by their capacity to tolerate and metabolize arsenic (Deng et al., 2018; Mirza et al., 2014; Serrano et al., 2017; Suhadolnik et al., 2017). We had anticipated that the type of arsenic-bearing mineral, as well as the addition of sulfate or molybdate, would be the driving factors in shaping the composition of the bacterial community, which agreed with the results (Figure 4.9). In the experiments performed with calcite and gypsum, the structure of the communities was similar among the three different bioassays. However, in the experiments performed with ferrihydrite, a different community developed in each bioassay (Figures 4.8C and 4.9), possibly because ferrihydrite is highly unstable and reactive under microbial activity and reducing conditions (Saalfield and Bostick, 2009).

The most redundant metabolic function, among the identified genera in all the assays, was As(V)-reduction, in agreement with the arsenic reducing activity observed with the three minerals (Figures 4.1 and 4.9). Arsenate respiring microorganisms are recognized by their metabolic versatility, as they can also use other electron acceptors such as sulfate or Fe(III), which allows them to proliferate in presence of distinct respiratory substrates (Suhadolnik et al., 2017).

Desulfosporosinus was the most flexible at the metabolic level because it can use As(V), S^0, Fe(III), and sulfate as electron acceptors, in addition to fermentation and being resistant to arsenic (Spring and Rosenzweig, 2006; Zhang et al., 2018). *Desulfosporosinus* is a sulfate reducer and represented up to 57% of the sequences in the assays amended with sulfate (A2), highlighting that this genus preferably used sulfate as electron acceptor. According to other authors,

one of the most representative sulfate-reducing bacteria associated with arsenic-rich sediments and their enrichments is *Desulfosporosinus* (Deng et al., 2018; Moon et al., 2017; Suhadolnik et al., 2017).

Significant increase of *Clostridium sensu stricto 7* was reported in anoxic arsenic enrichments, possibly playing an important role in the arsenic cycle since members of this group can reduce As(V) (Mirza et al., 2014). However, fermentation is the most known metabolism of *Clostridium sensu stricto 7*, explaining the high concentration of propionate in the assays with calcite and gypsum.

The enrichment of *Sulfurospirillum* in all the assays may be explained because, according to previous reports, some strains of the genus *Sulfurospirillum* can respire As(V) and Fe(III) (Yamamura and Amachi, 2014). *Sulfurospirillum* also couples the oxidation of lactate to the reduction of elemental sulfur (S^0) and their intermediates; this genus has also been associated with ferrihydrite reduction via a catalytic sulfur cycle (Hansel et al., 2015). The presence of *Sulfurospirillum* in the assays with ferrihydrite may be relevant because in the occurrence of biogenic sulfide, the abiotic reduction of ferrihydrite may take place with Fe(II) and S^0 as products, and *Sulfurospirillum* will reduce the S^0 again to sulfide, triggering the cycle. Furthermore, *Sulfurospirillum* can work synergistically with sulfate-reducing bacteria (Burton et al., 2011; Hansel et al., 2015).

Interestingly, the genus *Pseudomonas* was only enriched in ferrihydrite assays without sulfate (Fig. 5). Most probably, due to the fact that some species produce siderophores, that strongly chelate iron from the mineral surface and mobilize it to the cell surface, thereby increasing iron mineral dissolution rates (Garcia-Sanchez et al., 2005). Members of the genus *Pseudomona*s can reduce As(V) to As(III*)* by energy-conserving respiration pathways (Freikowski et al., 2010; Yamamura and Amachi, 2014). For instance, *Pseudomonas putida WB* could reduce As(V) using lactate or acetate as electron donor and produce H_2 (Freikowski et al., 2010); whereas some members of *Pseudomonas* can reduce Fe(III) using H_2 as electron donor, explaining its presence in the assays with ferrihydrite. Conversely, when sulfate was provided, *Desulfosporosinus* was the dominant genus promoting a

highly reduced redox potential (-137 mV) limiting the presence of *Pseudomonas,* a facultative anaerobe. Most probably, when sulfate-reduction was absent, *Pseudomonas* was favored due to its potential capacity to respire both As(V) and Fe(III). Deng et al. (2018) proposed that in Pleistocene aquifer sediments, iron-reducing bacteria such as *Pseudomonas* mobilized Fe(II) and As(III) from iron oxides in shallow sediments, whereas in deeper sediments sulfate-reducing bacteria (*Desulfosporosinus*) mitigated this mobilization by the formation of iron sulfides. Overall, the predominant detected genera (*Desulfosporosinus, Clostridium sensu stricto 7, Sulfurospirillum* and *Pseudomonas)* have been identified as highly resistant to arsenic and are very versatile, able to perform multiple metabolisms: As(V)/sulfate/Fe(III)-reduction and fermentation (Deng et al., 2018; Mirza et al., 2014; Suhadolnik et al., 2017).

The majority of the works addressing the use of arsenic-bearing ferrihydrite as solid electron acceptor have been achieved using pure cultures not consortia (Huang, 2018; Muehe et al., 2013; Ohtsuka et al., 2013). In the rare case when a consortium was used as inoculum, it was highly selected, only composed by the genera *Clostridium* and *Sporolactobacillus* (Burnol et al., 2007). To the best of our knowledge, the transformation of arsenic-bearing calcite and gypsum under reducing bioprocess and the description of the associated community has not been previously reported.

4.5. CONCLUDING REMARKS

Gypsum, calcite, and ferrihydrite were sources of electron acceptors and supported the microbial reduction of As(V), Fe(III), and sulfate using a consortium as inoculum. In systems with calcite-As(V), microbial As(V)-reduction had a negative effect because it enhanced the dissolution of arsenic incorporated into calcite and the produced As(III) remained in solution. In the case of gypsum-As(V), arsenate/sulfate-reduction contributed to arsenite remineralization up to 99%, possibly as arsenic sulfides; hence, there was a process of natural attenuation. In

the experiments with ferrihydrite-As(V), the solubilized As(III) in the biotic assays was adsorbed in iron secondary minerals (siderite, amorphous iron sulfides). However, sulfate-reduction had a negative effect because it triggered arsenic mobilization. The availability of the different electron acceptors shaped the dominant genera of the communities and promoted different metabolic pathways. In all the experiments As(V)-reduction took place, additionally fermentative metabolism was more favored with calcite or gypsum than with ferrihydrite.

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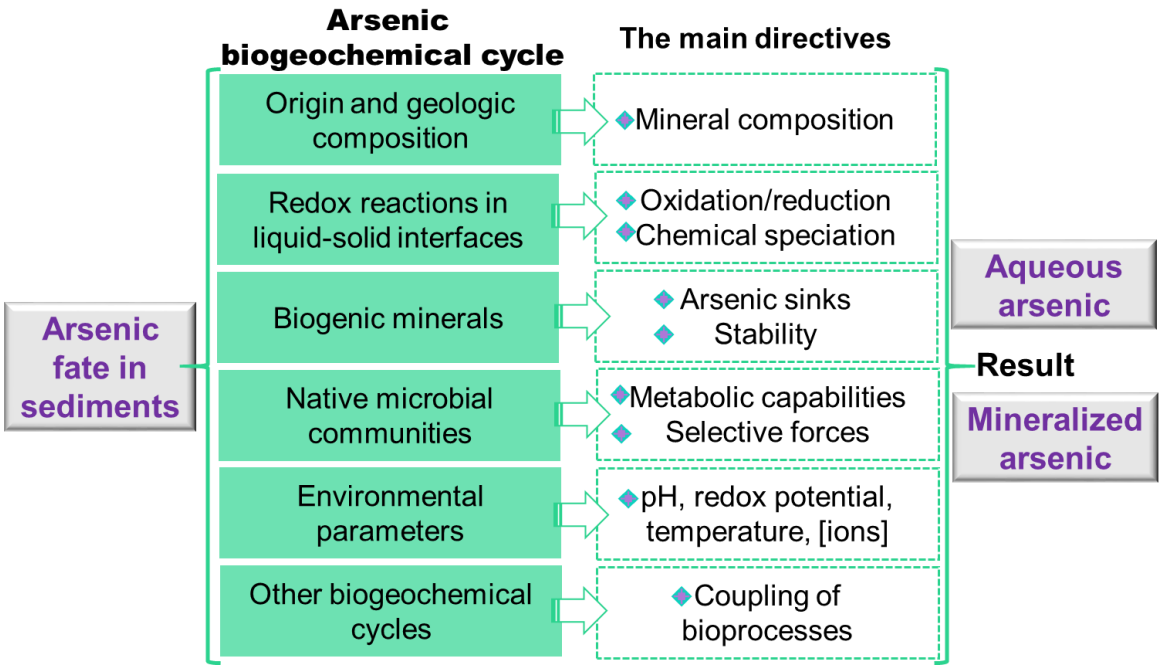
CHAPTER 5

Microbiota, biogenic minerals, and geochemical processes in arsenic biocycle: Conclusions and perspectives

Highlights

- The relevant findings of this thesis are underlined and discussed in general.
- The environmental significance and implications of arsenic bioremediation of this dissertation were addressed.
- Future directions are provided to contribute to the understanding of arsenic biocycle and respond to emerging questions.

Graphical Abstract



CHAPTER 5

5.1 OVERALL DISCUSSION AND CONCLUSIONS

The main goal of this thesis was to study microbial arsenotrophy, taking into account the influence of iron and sulfur biotransformations, and its role in arsenic speciation and transport in the solid-liquid interface. According to the bibliographic review we identified some unresolved gaps of knowledge and emerging questions, which were the main directives studied and discussed through this dissertation (Chapter 1).

The bioprocesses were studied in microcosms assays, the source of microorganisms were two arsenic-polluted sediments collected from freshwater bodies near a mining district in Matehuala, San Luis Potosi, Mexico. Sediment CB contained 238 ± 4.1 mg of As/kg and was collected from an artesian hydraulic complex, Sediment CT contained 10 times more arsenic (2263.1 ± 167.7 mg/kg) and was retrieved from a water spring. The majority of arsenic (more than 77%), in the two sediments, was bioavailable as it was associated to the soluble, carbonates, and ionically bound fractions. Despite the high concentration and bioavailability of arsenic, the native microbiota of both sediments was not impacted negatively, highlighting their tolerance to arsenic. Interestingly, the most diverse community (150 distinct bacterial genera) was found in the Sediment CT with the highest concentration of arsenic (Chapter 2). According to some authors, the long-term and high-level of arsenic contamination leads the microbiota toward adaptation to arsenic stress and the acquisition of genes responsible for arsenic resistance and metabolism by horizontal gene transfer (Cai et al., 2009; Costa et al., 2014).

In this work, the microbial community linked to biogenic minerals formed by As(V)/sulfate-reducing activity was explored for the first time. Surprisingly, the results showed that the biogenic arsenic-sulfide is a reservoir of arsenic resistant- and arsenic-respiring bacteria. The bacterial communities, recovered from the biominerals formed during the enrichment of the sediments, were dominated by the genus *Desulfosporosinu*s (*ca.*75%), which is an extremely versatile genus able to respire As(V), sulfate, Fe(III), nitrate and thiosulfate (Chapter 2). Most probably, the

activity of this genus was responsible of the formation of the arsenic-bearing sulfides. From the microcosms assays, it is clear that arsenic was reduced before sulfate, this fact seems to be the key to the formation of the minerals. Once arsenite was present in the media, the sulfate that was reduced gradually allowed the formation orpiment and then of realgar. At this stage, this was difficult to corroborate because as the microcosms assays contained sediment, the characterization of the biogenic minerals was not appropriate due to the "endogenous noise" provided by the sediment (Rios-Valenciana et al., 2017).

The next step in this work was to obtain biogenic minerals without the interference of the sediment composition. For this purpose, successive transfers helped to propagate the dissimilatory arsenate- and sulfate-reducing communities from both sediments (CB and CT), until the cultures were completely sediment-free. These consortia were essentially integrated by genera recognized by their ability to grow using As(V) as terminal electron acceptor. Concurrently with the enriched microbiota, the sediment-free communities were dominated by the genera *Desulfosporsinu*s and *Clostridium sensu stricto.* From the analysis of the functional marker for As(V)-respiration (*arrA* gene), it was found that both consortia contained *arrA* sequences homologous to *Desulfosporosinus sp. Y5* a strain isolated from sediments contaminated with aromatics compounds and mercury (Liu et al., 2004). Two selective forces were relevant to select the arsenic resistant/metabolizing bacterial consortia, the removal of the sediment matrix and the high arsenic concentration used in the microcosms assays (about 750 mg/L or 10 mM).

In summary, from Chapter 2 it can be highlighted that the study of the microbial communities associated with different microenvironments exposed to arsenic (sediments, enrichments, biogenic minerals, and consortia) revealed their great metabolic flexibility and the potential applicability in environmental biotechnologies.

As demonstrated in Chapter 3, biogenic minerals formed via sulfate- and arsenate-reduction in cultures from consortia CB and CT corresponded to As(III)/S(-II) nanostructures, which are only expected under limited supply of

biogenic sulfide. The majority of the works addressing the biological formation of nanomaterials of arsenic sulfide have been done with *Shewanella* strains (Jiang et al., 2009). In this dissertation we postulated that the precipitation of arsenic sulfides interferes with the performance of the community, decreasing the sulfate-reducing activity and allowing the dosage of sulfide. As proposed, the production of As(III)/S(-II) nanostructures starts with the formation of amorphous orpiment (Lee et al., 2007), we hypothesized that the precipitation of orpiment aggregates caused the cell deposition and trapping in the mineral matrix leading to bacterial inactivation (mineralization of cells) (Stanley et al., 2018). As a result, the metabolic activity of the microbial communities was constrained, promoting the development of As(III)/S(-II) nanostructures. In addition, the high concentration of aqueous As(III) from As(V)-reduction could inhibit partially the sulfate-reducing activity (Figure 5.1).

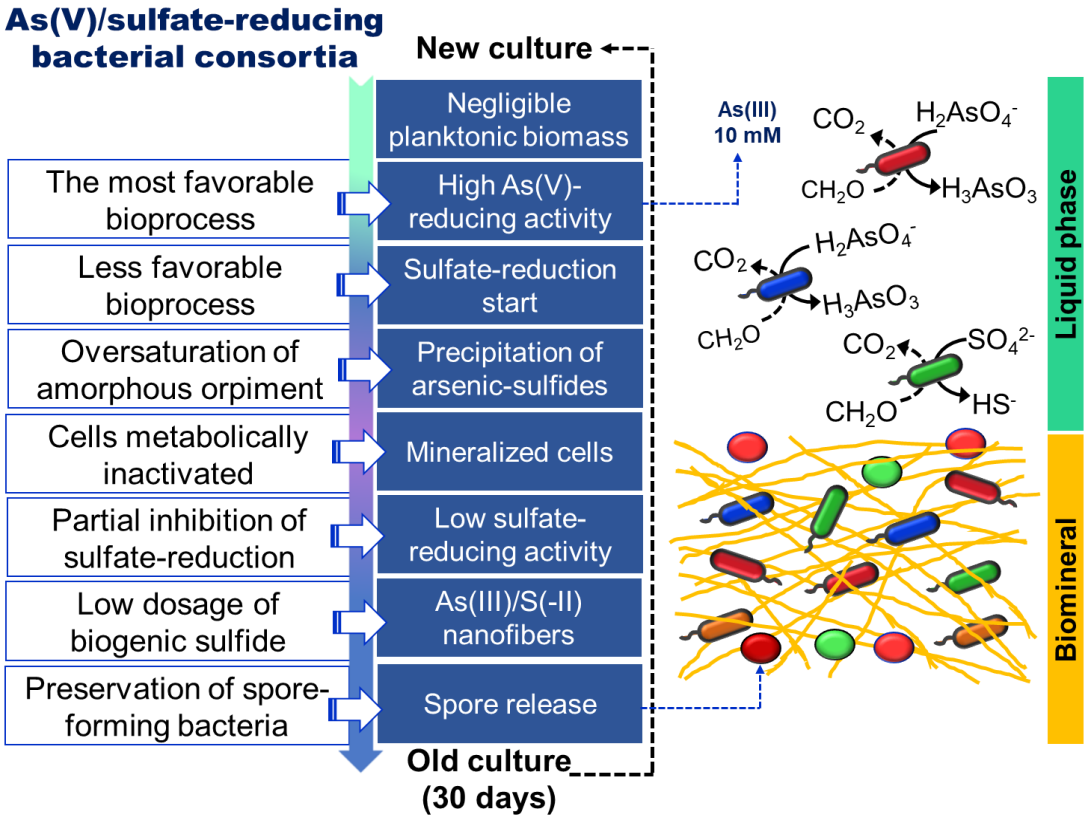


Figure 5.1. Possible systematic conditions for the development of As(III)/S(-II) nanostructures in cultures of the consortia obtained from sediment CB and CT.

Each bacterial consortium developed arsenic sulfides with different mineralogy and crystallinity; the biomineral CB was identified as bonazziite, and the biomineral CT was identified as realgar, being highly crystalline as demonstrated by XRD and Raman analysis (Figure 3.5, Chapter 3). The development of the different sulfide mineral phases was related to the composition of the microbial communities and therefore, with the rates of arsenate- and sulfate-reduction. *Desulfosporosinus*, *Clostridium sensu stricto*, *Pseudomonas*, *Exiguobacterium*, *Sedimentibacter*, *Fusibacter,* and *Anaerobacillus* represented 90% at genus level of the microorganisms associated to both arsenic sulfide biogenic minerals. All these genera include dissimilative As(V)-reducing species able to use arsenate as terminal electron acceptor (*arrA*-carriers). The most evident difference in the communities was the high relative abundance of *Desulfosporosinus* in biomineral CB (34%) or *Pseudomonas* in biomineral CT (36%). *Desulfosporosinus* can respire sulfate and arsenate and *Pseudomonas* can respire arsenate. In agreement, the results also shown that the sulfate-reduction rate was about 2.5 times higher in Consortium CB than in Consortium CT, while As(V)-reduction was *ca.* 2 times higher in Consortium CT. These results support the fact that the most crystalline biomineral (realgar) was recovered from consortium CT, where the sulfate-reduction rate was at least 5 times lower than the arsenate-reduction rate (Chapter 3).

The mineralogy of sediment CB consisted mainly of gypsum and quartz, whereas sediment CT was composed mainly of calcite and quartz (Rios-Valenciana et al., 2017). Both sediments were classified as iron-poor since they contain less than 1.3% (*wt.%*) of iron (Boggs, 2009). Up to date, few studies have focused on iron-depleted arsenic-polluted environments. While it is true that the possible role of calcite in sequestering arsenic has been considered in several studies, these studies have not considered the influence of microbial reducing processes such as As(V)-, sulfate-, and Fe(III)-reduction, that could occur in anoxic environments.

In Chapter 4, redox transformations and dissolution of arsenic were investigated when calcite, gypsum, and ferrihydrite coprecipitated with As(V) and were the source electron acceptors. Microbial-reducing processes caused the dissolution of arsenic bound to calcite, gypsum, and ferrihydrite. However, the final fate of arsenic largely derives from each mineral.

As(V)-reduction resulted in arsenic release from calcite, and the produced As(III) remained in solution. In this case, there was not a remineralization process, even when sulfate and lactate were available; the production of biogenic sulfide was negligible due to this the arsenic-sulfides remained under-saturated, resulting in the accumulation of aqueous As(III). In this system the fermentative metabolism plays an important role, lactate fermenting microbes outcompeted sulfate-reducing bacteria. In contrast, when gypsum-As(V) was the solid electron acceptor, As(V)- and sulfate-reduction lead to As(III) remineralization (99% was immobilized as arsenic-sulfide); hence, there was a process of autoregulation. In the presence of ferrihydrite, sulfate-reducing activity had a negative effect, despite iron secondary minerals were precipitated, sulfate-reduction triggered arsenic mobilization (Burton et al., 2011; Kocar et al., 2010).

Microbial communities were further investigated and the predominant genera were *Desulfosporosinus*, *Clostridium sensu stricto 7*, *Sulfurospirillum*, and *Pseudomonas*. All of them have been identified as extremely resistant to arsenic and are very versatile as they can use arsenic, sulfate, and iron as electron acceptors, besides being fermentative bacteria. These genera were present in different relative abundances depending on the availability of the electron acceptors (mineral type and sulfate), promoting different metabolic pathways (fermentation or respiration).

5.2 ENVIRONMENTAL RELEVANCE AND IMPLICATION IN ARSENIC BIOREMEDIATION

Biogenic minerals are relevant to several fields of research. For instance, in environmental biotechnology, the formation of biogenic minerals is crucial for the bioremediation of hazardous inorganic compounds (*i.e.* metals and metalloids). In the area of biogeochemistry, a better understanding of biogeochemical cycles may drive in-situ remediation strategies and prevention. Eventually, biogenic minerals can be applied in the industry. In this thesis, we propose that biogenic arsenic sulfides are an alternative to propagate and cultivate bacteria with potential applications in biotechnologies for arsenic remediation (*e.g.* bioaugmentation). The microbiota associated with the arsenic-sulfide biominerals offers several advantages that in the end help to mineralize arsenic as stable arsenic sulfide nanostructures. It is composed of indigenous bacteria, historically adapted to high arsenic concentrations, and the communities are resilient because they contain spore-forming bacteria. Bacteria can reduce both As(V) and sulfate, and the rates of As(V)-reduction were high. Overall, the evidence provided here is a good start to envision biominerals as an integral part of microbial communities.

To further investigate the transport and stabilization mechanisms of arsenic in sediments, two possible scenarios were considered. Iron-rich environments, where the fate of arsenic is controlled by adsorption and coprecipitation by iron-bearing minerals (*i.e.* iron oxides and iron sulfides), these have very low solubility products, hence their precipitation is highly favorable (O'Day et al., 2004). Iron-poor environments, where other players such as calcite and gypsum are relevant. In this framework, one important contribution of this dissertation was to find out the fate of arsenic when the iron is not the major scavenger for arsenic attenuation.

Calcite and gypsum can uptake arsenic, this phenomenon can be important in natural systems because of the ubiquity of these minerals in the Earth's crust, especially in semi-arid regions (Fernández-Martínez et al., 2006; Meng et al., 2016). The precipitation/dissolution of calcium carbonates and gypsum may be a controlling mechanism of arsenic transport in aquifers where calcium or sulfate instead of iron are the dominant ions, as was the case of sediments CB and CT.

The results of this thesis demonstrated that As(V)-, sulfate-, and Fe(III)-reduction enhance the arsenic mobilization from calcite, gypsum, and ferrihydrite, but other remineralization processes are triggered reversing the arsenic dissolution. In the presence of calcite, arsenate-reduction caused aqueous As(III) accumulation, and fermentation was favored over sulfate-reduction despite the availability of sulfate. Our results suggest the detrimental effect of As(V)-reduction in calcareous environments, it has been reported the low affinity of As(III) for calcite compared with As(V), due to this arsenite is very mobile in calcite bearing sediments (Sø et al., 2008). In the experiments with gypsum, remineralization occurred and As(III) formed part of arsenic sulfides. As(V)- and sulfate-reduction had a beneficial effect on the stabilization of arsenic in gypsiferous environments. Overall, the results highlight the influence of mineral composition over the performance of the microbial community and the final fate of arsenic.

5.3 PERSPECTIVES AND FUTURE DIRECTIONS

Many research efforts have been invested in understanding the biogeochemical arsenic cycle in association with other biogeochemical cycles (*e.g.* S and Fe), to comprehend the mechanisms that govern the chemical speciation and transport of arsenic in polluted-environments. However, due to the heterogeneity of the contaminated sites, either related to the sediment origin, geologic composition, environmental parameters, and native microbial communities, there are still loose ends that represent research opportunities. For instance, in-situ bioremediation is still a challenge and many investigations are necessary to optimize it. Strategies to improve and control biostimulation and bioaugmentation could be useful. For this purpose, it is fundamental a better understanding of the interconnections among the biogeochemical cycles.

5.3.1 Heterotrophic/autotrophic arsenotrophy as a complementary process in arsenic biocycle

The bioremediation of arsenic polluted-sediments is essentially directed by three main guidelines: microbial redox reactions at the water-solid interface, biominerals as arsenic sinks and their stability against environmental parameters, and microbial activity.

Considering that organic carbon in sediments is quickly depleted by native microbial communities that promote reducing conditions, anoxic sediments with minimal levels of organic carbon are somewhat common (Paul et al., 2015). Under these circumstances, autotrophic bioprocesses, such as denitrification, are very important. It is worth to note that, in sediments, the infiltration of nitrate has increased alarmingly due to the intensive use of fertilizers (Di Capua et al., 2015). In Chapter 1, it was roughly mentioned that denitrifying autotrophic microorganisms use reduced inorganic compounds such as Fe(II), H_2, reduced sulfur compounds, As(III) and iron sulfides as electron donors, by fixing inorganic carbon and reducing nitrate to nitrite and N_2 gas (Di Capua et al., 2015).

In reducing aquifers is common to find iron sulfides associated with arsenic (O'Day et al., 2004). Iron sulfides, such as mackinawite, pyrrhotite, and pyrite, have been tested as electron donors for autotrophic denitrifying bacteria resulting in the oxidation of sulfide to sulfate and Fe(II) to Fe(III). In this context, the bio-oxidation of iron sulfides would imply the dissolution of arsenic. However, the oxidation of Fe(II) to Fe(III) results in the formation of iron oxides (*e.g.*, ferrihydrite), which immobilizes arsenic. Therefore, this process is classified as a bioremediation strategy with potential applications in suboxic environments. (Sun et al., 2009). Eventually, arsenic sulfides (*i.e.* orpiment, realgar) could also serve as electron donors in autotrophic denitrification, moreover little is known about the bio-oxidation of arsenic sulfides and the fate of arsenic after this process (Chen et al., 2011). Possibly in the absence of iron, the reaction products (As(V) and sulfate) could remain in solution promoting arsenic mobilization (Figure 5.2).

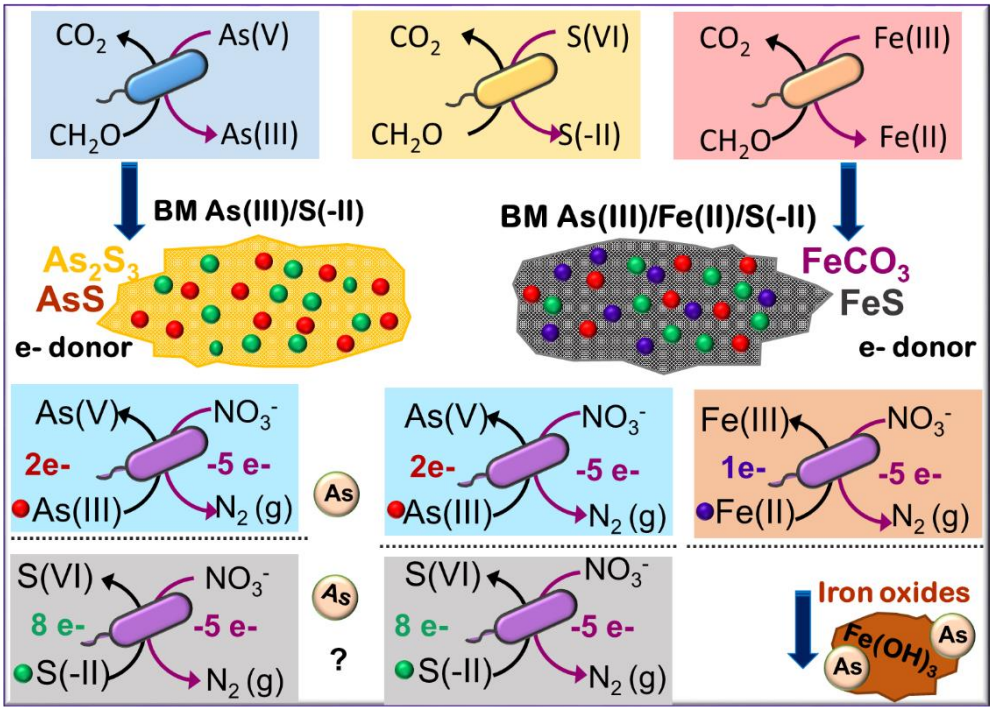


Figure 5.2. Diagram illustrating the role of arsenic-bearing biogenic minerals (BM) formed by As(III)/S(-II) and As(III)/Fe(II)/S(-II) as electron acceptors in autotrophic denitrification. Arsenite, As(III)); arsenate, As(V); Sulfide, S(-II), sulfate, S(VI), ferrous iron, Fe(II); ferric iron (Fe(III)).

As discussed in this thesis, arsenic-bearing biogenic minerals are considered arsenic sinks and a good strategy for the attenuation of this toxic element. Different biominerals ranging from amorphous to crystalline phases have been reported, under anoxic and mesophilic conditions at circumneutral pH: iron sulfides (mackinawite, pyrrhotite, and greigite; Burton et al., 2013; Picard et al., 2018); arsenic sulfides (orpiment, realgar, and their polymorphs; Mumford et al., 2013; Rodriguez-Freire et al., 2014); and even iron carbonates that adsorb arsenic like siderite (Muehe et al., 2013). All these biominerals could be oxidized through nitrate-reduction. Considering that biogenic minerals are arsenic sinks in polluted-systems, it is essential to assess their stability under microbial-oxidation processes, which eventually could re-contaminate the environments by arsenic destabilization. Under this perspective, there is a lack of studies that assess the stability of biogenic minerals, formed via As(V)-, Fe(III)- and sulfate-reduction as electron donors, in autotrophic denitrification. Figure 5.2 schematizes the general process to implement this study.

Finally, Figure 5.3 illustrates the heterotrophic arsenotrophy (evaluated in this thesis) and autotrophic arsenotrophy as a complementary process in the arsenic bio-cycle, considering anaerobic microcosms similar to the ones studied in this thesis. The proposed mechanisms could be relevant to understand the recycling of arsenic in sedimentary environments.

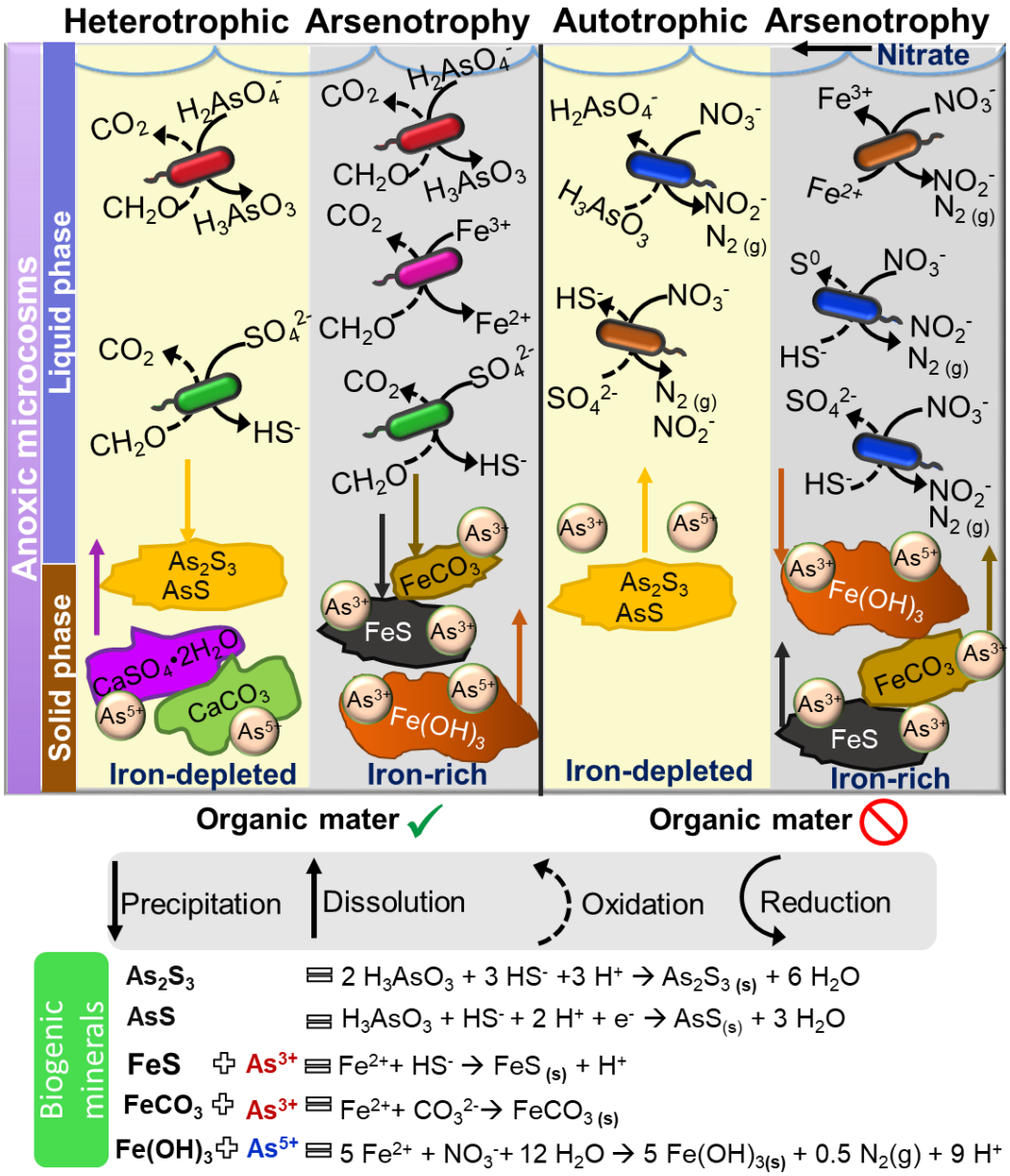


Figure 5.3. Schematic representation of the main redox transformations in anaerobic microcosms (neutral pH) either under heterotrophic arsenotrophy or autotrophic arsenotrophy in iron-depleted and iron-rich systems.

5.3.2 What else about arsenic-bearing biogenic minerals?

The nanostructures of arsenic-sulfides obtained from the consortia were characterized using XRD, Raman, SEM-EDS, and the microbiota linked to the biomineral was identified (Chapter 3). However, a more detailed characterization of As(III)/S(-II)-nanofibers is necessary to get better control of the biosynthesis process, we recommended to determine the growth kinetics of the biogenic nanofibers (precipitation rates), and analyze the nanostructures by transmission electron microscopy (TEM). These analyses will generate accurate information about the efficiency of bioprecipitation to compare with pure cultures (*i.e. Shewanella* strains), and TEM-EDX allows achieving an elemental analysis of cross-sections of nanostructures (Lee et al., 2007).

Another interesting aspect is to know more about the interrelationship between biominerals and microbiota. During the growth kinetics of arsenic biominerals, differential staining would be useful to visualize the cell distribution and investigate if mineralization reduces the metabolic activity of the bacteria. For instance, we could distinguish between life and dead cells, and follow the spore release through the kinetics using spore staining.

Another approach is to take advantage of the fluorescence in situ hybridization (FISH) technique, that uses 16S rRNA-target oligonucleotide probes to evaluate the phylogenetic identity, morphology, and spatial arrangements of microorganisms in environmental samples (Nguyen et al., 2017). The use of FISH could be helpful to identify the spatial and temporal distribution of arsenate- and sulfate-reducing bacteria in biogenic minerals and determine the occurrence of the bioprocesses through the growth kinetics of the biominerals. A complementary technique could be based on RNA extraction, cDNA amplification, and quantitative PCR of the *arrA* gene (Mirza et al., 2017).

5.3.3 Isolation of novel bacteria

Although the *arrA* gene is a useful molecular marker to corroborate the presence of arsenate-respiring bacteria, the majority of the *arrA* sequences reported so far are affiliated with uncultured bacteria. In agreement, 75% of the *arrA* gene OTUs found in the present study had similarities to uncultured bacteria. Several authors have discussed the existence of unique dissimilatory arsenate-reducing bacterial communities in arsenic-contaminated sites (Costa et al., 2014; Lu et al., 2017; Suhadolnik et al., 2017). For example, in studies based on the 16S rRNA gene the sediments with high arsenic levels and long-term contamination hold native communities highly diverse consistently. Accordingly, studies based on *arrA* gene also determined quite diverse arsenic resistant and metabolizing microbiota (Cai et al., 2009; Costa et al., 2014; Suhadolnik et al., 2017) (Suhadolnik et al., 2017). Therefore, more efforts are necessary to identify and cultivate novel arsenate-respiring microorganisms, which would contribute to characterize and describe arsenate reductase enzymes from novel strains, and even from these strains the presence of *ars* operon for arsenic respiration by detoxification could be confirmed. These novel microorganisms can be helpful to design primers and target a broader *arrA* gene diversity (Mirza et al., 2017).

Molecular markers that allow identifying pathways of arsenic metabolism and detoxification have been described in detail (Cai et al., 2009; Cavalca et al., 2013). However, the genes involved in anaerobic metabolism have received little attention since they were described more recently. Additional research must focus on the description of molecular markers in pure cultures, such as the genes (*arxA* and *aioA)* that codify for the enzymes that catalyze As(III)-oxidation in anaerobic and aerobic conditions, respectively. Several reports have demonstrated the use of As(III) as the electron donor in anoxic environments (Rhine et al., 2007; Sun et al., 2009; Zhang et al., 2015). However, the *arxA* type oxidase (arsenite as the sole electron donor) has been characterized only in three autotrophic microorganisms (Chapter 1). Thus, it is important to find more microorganisms that carry *the arxA* gene. Although *aioA* is related to aerobic metabolism, it could also have an

essential role in anaerobic As(III)-oxidation (Rhine et al., 2007; Zhang et al., 2015). Recently, Zhang et al. (2015) amplified the *aioA* gene from *Paracoccus sp.* SY, able to grow under anoxic conditions using nitrate as the electron acceptor. The authors found that *Paracoccus sp.* SY couldn't oxidize As(III), under both aerobic and denitrifying conditions, after deleting the *aioA* gene. Suggesting that the *aio* system was involved in anaerobic As(III)-oxidation; the identification of *aioA* gene in anaerobic bacteria could decipher the role of *aioA* gene in anaerobic systems.

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Appendix

Rarefaction curves of the 16S rRNA Illumina MiSeq analysis

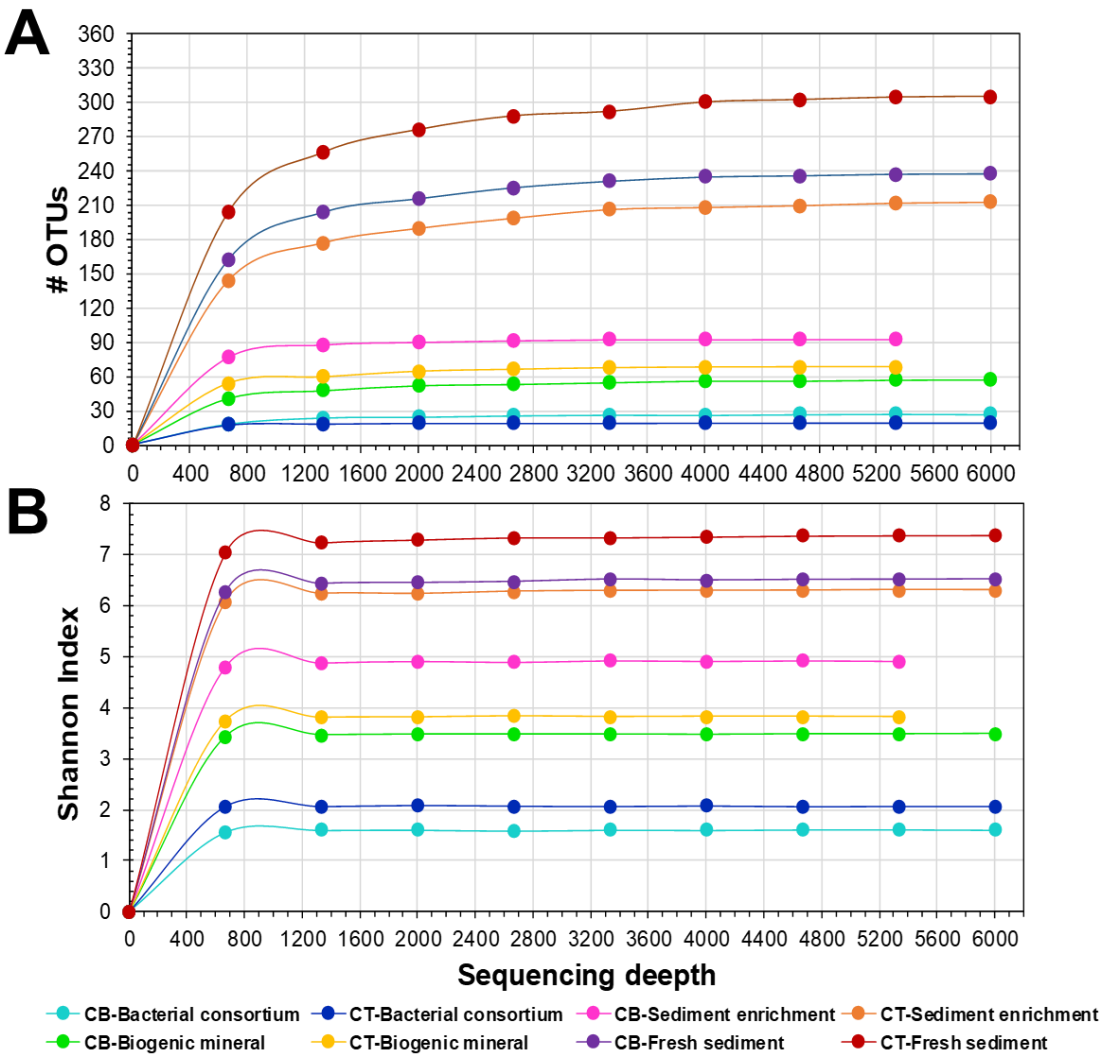

Figure A1. Alpha diversity based on 16S rRNA Illumina MiSeq analysis for the microenvironments from CB and CT sediments. A) Rarefaction curves based on operational taxonomic units (OTUs) at 99% of similarity; B) Rarefaction curves based in Shannon index.

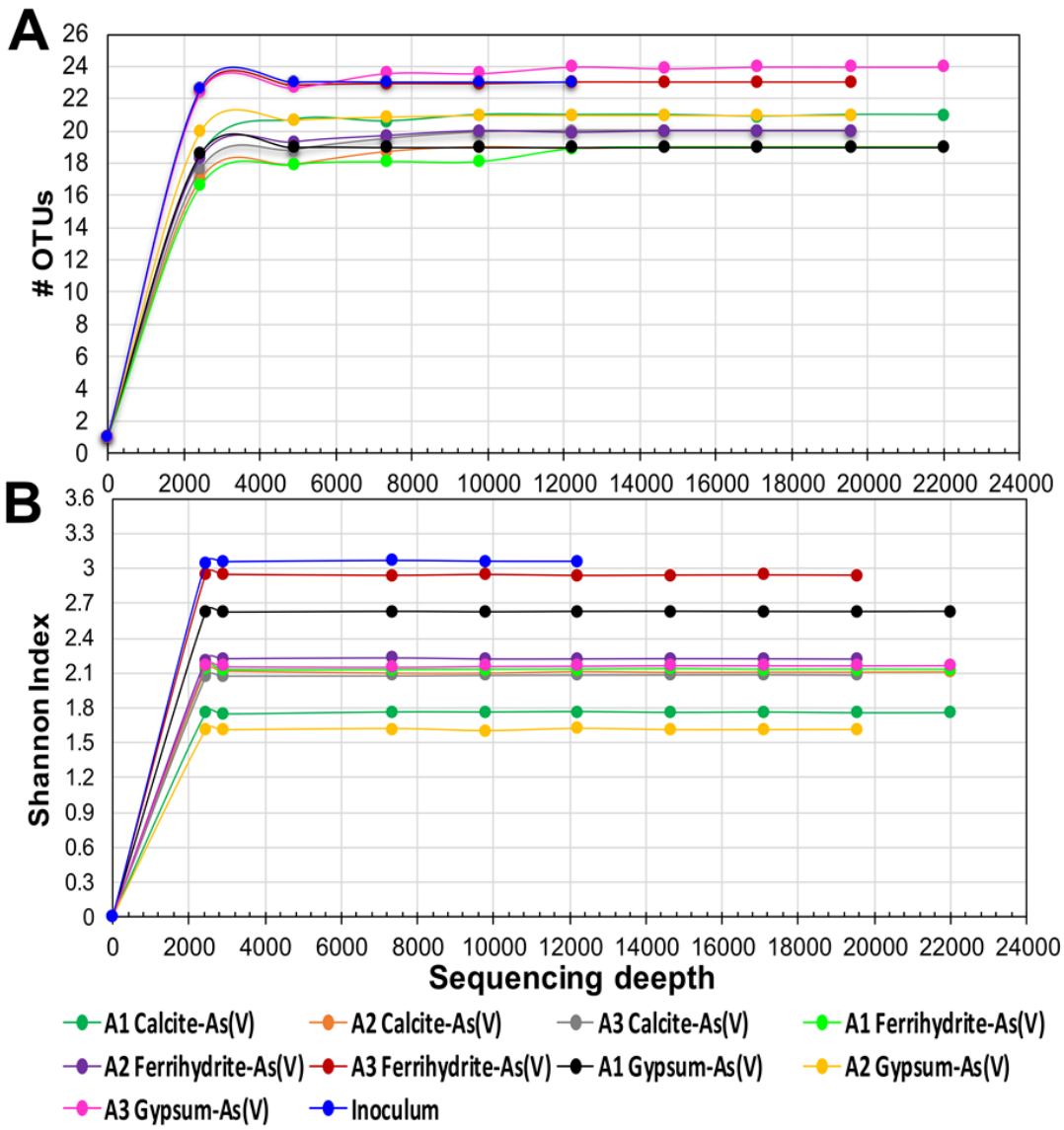

Figure A2. Alpha diversity analysis of the microbiota associated to batch systems with arsenic-bearing minerals. A) Rarefaction curves based on operational taxonomic units (OTUs) at 99% of similarity; B) Rarefaction curves based in Shannon index.

CV summary

About the author

Erika Elizabeth Rios Valenciana was born in Durango, Dgo. Mexico, she holds a Bachelor's in Biochemical Engineering at de Instituto Tecnológico de Durango (ITD), obtaining her degree with the thesis entitled “Biohydrogen production by continuous fermentation using acid hydrolysate of oat straw as substrate”, which she performed at the Instituto Potosino de Investigación Científica y Tecnológica (IPICyT, San Luis Potosi, Mexico) under the direction of Dr. Elias Razo-Flores. From 2012 to 2013, she worked as a Research Assistant in the project “Isolation and biochemical characterization of hydrogen-producing bacteria from anaerobic sludge and silages”. Afterward, she started her master's studies in Environmental Sciences (2013) at IPICyT under the direction of Dr. Lourdes B. Celis García with the thesis topic “Biological reduction of As(V) and sulfate in sediments of a hydraulic system contaminated with arsenic”. In January 2016, she joined to the Ph.D. program in Environmental Sciences also at IPICyT under the co-direction of Dr. Lourdes B. Celis García and Dr. Roberto Briones Gallardo (Universidad Autónoma de San Luis Potosí, UASLP, San Luis Potosi, Mexico). During the doctoral project, she explored microbial reducing processes involved in arsenic speciation and transport in arsenic-polluted sediments, considering the interconnections between biogeochemical cycles of key elements as carbon, sulfur, and iron.

Peer-Reviewed Research Papers

Published

Rios-Valenciana, E.E., Briones-Gallardo, R., Chazaro-Ruiz, L.F., Lopez-Lozano, N.E., Sierra-Alvarez, R., Celis, L.B., 2020. *Dissolution and final fate of arsenic associated with gypsum, calcite, and ferrihydrite: Influence of microbial reduction of As(V), sulfate, and Fe(III)*. ***Chemosphere 239, 124823*****.** DOI: 10.1016/j.chemosphere.2019.124823.

Rios-Valenciana EE., Briones-Gallardo R, Cházaro-Ruiz LF, Martínez-Villegas N, Celis L.B., (2017). *Role of indigenous microbiota from heavily contaminated sediments in the bioprecipitation of arsenic.* ***Journal of Hazardous Materials 339, 114-121***. DOI: 10.1016/j.jhazmat.2017.06.019

In preparation

Rios-Valenciana, E.E., et al. *From arsenic rich sediments to sulfate/As(V)-reducing bacterial consortia: Transition of the microbial communities*.

Rios-Valenciana, E.E., et al. Biogenic minerals and extreme bacteria a team in arsenic-polluted environments.

Presentations/symposia/seminars significant to this thesis

Rios-Valenciana, E.E., Briones-Gallardo, Celis, L.B. *Potential colonizers in a world of arsenic*. Oral presentation at the **XVIII National Congress of Biotechnology and Bioengineering, Leon, Guanajuato, Mexico, 2019**.

Rios-Valenciana, E.E., Briones-Gallardo, Celis, L.B. *Anaerobic arsenotrophy linked to the bioprocesses of reduction of As(V), sulfate and Fe(III): implications in the fate of arsenic in a solid-liquid interface*. Oral presentation at the **Divisional Seminar, División de Ciencias Ambientales, IPICyT, SLP, Mexico, 2019.**

Rios-Valenciana, E.E., Briones-Gallardo, Celis, L.B. *High arsenic concentrations in nature: a toxic environment for some microorganisms and optimum for others.* Poster presentation at the **International symposium of Extreme Ecosystems & Extremophile Organisms: Biodiversity, Physiology, Biochemistry & Biotechnology, Cuernavaca, Morelos, México, 2018.**

Rios-Valenciana, E.E., Briones-Gallardo, Celis, L.B. *From the native microbiota of arsenic-polluted sediments to As(V)-respiring microbial consortia: Transformation and mobilization of arsenic*. Oral presentation at the **IX Symposium of Thesis Advances of the División de Ciencias Ambientales, IPICYT, SLP, Mexico, 2018.**

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Rios-Valenciana EE, Briones-Gallardo R, Cházaro-Ruiz LF, Martínez-Villegas N, Celis LB. *Microbial processes of As(V)- and sulfate-reduction in arsenic mobilization from sediments of a hydraulic system: effect in arsenic precipitation.* Oral presentation at the **XVI National Congress of Biotechnology and Bioengineering, Guadalajara, Mexico, 2015.**