

THE ROLE OF MICROORGANISMS IN THE BIOGEOCHEMICAL CYCLE OF
ARSENIC IN THE ENVIRONMENT

by

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“He aprendido a derrapar y a chocar con la pared”

Antes de que cuente diez. Fito Cabrales

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ABSTRACT

Arsenic (As) is a highly toxic chemical that is widely distributed in groundwater around the world. As-bearing sulfide minerals (ASM) are known to contribute to high background concentrations of As in groundwater in regions where the geochemistry of the parent material is dominated by sulfide minerals. The fate of As in groundwater depends on the activity of microorganisms which can oxidize arsenite (As^{III}), or reduce arsenate (As^{V}). In oxidizing environments, As^{V} is the predominant species, and the accumulation of As is limited by the sorption of As onto iron (Fe) oxides and hydroxides. Under reducing environments, As^{III} is the predominant specie, and while the sorption strength of As^{III} on the Fe-surface of Fe (oxy)hydroxides is weaker, the accumulation of As in water can be limited by the precipitation of As as part of an ASM. The main aim of this research is to study the impact of microbial activity on the mobilization and immobilization of As in the environment.

The first objective of this research was to characterize the metabolic activity of three As^{III} -oxidizing bacteria, *Azoarcus* sp. pb-1 strain EC1, *Azoarcus* sp. pb-1 strain EC3 and *Diaphorobacter* sp. pb-1 strain MC, isolated from a non-contaminated, pristine environment. These As^{III} -oxidizing bacteria demonstrated a great metabolic flexibility to

use oxygen and nitrate to oxidize As^{III} as well as organic and inorganic substrates as alternative electron donors (e-donors) explains their presence in non-As-contaminated environments. The findings suggest that at least some As^{III} -oxidizing bacteria are flexible with respect to electron-acceptors and e-donors and that they are potentially widespread in low As concentration environments.

The second objective of this research was to investigate the stability of orpiment (As_2S_3) and arsenopyrite (FeAsS), at circumneutral pH and 30°C , under aerobic- and or anoxic conditions (nitrate amended as electron acceptor (e-acceptor)), in order to assess the feasibility of immobilizing As by formation of ASM as a long-term option for the bioremediation of As contamination. The percentage of As released from the minerals ranged from zero when FeAsS was biologically incubated to 87% for $\text{As}_2\text{S}_{3(\text{s})}$ under anoxic abiotic conditions. While the dissolution of ASM was greater in biological conditions, the presence of inoculum provided as sludge served as a sink for As, limiting the mobilization of As into aqueous phase. Thus, the mobilization of As from ASM can be controlled by altering the environmental conditions such as the redox conditions or by stimulating microbial activity.

Further research investigated the formation of ASM catalyzed by biological reduction of As^{V} and sulfate (SO_4^{2-}). In particular, the third objective of this research was to study the effect of the pH on the removal of As due to the biological-mediated

formation of ASM in an iron-poor system. A series of batch experiments were performed to study the reduction of SO_4^{2-} and As^{V} by an anaerobic mixed culture in a range of pH conditions (6.1-7.2), using ethanol as the e-donor. A marked decrease of the total aqueous concentrations of As and S and the formation of a yellow precipitate was observed in the inoculated treatments amended with ethanol, but not in the non-inoculated controls, indicating that the As-removal was biologically mediated. The pH dramatically affected the extent and rate of As removal, as well as the stoichiometric composition of the precipitate. The precipitate was composed of a mixture of orpiment and realgar, and the proportion of orpiment in the sample increased with increasing pH. The results suggest that ASM formation is greatly enhanced at mildly acidic pH conditions.

The fourth objective was to investigate the biomineralization of As through simultaneous As^{V} and SO_4^{2-} reduction in a minimal iron environment for the As-contaminated groundwater bioremediation. A continuous bioreactor, inoculated with an anaerobic sludge was maintained at circumneutral pH (6.25-6.50) and fed with As^{V} and SO_4^{2-} , utilizing ethanol as an e-donor for over 250 d. A second bioreactor running under the same conditions but lacking SO_4^{2-} was operated as a control to study the fate of As removal. The reactor fed with both SO_4^{2-} and As^{V} removed on the average 91.2% of the total soluble As, while less than 5% removal was observed in the control bioreactor without S. The biomineralization of As in the bioreactor was also evident from the formation of a yellow precipitate made of a mixture of As_2S_3 and AsS minerals. These

results taken as a whole indicate that a bioremediation process relying on the addition of a simple, low-cost e-donor offers potential to promote the removal of As from groundwater by precipitation of ASM.

The fifth objective was to evaluate the toxic impact that the exposure to soluble As or the formation of ASM could have on the anaerobic mixed culture used as inocula. The methanogenic community on the reactors was impacted by addition of As. The biogenic ASM inhibited the acetoclastic methanogens causing an accumulation of acetate. In the SO_4^{2-} -free bioreactor, the methanogens were initially highly sensitive to As^{III} (formed from As^{V} reduction) but quickly adapted to its toxicity. Consequently, the formation of ASM would impact the methanogenic activity of an anaerobic biofilm, while the exposure to As^{III} would not have a negative impact if the biofilm undergoes adaptation.

The sixth and final objective was to study the stability of a biogenic ASM at two different pH values (6.5 and 7.5) and under different redox conditions. The long-term stability was evaluated in three different bioreactors that operated for 145 d: aerobic (R1), anoxic (nitrate as alternative e-acceptor (R2) and anaerobic (R3). The dissolution of ASM was greatly affected by the pH, and slightly by the presence and nature of the e-acceptor. The ASM was very stable at pH 6.5, however, the As mobilization rate was up to 7-fold higher at pH 7.5, likely due to the formation of thioarsenic species. The stability of ASM

was also impacted by the e-acceptor present. The As mobilization rate was 77% higher under anaerobic conditions than under aerobic conditions, most likely due to the formation of secondary As-bearing minerals. Therefore, the stability of ASM depends on the conditions of the operation, and it can be controlled by altering the environmental conditions, such as the pH or the presence of the e-acceptor.

CHAPTER I:

INTRODUCTION

1.1. Arsenic occurrence in the environment and global concern

1.1.1. Overview

Arsenic (As) is a toxic metalloid which is naturally present in the environment and it can be found at a wide range of concentrations in the groundwater around the world, from less than 1 µg/L to more than 5,000 µg/L (Murcott 2012). Chronic As exposure increases the risk of cancer and cardiovascular diseases, skin lesions, and damages to the hematological, neurological, renal, hepatic, pulmonary and reproductive systems (ATSDR 2007, Ng et al. 2003). The World Health Organization (WHO) (WHO 1993) and the United States Environmental Protection Agency (US-EPA) (US-EPA 2001) have agreed that an As concentration of 10 µg/L in water can be considered an acceptable risk. Hence, the presence of As in drinking water supplies poses a serious health risk for the people where As levels are well above the acceptable limit. Fig. 1.1 illustrates the widespread occurrence of As in the world and the amount of people at risk of As poisoning. Arsenic groundwater concentration ranges from a few ppb to a thousand

depending on anthropogenic activities (Garelick et al. 2008) and different geological factors (Smedley and Kinniburgh 2002). However, whichever the source, microorganisms will play a main role controlling the fate of As in the environment, by mobilizing or immobilizing As. Therefore, this study has focused in enhancing the understanding of such microorganisms and their interactions with the As cycle.

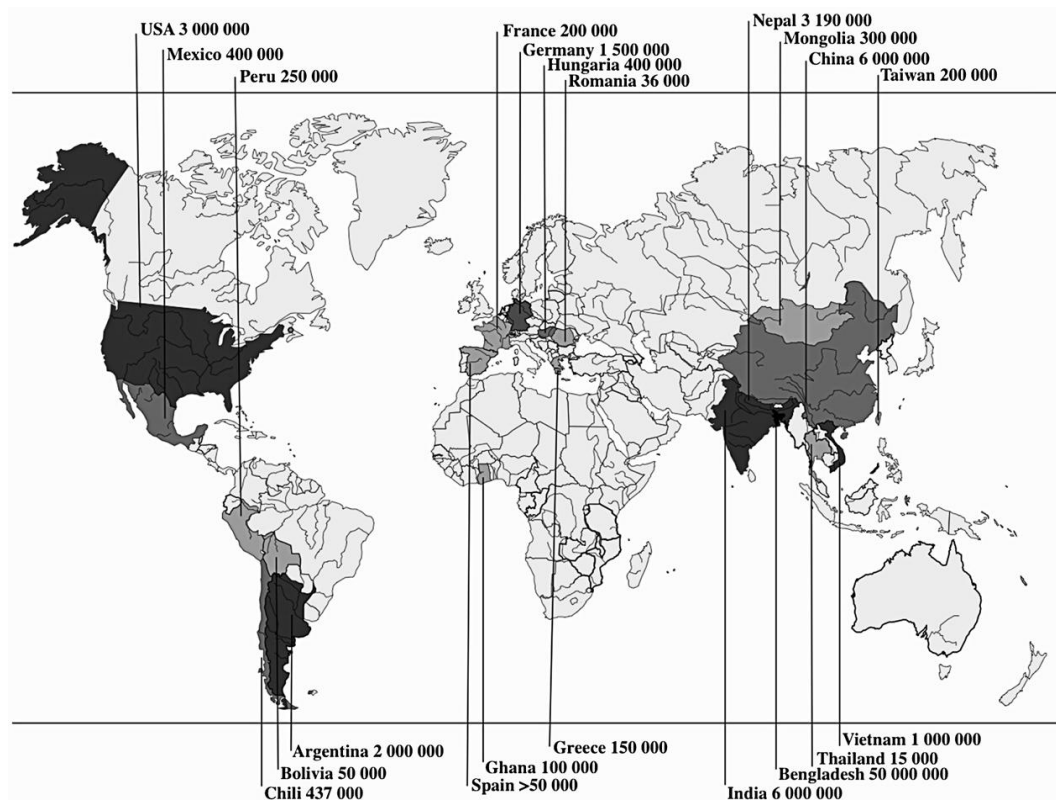


Fig. 1.1. Arsenic contamination in the world. The number of people exposed to As levels above 10 µg/L through their drinking water supply is indicated in the map. The shades of grey indicate the number of persons contaminated, working from the palest (the lowest numbers) to the darkest shade (the highest number) (Lievremont et al. 2009).

1.1.2. Arsenic sources in the environment

1.1.2.1. Natural sources of As

Arsenic ranks 20th in abundance in the Earth's crust, with an average concentration of 1-1.8 mg/kg (Henke 2009). The highest As concentrations are found in hydrothermal veins and sedimentary rocks, where As is associated with iron (Fe) and sulfur (S) minerals (Mandal and Suzuki 2002). The concentration of As in hydrothermal veins is usually three orders of magnitude greater than the global average As concentration (Webster and Nordstrom 2003). There are more than 300 As minerals, among them, 60% are arsenates, 20% are S-minerals, 10% are oxides, and the rest is composed of arsenates, arsenides, elemental As and metal alloys (Drahota and Filippi 2009). As-minerals are commonly associated with other minerals of cadmium, copper, nickel, silver, gold, antimony, phosphorous, tungsten and molybdenum (Garellick et al. 2008, Mandal and Suzuki 2002). The concentration of As in these mineralized areas can be as high as 40,000 mg/kg, in arsenical pyritic copper deposits (Mandal and Suzuki 2002). Arsenic abundance in sedimentary rocks made it more vulnerable to weathering processes that can dissolve the mineral and release the immobilized As into the environment (Welch et al. 2000). The concentration of As in groundwater depends on the local biogeochemistry of the aquifer sediments. High As concentrations are usually associated with to the oxidation of As-minerals, reductive dissolution or the desorption of

As from oxides and hydroxides, and the release of As from geothermal waters (Garelick et al. 2008). A more comprehensive understanding of complex processes governing the mobilization and immobilization of As is necessary in order to control and remediate As contamination.

1.1.2.2. Anthropogenic sources of As

The release of As in the environment due to anthropogenic activity can be direct (intentional use of As) or indirect (by-product of other industries). 52,800 t of As were globally produced in 2012 to use in agriculture as pesticide (4%), in glass manufacturing (3%), in the semiconductor industry (3%), wood preservative (89%), and other uses (1%) (Kelly and Matos 2003). The wood-preservation industry is the major consumer of As. Chromated copper arsenate (CCA) the most widely used wood preservative in the world, however the industry is trying to replace the use of CCA, since it is known to have contributed to the contamination of soil and water bodies. Arsenic use as pesticide in agriculture has substantially decreased since the 1980s; inorganic (lead and calcium arsenates, sodium arsenates and arsenic acid) and organic arsenicals (calcium acid methanearsonate, cacodylic acid) are applied to a variety of crops (ATSDR 2007). The use of poultry and swine manure as fertilizer could also contribute to As contamination, since roxarsone, 4-hydroxy-3-nitrophenylarsonic, a growth additive, has been used in animal and poultry feeds (Sierra-Alvarez et al. 2006). The mining and smelting industry

is the major responsible for the indirect release of As. The extraction processes of valuable metals (copper, gold) will expose reduced As-minerals to the environment and As will become mobilized (Williams 2001).

1.1.3. Arsenic speciation

1.1.3.1. Arsenic chemistry

Arsenic is classified as a transition metal or metalloid, it has atomic number 22 and its molecular weight is 74.92. Arsenic is a member of the group 15 of the periodic table, along with nitrogen, phosphorous, antimony and bismuth. Arsenic has four major oxidation states (-3), (0), (+3) and (+5). In crystalline structures, As can be found with the oxidation state of (-1) when forming covalent bonds with itself and most other elements, like Fe and S. Arsenic can also bond covalently with oxygen, carbon and hydrogen. Arsenic can also form complexes with metals like Fe or aluminum (Al) (Henke and Hutchison 2009). These chemical properties explains the association of As with Fe and S in minerals (Saunders et al. 2008), the formation of organoarsenicals (Reimer et al. 2010); and, the ability of As to adsorb in Fe (Root et al. 2007) and Al (Ghosh and Yuan 1987) sediments; and therefore, explaining the role of As in the environment.

1.1.3.2. Organoarsenicals

Arsenic biotransformation by microorganisms, plants and fungi can methylate As and produce organoarsenicals, monomethylarsonous acid (MMA^{III}), dimethylarsinous acid (DMA^{III}), methylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), and trimethylarsine oxide (TMAO^{V}). In strongly reducing conditions, methyl-As can be reduced producing volatile As compounds, arsine (AsH_3 , As with (-1) oxidation state), monomethylarsine ($\text{MMA}^{-\text{III}}$), dimethylarsine ($\text{DMA}^{-\text{III}}$), and trimethylarsine ($\text{TMA}^{-\text{III}}$). Other more complex organoarsenicals are arsenobetaine (AsB), arsenocholine (AsC), arsenosugars, arsenoriboses, and phenylarsonics. Intense biological activity will promote the formation of organoarsenical, which could account up to 70% in peatland porewater or 30% in forest soils. The use of organoarsenicals as pesticides and in the poultry industry also contributes to higher organoarsenical concentrations. However, the abundance of organoarsenicals in the environment is generally low, accounting for less than 5% of the total As concentrations (Huang et al. 2011).

1.1.3.3. Inorganic As

The oxyanions arsenate (As^{V}) and arsenite (As^{III}) are the predominant As aqueous species in circumneutral environments. Arsenic speciation is controlled mainly by pH (Henke and Hutchison 2009) and redox conditions (Ascar et al. 2008, Beauchemin and

Kwong 2006). As^{V} and As^{III} are compounds that have acid/base characteristics. The dissociation reactions and the corresponding dissociation constants are presented in Table 1.1. Hence, in circumneutral pH environments, As^{V} will be deprotonated (H_2AsO_4^- , HAsO_4^{2-}), and As^{III} would be present as the uncharged form (H_3AsO_3). The standard redox potential (E_h) for the $\text{As}^{\text{V}}/\text{As}^{\text{III}}$ pair is also dependent on the pH. E_h is approximately +300 mV at pH 4, -200 mV at pH 9 (O'Day 2006), and, 60 mV at pH 7 (Hoeft et al. 2004). In oxidizing environments, As^{V} is the predominant species, and the accumulation of As is limited by sorption processes of As on Fe oxides and oxyhydroxides surfaces (Jonsson and Sherman 2008); in reducing environments, As^{V} can be microbially reduced to As^{III} (van Lis et al. 2013). While As^{III} is also adsorbed onto Fe oxides and oxyhydroxides, its sorption strength with Fe surface complexation is weaker than As^{V} (Jonsson and Sherman 2008). In environments where Fe is lacking and S is present, the solubility of As is potentially controlled by the precipitation of As in ASM (O'Day et al., 2004). At circumneutral pH, As^{V} will be the dominant species in oxidizing environments, under As^{III} predominates in reducing conditions. The transformations between As^{III} and As^{V} are the key mechanisms dictating the mobilization or immobilization of As in the environment, and microorganisms play the main role on controlling them. (van Lis et al. 2013).

Table 1.1. Arsenic acid-basic chemistry (Henke and Hutchison 2009)

Arsenic specie	Dissociation reactions	pKa
As ^V reactions	$\text{H}_3\text{AsO}_4 \rightarrow \text{H}_2\text{AsO}_4^- + \text{H}^+$	2.3
	$\text{H}_2\text{AsO}_4^- \rightarrow \text{HAsO}_4^{2-} + \text{H}^+$	6.8
	$\text{HAsO}_4^{2-} \rightarrow \text{AsO}_4^{3-} + \text{H}^+$	11.6
As ^{III} reactions	$\text{H}_3\text{AsO}_3 \rightarrow \text{H}_2\text{AsO}_3^- + \text{H}^+$	9.2
	$\text{H}_2\text{AsO}_3^- \rightarrow \text{HAsO}_3^{2-} + \text{H}^+$	12.1
	$\text{HAsO}_3^{2-} \rightarrow \text{AsO}_3^{3-} + \text{H}^+$	13.4

1.1.4. Arsenic toxicity

Arsenic bioavailability and toxicity will depends on the specific As compound. The toxicity of arsenic methylated compounds is not well understood. The methylation of As was suspected to be a detoxification mechanism (Bentley and Chasteen 2002, Tchounwou et al. 2004), but it has been proven that trivalent methylated arsenicals are more toxic that inorganic As^{III} (Dopp et al. 2010). Among the inorganic As species, As^{III} is usually more toxic than As^V. The 50% lethal concentration, LC₅₀, of different As species in human cells is 2.16 μM for DMA^{III}, 5.49 μM for As^{III}, 571 μM for As^V, and 843 μM for DMA^V (Naranmandura et al. 2007). The higher toxicity of DMA^{III} (and

MMA^{III}) is believed to be associated with the formation of reactive oxygen species that will induce DNA damaging (Dopp et al. 2010).

Inorganic As toxicity mechanisms to cells can be explained by its chemical characteristics. First, the structure of As^V, analogue to phosphate, facilitate its uptake into the cell by phosphate transporters (Kruger et al. 2013). Second, As^V can replace phosphate in the oxidative phosphorylation disrupting ATP synthesis; or, it can substitute phosphate in DNA inhibiting DNA repair mechanism, which might explain As carcinogenesis (Mandal and Suzuki 2002). Third, the uncharged As^{III} (pKa 9.2) can passively enter the cell using aquaglyceroporins, which allow the transport of water, glycerol and other uncharged molecules (Slyemi and Bonnefoy 2012). Lastly, the affinity of As^{III} with S which caused its association with thiol groups on enzymes, impeding its normal function and expression. As^{III} is known to inhibit more than 200 enzymes in human cells (Lievremont et al. 2009). As^{III} will also bind with thiol groups on glutathione, glutaredoxin and thioredoxin, affecting the intracellular homeostasis, deoxyribonucleotide synthesis and repair, protein folding, sulfur metabolism, and xenobiotic detoxification (Slyemi and Bonnefoy 2012).

1.2. Microorganisms and the biogeochemical cycle of As

1.2.1. Overview

The different properties that As species have causes that the processes governing the transformations between the different As species will have an important impact on the fate of As in the environment. Microbial activity plays a major role in the transformations between As^{V} and As^{III} (Oremland and Stolz 2003, Paez-Espino et al. 2009, Rhine et al. 2005). Some heterotrophic bacteria will oxidize As^{III} or reduced As^{V} as a detoxifying mechanism, heterotrophic As^{III} -oxidizing bacteria (HAO) and As^{V} -resistant bacteria (ARB), respectively; and, numerous microorganisms can gain energy using As either as electron donor (chemolithotrophic As^{III} -oxidizing bacteria, AOB) or as electron acceptor (dissimilatory As^{V} -reducing bacteria, DARB). The microorganisms able to utilize As have demonstrated a great phylogenetic diversity, and they can be found in aquatic and terrestrial environments, as well as, in a wide range of environmental conditions. Fig. 1.2 illustrates the rich diversity of As-utilizing microorganisms (Cavalca et al. 2013).

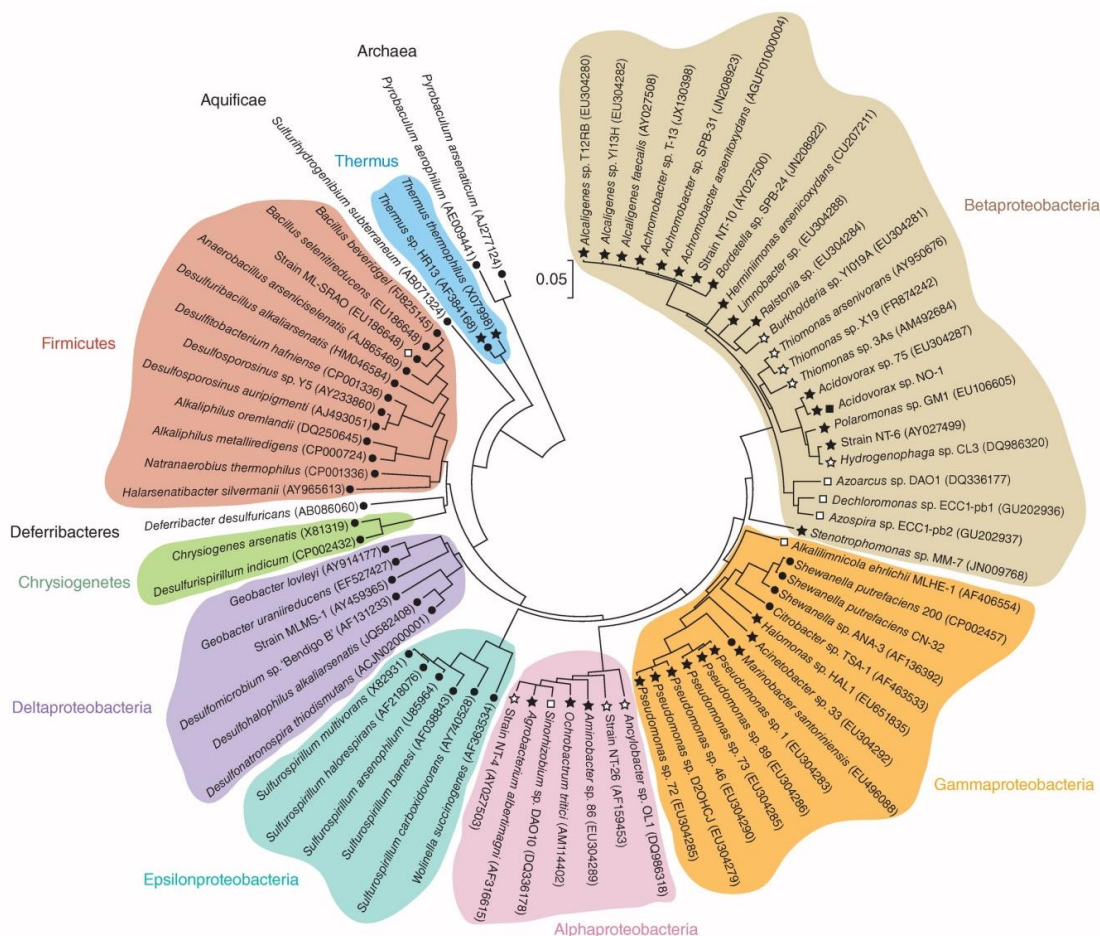


Fig. 1.2. Phylogenetic tree based on 16S rRNA sequences of Archaea and Bacteria that are involved in microbial transformations of arsenic. Organisms that can oxidize As^{III} aerobically (autotrophic, open stars; heterotrophic, solid stars), can oxidize As^{III} anoxically (autotrophic, open squares; heterotrophic, solid squares) and can respire As^V (solid circles). The scale bar represents 5% nucleotide change (Cavalca et al. 2013).

1.2.2. As^{III} oxidation

The great toxicity of As compounds has induced the development of detoxification mechanisms in microorganisms exposed to high As concentrations. HAO can oxidize the uncharged As^{III} to As^{V} , in order to impede the cellular uptake through the aquaglyceroporins. Since 1918, when bacteria capable of oxidizing As^{III} in aerobic environments were first recognized (Green 1918), numerous aerobic HOA have been identified in a wide variety of environments, from cattle-dipping fluids (Ehrlich 2002, Turner 1949) and mining sites (Santini et al. 2002) to garden soils (Bachate et al. 2012). This process was initially thought to be a detoxification mechanisms, however the aerobic oxidation of As^{III} is thermodynamically feasible ($E_{\text{h}} \text{O}_2/\text{H}_2\text{O} = + 820 \text{ mV}$) (Rhine et al. 2006) suggesting that microorganisms could gain energy from As^{III} oxidation. Furthermore, a study by Cruveiller et al. (2007) demonstrated that the swimming speed of *Herminiimonas arsenicoxydans* decreased after disrupting the As^{III} oxidation genes, confirming the energetic contribution from oxidizing As^{III} .

The other group of As^{III} -oxidizers, AOB, can take advantage of the energy released by the As^{III} oxidation to promote their growth and fix CO_2 as organic carbon. The first aerobic AOB was studied in 1982, *Pseudomonas arsenitoxidans* (Ilyaldinova and Abdrashitova 1981), followed by the isolation of the strain NT-26, an α -Proteobacterium isolated from a gold mine (Santini et al. 2000). Some AOB have shown

flexibility in using electron donors other than As^{III} , and to use alternative electron acceptors in environments where O_2 might have been depleted. The aerobic strains *Ancylobacter* OL-1, *Thiobacillus* S-1 and *Hydrogenophaga* CL-3, isolated by Garcia-Dominguez (Garcia-Dominguez et al. 2008), and, the aerobic strain *Thiobacillus denitrificans* WAO (Rhine et al. 2008) were able to growth using S compounds (elemental sulfur, sulfide or thiosulfate) as electron donors. As^{III} oxidation under anoxic conditions utilizing nitrate (NO_3^-) as an efficient alternative electron acceptor has been studied ($E_h \text{ NO}_3^-/\text{N}_2 = +740 \text{ mV}$, (Rhine et al. 2006)). In 2002, Oremland et al. (2002) isolated the strain *Alkalilimnicola ehrlichii* sp. MLHE-1 from an As-contaminated soda lake in California. MLHE-1 is capable of oxidizing As^{III} when linked to NO_3^- reduction to nitrite (NO_2^-). More recently, in 2006, Rhine et al. (2007, 2006), identified two facultative autotroph anaerobic As^{III} oxidizing strains from an As-contaminated soil, *Azoarcus* sp. DAO1 and *Sinorhizobium* sp. DAO10 that can link the As^{III} oxidation to the complete denitrification of NO_3^- to dinitrogen gas (N_2). DAO1 and DAO10 were also able to use a wide variety of organic compounds as electron donors. Sun isolated two pure cultures, *Dechloromonas* strain ECC1-pb1 and *Azospira* strain ECC1-pb2, which were able to link As^{III} oxidation to chlorate reduction to chlorine ($E_h \text{ ClO}_3^-/\text{Cl}_2 = +618 \text{ mV}$, (Sun et al. 2010).

Two different heterodimeric molybdoenzyme from the DMSO reductase family have been identified as responsible for As^{III} oxidation. As^{III} oxidase (Aio, also known as

Aox, Aro or Aso) was initially isolated from the strain *Alcaligenes faecalis* (Anderson et al. 1992, Ellis et al. 2001). Aio has been detected in various aerobic AOB in a great variety of environments (Heinrich-Salmeron et al. 2011, Inskeep et al. 2007, Yamamura et al. 2014), however, attempts to detect Aio in anaerobic AOB As^{III} have been unsuccessful (Hoeft et al. 2007, Rhine et al. 2006) suggesting that an alternative enzyme should be the responsible for the anaerobic As^{III} oxidation. The As^{V} reductase As^{III} oxidase (Arx) has been recently known for being an enzyme responsible for anaerobic As^{III} oxidation in *Alkalimnicola ehrlichii* MLHE-1 (Richey et al. 2009, Zargar et al. 2012, Zargar et al. 2010) as well as in *Ectothiorhodospira* strains PHS-1 and MLP2 (Zargar et al. 2012). Initially, Arx was mistaken with Arr (see section 1.2.3) which was thought to be a bidirectional enzyme for its ability to oxidize and reduce As *in vitro* (Richey et al. 2009) Further *in vivo* studies demonstrated that Arx is a unique enzyme closely related to Arr but unable to reduce As^{V} ; it is an As^{III} oxidize but considerably different to Aio (Zargar et al. 2012).

1.2.3. As^{V} reduction

The reduction of As^{V} to As^{III} as a detoxification mechanism is controlled by an As^{V} reductase. Three different families of As^{V} reductases have been recognize. First, the *arsC* gene from the *ars* operon of *Escherichia coli* plasmid R773, which will reduce As^{V} to As^{III} using glutaredoxin and glutathione as reducers. Second, a different *arsC* from the

Staphylococcus aureus plasmid pI258 which uses thioredoxin as reducer. Lastly, Acr2p from *Saccharomyces cerevisiae* that will also use glutaredoxin and glutathione as reducers. After As^{V} is oxidized to As^{III} , it can be then extruded from the cell by the transmembrane carrier protein or by an As^{III} -translocating ATPase (Rosen 2002).

The redox potential of the As redox pair will allow, not only As^{III} to be used as electron donor, but for As^{V} to be used as terminal electron acceptor by DARB, with several electron donors ($E_{\text{h}} \text{SO}_4^{2-}/\text{H}_2\text{S} = -220 \text{ mV}$ (Hoeft et al. 2004); $E_{\text{h}} \text{H}^+/\text{H}_2 = 0 \text{ mV}$). DARB were not first studied until 1994, when Ahmann et al. (1994) and Oremland et al. (1994) identified two closely related heterotrophic bacteria, *Sulfurospirillum arsenophilum* MIT-13 and *Sulfurospirillum barnessi* SES-3, respectively, that were able to respire As^{V} using lactate as electron donor. Nowadays, a wide number of DARB have been identified to be facultative As^{V} respirers (Huber et al. 2000, Krafft and Macy 1998, Macy et al. 1996, Macy et al. 2000, Newman et al. 1997b, Saltikov and Newman 2003), and the strain MLMS-1 (Hoeft et al. 2004), which will is an obligate As^{V} reducer. These microorganisms have the flexibility to utilize different substrates as electron donors, from lactate to sulfide (H_2S), as well as various electron acceptors other than As^{V} . The strain *Bacillus* JMM-4 (Santini et al. 2000, Santini et al. 2004) isolated from gold mine soil could reduce As^{V} or NO_3^- , *Bacillus arsenicoselenatis* EIH (Blum et al. 2009) can also utilize selenium, and a great number of them can respire both sulfate (SO_4^{2-}) and As^{V} ,

like the strain *Desulfotomaculum auripigmentum* OREX-4 (Newman et al. 1997a), including the strains MIT-13 and SES-3 described earlier in this paragraph.

Dissimilatory As^V reductase (Arr) is also a heterodimeric molybdoenzyme from the DMSO reductase family and it is the only enzyme recognize as responsible for the ability to use As as a terminal electron acceptor. There is not many information about Arr, since it has been characterized just in three DARB *Chrysiogenes arsenates* (Krafft and Macy 1998), *Bacillus selenitireducens* (Afkar et al. 2003) and *Shevanella trabarsenatis* ANA-3 (Malasarn et al. 2004, Saltikov and Newman 2003), despite the high number of As^V reducers have been identified in the recent years (Slyemi and Bonnefoy 2012, van Lis et al. 2013), suggest that the Arr sequence is not well conserved among the DARP (Malasarn et al. 2008).

1.3. Interaction between the As and sulfur cycles

The tendency over the past years has been to study the As and iron cycle relationship, but little research has addressed the impact of microbial reactions in the As biogeochemistry dominated by sulfur (S). Realgar (AsS), arsenopyrite (FeAsS) and orpiment (As₂S₃) are natural As- S²⁻ minerals (ASM), and their biogenesis constitute a clear example of the relationship between As and S in the environment (Welch et al. 2000). Fig. 1.3 describes the As and S biogeochemical cycles and the ASM formation, in

this case represented as As_2S_3 . The predominant species of S are H_2S and SO_4^{2-} , the most reduced and oxidized species, respectively. In the same fashion that the As biogeochemical cycle, microorganisms can catalyze the oxidation of H_2S (sulfoxidizing bacteria, SOB), under aerobic and anoxic conditions, and they are also able to gain energy from SO_4^{2-} reduction (dissimilatory SO_4^{2-} reducing bacteria, DARB) (Tang et al. 2009). The natural affinity of As towards S compounds has facilitated that As and S utilizing bacteria can be found associated in microbial communities. The aerobic strains OL-1, S-1, CL-3, WAO were able to growth using As^{III} or S compounds as electron donors (Garcia-Dominguez et al. 2008, Rhine et al. 2008). Also, some bacteria have been reported to have the ability to reduce As^{V} and SO_4^{2-} , like the *Sulfurospirillum arsenophilum* MIT-13 (Ahmann et al. 1994) or the *Desulfomicrobium* sp. str. Ben-RB and *Desulfovibrio* sp. str. Ben-RA (Macy et al. 2000). Therefore, the microbial activity can potentially mobilize As from the ASM, but it could also promote the precipitation of the mineral.

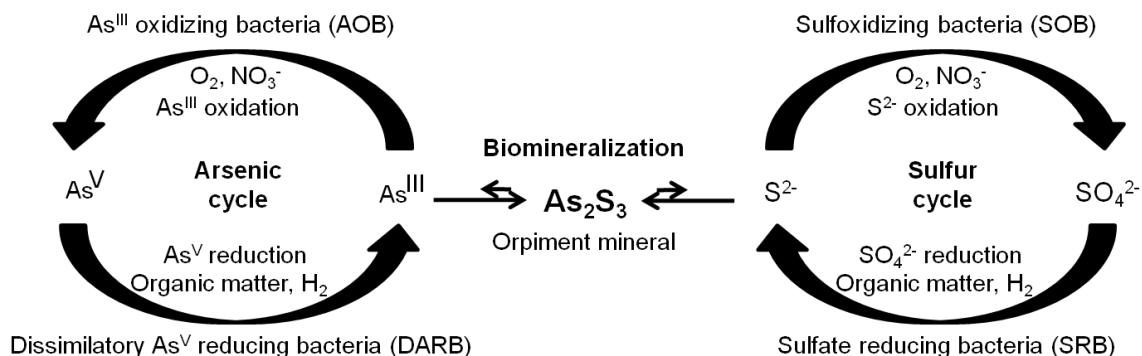
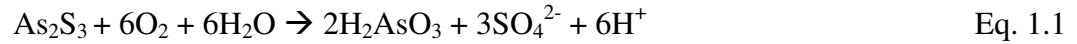


Fig. 1.3. The interaction between the As and S cycles can contribute to the immobilization of As and, consequently, to the prevention of As groundwater contamination.

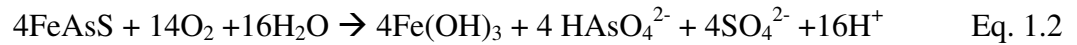
1.3.1. ASM as a source for As contamination

There are several factors that can affect the mobilization of As from ASM, among them, the pH and the redox conditions are the main ones to consider. In alkaline pH, ASM are not thermodynamically stable and it will lead to the formation of As-S aqueous species, thioarsenites (Suess and Planer-Friedrich 2012, Wilkin et al. 2003). In oxidizing environments, the minerals will undergo weathering liberating the soluble species of As and S into water. Microbial activity could enhance the mobilization of As from ASM (eg. *Acidithiobacillus ferrooxidans* (Jiang et al. 2008, Rhine et al. 2008, Tuovinen et al. 1994), however there are also evidences that some microorganism can decrease the dissolution rate of some ASM (e.g. *Thiobacillus caldus* (McGuire et al. 2001)).

The reaction that describe the dissolution process of amorphous orpiment in aerobic conditions was defined by Lengke and Tempel (2001) as the following:



For arsenopyrite, the reaction of dissolution can be described as:



There are many publications on the dissolution of ASM in extreme pH environments, with very low pH (Ehrlich 1963, 1964, Jiang et al. 2008, McKibben et al. 2008, Tuovinen et al. 1994). Just a few studies focus on the dissolution in circumneutral pH. Lengke and Tempel (2001) evaluated the dissolution of As from orpiment in the neutral to alkaline range of pH in aerobic conditions without any inoculum addition, using a continuous reactor. In 2002, Lengke and Tempel (2002) analyzed the dissolution of As from natural orpiment, in order to evaluate the effect of the orpiment solid state in the release of As. They obtained that the dissolution rate is lower by a factor of 0.002 to 0.560 in natural orpiment than in its amorphous form. These evidences suggest that orpiment is relatively stable in aerobic conditions over time in a circumneutral pH. Walker et al. (2006) studied the dissolution rate of arsenopyrite in aerobic conditions and circumneutral pH. Nevertheless, arsenopyrite seems to be relatively stable in aerobic

conditions over time. Lengke in his review of ASM dissolution rates (Lengke et al. 2009) suggested that an increase in dissolution rate with other oxidants than O₂ could be due to complexation reactions at the surface of the mineral that could promote the release of As from the solid.

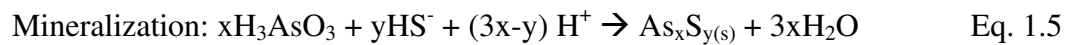
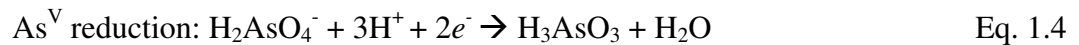
1.3.2. ASM as a sink for As in the environment

The interaction of the As and S biogeochemical cycles can also be harnessed to promote the immobilization of As. Recent evidence demonstrates the biological nature of the formation of ASM. Demergasso (2007) demonstrated the biological origin of As₂S₃ in Andean sediments by analyzing the sulfur isotope ratios (³⁴S/³²S) in chemically and biologically formed ASM, and comparing it with the minerals found in the sediments. In addition, Saunders (2008) evaluated the effect of SO₄²⁻ and electron donor addition on the As mobility in As contaminated groundwater, which resulted in a decrease of the dissolved As in the aquifer, most likely due to the formation of FeAsS. But the microbial role on the precipitation of ASM has not only been reported in mixed cultures, but also in pure cultures.

Newman (1997a) discovered a new bacterial strain *Desulfotomaculum auripigmentum* sp. OREX-4 which was able to precipitate As₂S₃ through the heterotrophic reduction of As^V and SO₄²⁻. Furthermore, *Caloramator* (Ledbetter et al.

2007) and *Shewanella* (Lee et al. 2007) have been reported to precipitate As_2S_3 , AsS and As-S nanotubes, respectively; and, a hyperthermophilic archaea genus, *Pyrobaculum* (Huber et al. 2000) can precipitate AsS. However, not every microorganism capable of reducing both As^{V} and SO_4^{2-} will precipitate ASM, the strain *Desulfobulbus propionicus* (Newman et al. 1997a) is unable to form an ASM because it quickly reduces SO_4^{2-} before reducing As^{V} , leading to the accumulation of H_2S in the medium and favoring the formation of thioarsenites (Wilkin et al. 2003); while, strain OREX-4 starts reducing As^{V} and then SO_4^{2-} . Consequently, the microbial reduction of As^{V} and SO_4^{2-} can enhance or limit the formation of ASM by controlling the rate of formation of As^{III} and H_2S .

The formation of ASM throughout the reduction of As^{V} and SO_4^{2-} can be summarized by the following reactions:



where $x = y = 1$, if realgar AsS is being formed; or, $y = 1.5x$ if As_2S_3 is the mineral formed.

Several lab-scale experiments, conducted in microcosm or bioreactors, have been performed to study the biological precipitation of iron-bearing ASM at circumneutral or acidic pH (Barringer and Reilly 2013, Keimowitz et al. 2007, Kirk et al. 2010, Omoregie et al. 2013, Onstott et al. 2011). However, the formation of ASM in systems lacking Fe will be relevant in SO_4^{2-} rich waters (O'day et al. 2004), since Fe-sulfide minerals, such as pyrite (FeS_2) or mackinawite (FeS) have lower solubility than the ASM and it will become immobilized the first. Battaglia-Brunet et al. (2012) studied the precipitation of ASM in a Fe-free bioreactor in acidic conditions (pH 5). Since the solubility of ASM is lower under acidic conditions and increases drastically around pH 7 due to the formation of thioarsenite species (Lengke et al. 2009, Wilkin et al. 2003), it is necessary to understand the process of formation of ASM in near neutral pH range.

CHAPTER II:

OBJECTIVES

2.1. General aim

Microorganisms play a major role in the fate of arsenic in the environment by controlling its speciation and therefore the mobilization of As in the water. The general aim of this research project is to investigate the impact of microbial activity on the mechanisms that drive the (im)mobilization of As in the environment.

2.2. Specific objectives

1. To understand the physiological role of microorganisms involved in the cycle of As in the environment, by the characterization of three As oxidizing bacterial strains, isolated from pristine, non-contaminated environments.

2. To study the stability of commercial orpiment and arsenopyrite under circumneutral pH, and the effect that the redox conditions or the presence of microorganisms could have in the dissolution of As-bearing minerals.

3. To investigate the biological formation of arsenic-sulfide minerals due to the microbial reduction of As^{V} and sulfate (SO_4^{2-}) and the effect of the pH on the effectiveness of the process.

4. To assess the feasibility of As biomineralization in continuous anaerobic bioreactors stimulating the concomitant As^{V} and SO_4^{2-} as a technique for the bioremediation of As-contaminated groundwater.

5. To evaluate the potential microbial toxicity of soluble As species and As-containing minerals formed in bioreactors operated to investigate the biomineralization of As-contaminated water.

6. To determine the stability of the biogenic As-bearing mineral under changing environmental conditions in order to understand the effect that changes in the pH or the redox conditions could have on the mobilization of As from the mineral.

CHAPTER III:

**FLEXIBLE BACTERIAL STRAINS THAT OXIDIZE ARSENITE IN
ANOXIC OR AEROBIC CONDITIONS AND UTILIZE HYDROGEN OR
ACETATE AS ALTERNATIVE ELECTRON DONORS**

3.1. Abstract

Arsenic is a carcinogenic compound widely distributed in the groundwater around the world. The fate of arsenic in groundwater depends on the activity of microorganisms either by oxidizing arsenite (As^{III}), or by reducing arsenate (As^{V}). Because of the higher toxicity and mobility of As^{III} compared to As^{V} , microbial-catalyzed oxidation of As^{III} to As^{V} can lower the environmental impact of arsenic. Although aerobic As^{III} -oxidizing bacteria are well known, anoxic oxidation of As^{III} with nitrate as electron acceptor has also been shown to occur. In this study, three As^{III} -oxidizing bacterial strains, *Azoarcus* sp. pb-1 strain EC1, *Azoarcus* sp. pb-1 strain EC3 and *Diaphorobacter* sp. pb-1 strain MC, have been characterized. Each strain was tested for its ability to oxidize As^{III} with four different electron acceptors, nitrate, nitrite, chlorate and oxygen. Complete As^{III} oxidation was achieved with both nitrate and oxygen, demonstrating the novel ability of these bacterial strains to oxidize As^{III} in either anoxic or aerobic conditions. Nitrate was only reduced to nitrite. Different electron donors were used to study their suitability in supporting nitrate reduction. Hydrogen and acetate were readily utilized by all the

cultures. The flexibility of these As^{III} -oxidizing bacteria to use oxygen and nitrate to oxidize As^{III} as well as organic and inorganic substrates as alternative electron donors explains their presence in non-arsenic-contaminated environments. The findings suggest that at least some As^{III} -oxidizing bacteria are flexible with respect to electron-acceptors and electron-donors and that they are potentially widespread in low arsenic concentration environments.

3.2. Introduction

Arsenic (As) is a toxic metalloid that is found in groundwater by the natural weathering of rocks (Smedley and Kinniburgh 2002). Long term exposure to As contaminated drinking water increases the risk of cancer in the skin, liver, bladder and lungs (ATSDR 2007). In response to an ever increasing awareness of the health risks associated with As, the maximum concentration level (MCL) of drinking water standard in the United States was made stricter by lowering it from 50 to 10 $\mu\text{g/L}$ in 2006 (USEPA 2001).

Arsenate (As^{V} , H_2AsO_4^- and HAsO_4^{2-}) and arsenite (As^{III} , H_3AsO_3) are the predominant As species in circumneutral environments. As speciation is controlled mainly by redox conditions (Ascar et al. 2008, Beauchemin and Kwong 2006) with As^{V} being the dominant species in oxidizing environments while As^{III} predominates in

reducing environments. As^{III} is adsorbed less than As^{V} in soil common metal oxides such as aluminum oxides (Hering 2005) and clay minerals (Goldberg 2002, Lin and Puls 2000), likewise As^{III} desorbs faster from iron (hydr)oxides than As^{V} (Tufano et al. 2008), therefore As^{III} is more mobile compared to As^{V} . Thus processes governing the transformations between the different As species will have an important impact on the fate of As in the environment. Microbial activity plays a major role in the transformation between As^{V} and As^{III} (Oremland and Stolz 2003, Paez-Espino et al. 2009, Rhine et al. 2005). As^{V} can be reduced to As^{III} by dissimilatory As^{V} reducing bacteria, when an electron donor, such as organic matter or hydrogen (H_2), is present in the environment. The dissimilatory As^{V} reductase (*arrA*) are the responsible genes found in the As^{V} reducing bacteria (Malasarn et al. 2004, Saltikov and Newman 2003). On the other hand, As^{III} also can be oxidized to As^{V} by As^{III} oxidizing bacteria when an electron acceptor becomes available. A large variety of bacteria have been reported to contain the As^{III} oxidase (*aroA*) responsible for oxidation of As^{III} under aerobic (Inskeep et al. 2007, Santini and vanden Hoven 2004, Silver and Phung 2005) or anaerobic conditions (Hoeft et al. 2007, Rhine et al. 2006, Sun et al. 2010). The oxidation readily occurs under aerobic conditions. Since 1918, when bacteria capable of oxidizing As^{III} in aerobic environments was first recognized (Green 1918), numerous aerobic As^{III} oxidizing bacteria have been identified (Krumova et al. 2008, Turner 1949, Wang and Suttigarn 2007). A biodiversity of As metabolizing bacteria isolated from a variety of soil water

systems have detectable As^{III}-oxidizing genes (Inskeep et al. 2007), indicating As^{III} oxidation plays an important role in the biogeochemical cycle of As.

Recently, As^{III} oxidation under anoxic conditions utilizing nitrate (NO₃⁻) as an efficient alternative electron acceptor has been studied. Results from an urban lake have shown that nitrate (NO₃⁻) levels in the anoxic zone are positively correlated with the formation of both As^V and As^V-adsorbing hydrous ferric oxides (Senn and Hemond 2002). NO₃⁻ injected into As-contaminated groundwater of Bangladesh was also shown to effectively lower the aqueous As concentration (Harvey et al. 2002). These results demonstrated the importance of NO₃⁻ as a controlling factor of As mobility in anoxic environments. In 2001, Hoeft *et al.* reported microbial As^{III} oxidation coupled with the reduction of NO₃⁻ to nitrite (NO₂⁻) in an arsenic-contaminated soda lake in California (Hoeft et al. 2002). Oremland and coworkers identified the strain *Alkalilimnicola ehrlichii* sp. MLHE-1 as a bacterial species responsible for oxidizing As^{III} when linked to NO₃⁻ reduction to NO₂⁻ (Oremland et al. 2002). More recently, in 2006, Rhine *et al.*, identified two anaerobic As^{III} oxidizing strains from an As-contaminated soil, *Azoarcus* sp. DAO1 and *Sinorhizobium* sp. DAO10 (Rhine et al. 2007, Rhine et al. 2006). Based on the stoichiometry of As^V-formed to NO₃⁻-consumed as well as the presence of nitrous oxides reductase (*nosZ*) genes, these strains, DAO1 and DAO10, appear to link the As^{III} oxidation to the complete denitrification of NO₃⁻ to dinitrogen gas (N₂).

The three strains characterized in this paper are *Azoarcus* sp. EC1-pb1, *Azoarcus* sp. EC3-pb1 and *Diaphorobacter* sp. MC-pb1 (to be referred as EC1, EC3 and MC in the rest of the manuscript). These strains were isolated from enrichment cultures (ECs) which were originally derived from sediments or sludge from pristine environments as inoculum (Sun et al. 2009). The ECs, from which strains EC1 and EC3 were isolated, linked As^{III} oxidation with complete denitrification as evidenced from measurements of N₂ production (Sun et al. 2009). The fact that these strains originate from environments with no known As contamination suggests they must have metabolic flexibility. The scope of this research was to better understand the flexibility of EC1, EC3 and MC to utilize different electron acceptors in the oxidation of As^{III} as well as different electron donors for the reduction of nitrate.

3.3. Materials and methods

3.3.1. As^{III}-oxidizing pure cultures

Azoarcus sp. EC1-pb1, *Azoarcus* sp. EC3-pb1 and *Diaphorobacter* sp. MC-pb1 were isolated from enrichment cultures EC1, EC3 and MC, respectively (Sun et al. 2009). The sequences of three isolated have been deposited in the GenBank with accession numbers: HM177479, FJ514096 and FJ514095, respectively.

3.3.2. Medium composition

The standard basal medium (pH 7.0-7.2) was prepared using ultra pure water (Milli-Q system; Millipore). The final composition of the basal medium in the batch experiments was (mg/L) K_2HPO_4 (8.33); NH_4Cl (617.5); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (173.3); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (23.3); CaCl_2 (23.3), and trace elements in concentration (mg/L): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (0.4); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.1); $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (0.018); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.006); ZnCl_2 (0.01); H_3BO_3 (0.01); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.01); $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ (0.032); $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01); EDTA (0.2); resazurin (0.04); HCl 36% (0.2 μL). 10 mM HCO_3^- (NaHCO_3) was used to buffer the pH of the medium.

Two different sets of experiments were carried out. In the first set of experiments, the electron acceptor was NO_3^- as KNO_3 (1.5 mM) and different electron donors were tested to be able to reduce NO_3^- , including NaAsO_2 (0.5 mM), CH_3COONa (0.11 mM), H_2 (0.4 mmol/ L_{liq}), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1mM), $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.125 mM) or $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.5 mM). In the second set of experiments, the electron donor was As^{III} added as NaAsO_2 (0.5 mM) and different electron acceptor were tested as potential As^{III} oxidants, at the concentration of KNO_3 (1.5 mM), NaNO_2 (0.5 mM), O_2 (0.4 mmol/ L_{liq}) or NaClO_3 (0.25 mM), respectively. The basal medium with the electron acceptor was sterilized by autoclaving, 20 min at 121°C, while NaHCO_3 and the electron donor solution were sterilized using membrane filtration (0.22 μm).

3.3.3. Pure cultures maintenance

The three pure cultures were maintained in the absence of oxygen (O_2) in a basal medium amended with 0.5 mM As^{III} as electron donor and 5 mM NO_3^- as electron acceptor. The pure cultures were incubated in 160 mL serum bottles, with a total liquid volume of 110 mL. After the medium was autoclaved, bottles were closed using butyl rubber septa in order to ensure an anaerobic atmosphere. A flush gas mixture of N_2/CO_2 (80%:20%) was used to purge the headspace and the medium for exclusion of O_2 . The gas was introduced through a 0.22 μm filter to sterilize it (needle in-needle out). As^{III} solution was added by using a 0.22 μm filter in order to sterilize the solution and then the medium was inoculated with the pure culture (6.4% v:v). The culture bottles were incubated in the dark on an orbital shaker (115 rpm) at 30°C. Pure cultures were transferred to fresh medium every 10-14 d once the complete oxidation of As^{III} to As^V was proved with 85% As^{III} converted to As^V .

3.3.4. Experimental incubations

Batch experiments were performed in 160 mL serum bottles. All assays were conducted in duplicate. In order to avoid the contamination of carry-over NO_3^- from old cultures to fresh medium during the inoculation, the cultures (10% volume of previous culture) were centrifuged in 15 mL sterilized centrifuge tubes at 1,400 g for 20 min. The

pellets were resuspended into same volume of sterilized MiliQ water, and after two cleaning cycles they were transferred to the experiments. Several controls were run in parallel. Abiotic controls were prepared without adding pure culture to prove biological nature of the reaction. Controls lacking the electron donor or the electron acceptor but inoculated were included to demonstrate that the reaction just occurs when both reactants are added to the medium. The culture conditions were the same as those described for culture maintenance. He/CO₂ (80%:20%) was utilized as an alternative flushing gas in the experiment in which aerobic oxidation of As^{III} was studied to avoid N₂ interference when measuring O₂. In the experiments in which H₂ was added as electron donor, a H₂ stock bottle was prepared. An empty 160 mL serum bottle closed with a butyl rubber septa was flushed through a 0.22 µm filter with pure N₂ gas during 20 min and with H₂/CO₂ (80%:20%) for an extra 20 min. In a similar way, a 100% O₂ stock bottle was prepared to use in the aerobic experiments, but the serum bottle was flushed with pure O₂ for 30 min. From these bottles, the adequate volume of gas for each experiment (1.6 mL H₂/CO₂ and 1 mL O₂) was taken with a syringe and injected in the corresponding assays.

3.3.5. Analytical Methods

Aliquot samples were taken from sealed anaerobic serum flasks by piercing the stoppers using sterile syringes with 16-gauge needles. All samples were centrifuged (10 min, 14,000 g) immediately after sampling and stored in polypropylene vials. As^V, sulfate

(SO_4^{2-}), chlorate (ClO_3^-), NO_3^- and NO_2^- were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 analytical column (4 x 250 mm) and AG16 guard column (4 mm x 40 mm). During each injection the eluent (KOH) used was 30 mM for 10 min. Mn^{2+} was measured by using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system model Optima 2100 DV from Perkin–Elmer TM (Shelton, CT, USA). Fe^{2+} was quantified by the 5-ortho-phenanthroline colorimetric method, using an UV-visible spectrophotometer (Agilent 8453, Palo Alto, CA, USA). Total Fe was obtained by reducing Fe^{3+} with a hydroquinone solution in pH 4.5 (0.05 M acetate buffer) (Fortune and Mellon 1938).

Headspace samples were taken with a pressure lock gas tight syringe (1710RN, 100 μL (22s/2"/2), Hamilton Company). N_2 , O_2 and nitrous oxide (N_2O) were analyzed using a Hewlett Packard 5890 Series II gas chromatograph fitted with a CarboxenTM 1010 Plot column (30 m x 0.32 mm) and a thermal conductivity detector. Helium (He) was used as the carrier gas. H_2 and acetic acid were detected in an Agilent Technologies 7890A gas chromatography system. A CarboxenTM 1010 Plot Fused Capillary Column (30 m x 0.53 mm) with a thermal conductivity detector was used to analyze H_2 with N_2 used as the carrier gas. A Restek Stabilwax®-DA Column (30 m x 0.35 mm, ID 0.25 μm) with flame ionization detector, and He used as a carried gas, was used to detect acetic acid.

DNA concentration was measured with a TBS-360 Mini-Fluorometer (Turner Biosystems, Sunnyvale, CA, USA) for DNA quantification using PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Inc., Eugene, OR, USA) (Ahn et al. 1996).

Metabolizing genes including *nosZ*, RuBisCO genes, *aroA* and *arrA* genes were PCR amplified with specifically designed primer sets. Successfully purified PCR products were cloned to identify the sequences. The methodology and conditions for these techniques are described in the Appendix A.

Table 3.1 Stoichiometric calculations of the biological catalyzed reactions mediated by three pure culture strains EC1, MC and EC3 *

Part A	$\Delta(\text{NO}_3^-)$	$\Delta(\text{NO}_2^-)$	$\Delta(\text{As}^{\text{V}})$	$\frac{\Delta(\text{As}^{\text{V}})}{\Delta(\text{NO}_3^-)}$
	mM	mM	mM	
EC1	0.480	0.487	0.481	1.00
MC	0.531	0.494	0.483	0.91
EC3	0.583	0.490	0.468	0.80

Part B	$\Delta(\text{O}_2)$	$\Delta(\text{As}^{\text{V}})$	$\frac{\Delta(\text{As}^{\text{V}})}{\Delta(\text{O}_2)}$
	mmol/L _{liq}	mM	
EC1	0.217	0.488	2.25
MC	0.2436	0.556	2.28
EC3	0.233	0.561	2.41

Part C	$\Delta(\text{H}_2)$	$\Delta(\text{NO}_2^-)$	$\Delta(\text{NO}_3^-)$	$\frac{\Delta(\text{H}_2)}{\Delta(\text{NO}_3^-)}$
	mmol/L _{liq}	mM	mM	
EC1	0.355	0.353	0.387	0.92
MC	0.352	0.364	0.328	1.07
EC3	0.352	0.359	0.336	1.05

*: The stoichiometry has been calculated as the moles of electron donor consumed (moles of As^{V} produced are equivalent to the moles of As^{III} oxidized) per mole of electron acceptor utilized. Molar relationship of the As^{III} oxidation under denitrifying conditions (Part A), and aerobic conditions (Part B). Molar relationship of the H_2 oxidation under denitrifying conditions (Part C)

3.4. Results

3.4.1. Anoxic As^{III} oxidation linked to NO_3^- reduction

Fig. 3.1A shows that As^{III} was oxidized to As^V in the full treatments amended with 0.5 mM As^{III} as electron donor and 1.5 mM NO_3^- as electron acceptor in the presence of inoculum. As^V formation began after a lag phase of 1.5 d, and complete oxidation was achieved after 3 d of incubation by observing the formation of 0.49 ± 0.01 mM of As^V as an average for the three pure cultures. No conversion of As^{III} to As^V was observed in control treatments run in parallel.

The end product of the NO_3^- reduction was evaluated. NO_3^- and NO_2^- concentrations were measured throughout the experiment (shown in Fig. 3.1B and 3.1C, respectively). NO_3^- concentration decreased from 1.7 ± 0.2 mM to 1.1 ± 0.1 mM, which occurred concomitantly with NO_2^- production of 0.49 ± 0.01 mM in the full treatments, whereas no appreciable NO_2^- formation was observed in control assays. These results imply that for each mole of NO_3^- reduced, approximately one mole of As^{III} is oxidized and one mole of NO_2^- is formed (Table 3.1A) in accordance with the reaction shown in equation 3.1:



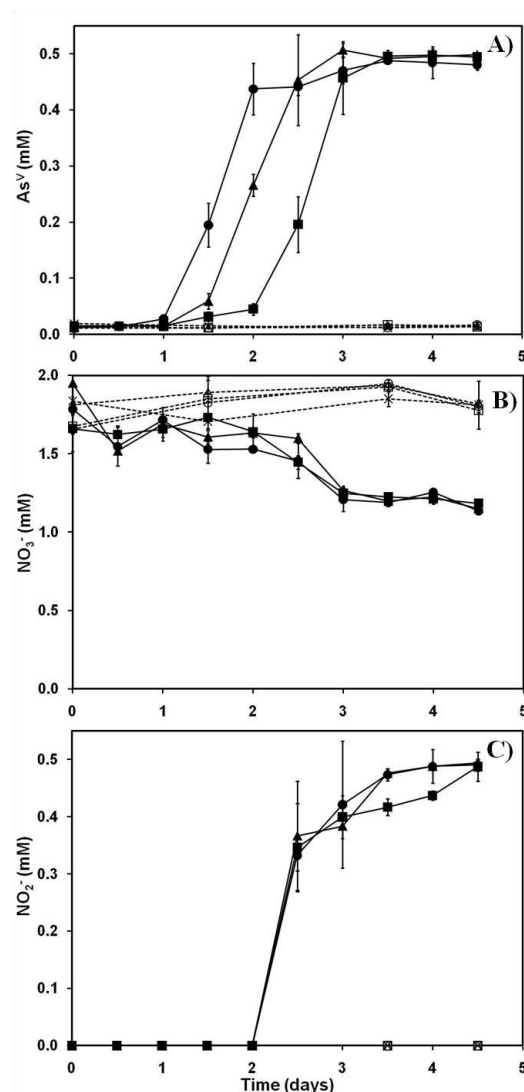


Fig. 3.1 As^V formation by pure cultures EC1, EC3 and MC for oxidation of As^{III} (0.5 mM) linked to NO₃⁻ (1.5 mM) reduction. Legends: panel A, As^V formation, complete treatments containing As^{III} and NO₃⁻ and inoculated with EC1 (■), EC3 (●) and MC (▲); biological controls (dashed lines) containing As^{III} but lacking NO₃⁻: EC1 (□), EC3 (○) and MC (Δ); non inoculated control (x); panel B, NO₃⁻ consumption; panel C, NO₂⁻ formation. Biological treatments supplied with NO₃⁻ and As^{III}, EC1 (■), EC3 (●) and MC (▲); biological controls (dashed lines) supplied with As^{III} but no NO₃, EC1 (□), EC3 (○) and MC (Δ); non inoculated control (x).

3.4.2. Aerobic As^{III} oxidation

The three pure cultures were tested for their capacity to oxidize As^{III} in aerobic conditions. As^V formation was observed when 0.35 ± 0.06 mmol O₂/L_{liq} was added as electron acceptor as shown in Fig. 3.2. In Fig. 3.2A, the first evidence of As^V formation was observed after only 1 d of incubation and the complete oxidation was achieved after 2.5 d in all the cultures. No As^{III} conversion was observed in the control assays (lacking either inocula or O₂). The O₂ concentration was also monitored (Fig. 3.2B). As shown in Table 3.1B, O₂ concentration was 0.32 ± 0.03 mmol/L_{liq} in the controls after 3.5 d while the concentration in the full treatments was 0.112 ± 0.01 mmol/L_{liq}, confirming the fact that biological As^{III} oxidation is linked to the O₂ consumption. The measured molar ratios (As^{III}:O₂) of the reaction were 2.25 to 2.4, depending on the strain, approximately corresponding to the reaction shown in equation 3.2:



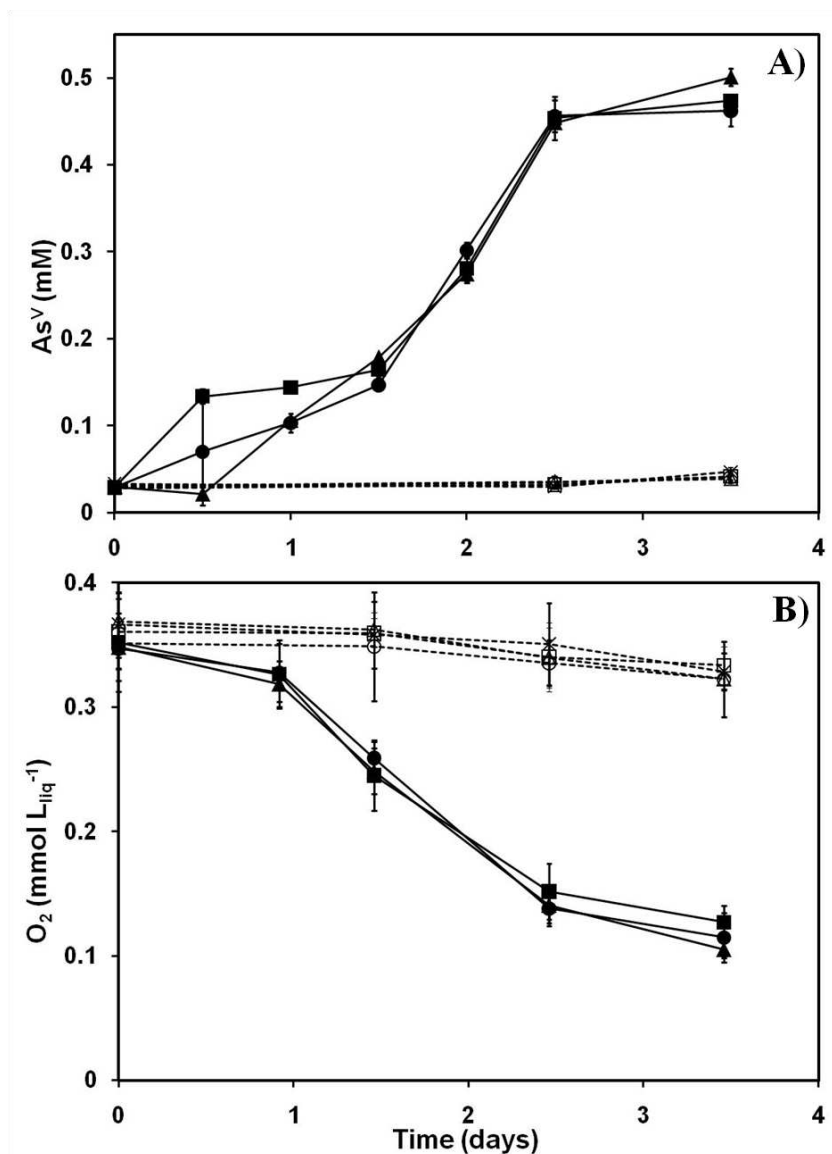


Fig. 3.2 As^V production by pure cultures EC1, EC3 and MC with O₂ as electron acceptor. Oxidation of 0.5 mM of As^{III} with 0.35 mmol O₂/L_{liq}. Legends: panel A, As^V formation. Biological treatments supplied with O₂ and As^{III}, EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) supplied with As^{III} but no O₂, EC1 (□), EC3 (○) and MC (Δ); non inoculated control (x); panel B, O₂ consumption. Full treatments supplied with O₂ and As^{III}, EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) supplied with O₂ but no As^{III}, EC1 (□), EC3 (○) and MC (Δ); non inoculated control (x).

3.4.3. Oxidation of As^{III} by alternative electron acceptors

Aside from NO₃⁻ and O₂, NO₂⁻ and ClO₃⁻ were also tested as potential electron acceptors. The summary of the results obtained from testing electron acceptors is shown in Table 3.2. Strains MC and EC3 were able to link a partial oxidation of As^{III} when NO₂⁻ was present in the active treatments as electron acceptor. An As^V concentration of 0.24 ± 0.04 mM was reached in less than 18 d, and no further increase was observed after an additional 7 d of incubation. As^V formation was not detected when strain EC1 was used as the inoculum. No changes in As speciation occurred in the control bottles (lacking either NO₂⁻ or inocula) with an As^V concentration of 0.068 ± 0.01 mM at the end of the experiment. ClO₃⁻ was not utilized as an electron acceptor by any of the pure cultures even after 24 d of incubation for strains MC and EC3. A small As^{III} oxidation was observed by strain EC1, but it accounted for less than a 10% in 54 d of incubation; the concentration of As^V in the full treatment was 0.121 ± 0.051 mM, while in the control bottles was constant at 0.070 ± 0.02 mM throughout the entire experiment.

Table 3.2 Summary of As^{III} oxidation * with various electron acceptors tested

Pure Cultures	Electron acceptors ⁺							
	KNO ₃		NaNO ₂		O ₂		NaClO ₃	
	As ^V formation (mM)	Time to completion (days)	As ^V formation (mM)	Time to completion (days)	As ^V formation (mM)	Time to completion (days)	As ^V formation (mM)	Time to completion (days)
EC1	0.496 ± 0.011	4	0.053 ± 0.052	54	0.473± 0.002	2.5	0.121 ± 0.051	54
MC	0.496 ± 0.004	3	0.240 ± 0.008	24	0.500 ± 0.010	2.5	0.072 ± 0.002	24
EC3	0.485 ± 0.028	3	0.284 ± 0.116	24	0.472 ± 0.018	2.5	0.063 ± 0.021	24

*: As^{III} concentration was 0.5 mM.

⁺: Concentrations of the different electron acceptor tested were 1.5 mM KNO₃, 0.5 mM NaNO₂, 0.35 mmol O₂/L_{liq} and 0.25 mM NaClO₃. Electron acceptors that have been positively utilized by the cultures to oxidize As^{III} are shown in bold text.

3.4.4. Use of alternative electron donors under NO_3^- -reducing and aerobic conditions

In order to test the ability of the three pure cultures to reduce NO_3^- with different substrates under anaerobic conditions, four inorganic electron donors and an organic electron donor were evaluated. The three strains were able to reduce NO_3^- when H_2 or acetate was amended to the treatment, but the other electron donors tested, ferrous iron (Fe^{2+}), sulfide (S^{2-}) and manganese (Mn^{2+}), were not utilized by the cultures to reduce NO_3^- .

Fig. 3.3A shows the consumption of H_2 linked to the NO_3^- consumption represented in Fig. 3.3B. H_2 and NO_3^- removal were first noticeable after 1 d, and the reaction was completed by day 1.5. H_2 concentration decreased from 0.356 ± 0.014 mmol $\text{H}_2/\text{L}_{\text{liq}}$ to 0.0036 ± 0.0001 mmol $\text{H}_2/\text{L}_{\text{liq}}$ in the complete treatments, while it was not oxidized in the controls treatments (lacking either NO_3^- or inocula). In parallel, NO_3^- concentration decreased an average of 0.350 ± 0.024 mM in the course of the experiment in the complete treatments and no NO_3^- removal was observed in the control treatments (lacking either H_2 or inocula). NO_3^- was reduced reduction to NO_2^- which accumulated to a concentration of 0.359 ± 0.004 mM at the end of the experiment (Fig. 3.3C). The experimental results indicated a molar ratio of H_2 consumed and NO_3^- reduced of 1.12 (Table 3.1C). This was close to the stoichiometric molar ratio of 1 shown in equation 3.3:



H_2 was also tested as a possible electron donor under aerobic conditions. To pursue this experiment, the three strains were incubated with an atmosphere consisting of 0.503 mmol $\text{H}_2/\text{L}_{\text{liq}}$ and 0.251 mmol $\text{O}_2/\text{L}_{\text{liq}}$ and the H_2 consumption was monitored. The initial H_2 concentration was completely consumed in less than 20 hours in bottles containing O_2 and inoculated with pure cultures. No H_2 consumption was observed in the controls (lacking either inoculum or O_2), confirming a biological mediated oxidation of H_2 by O_2 .

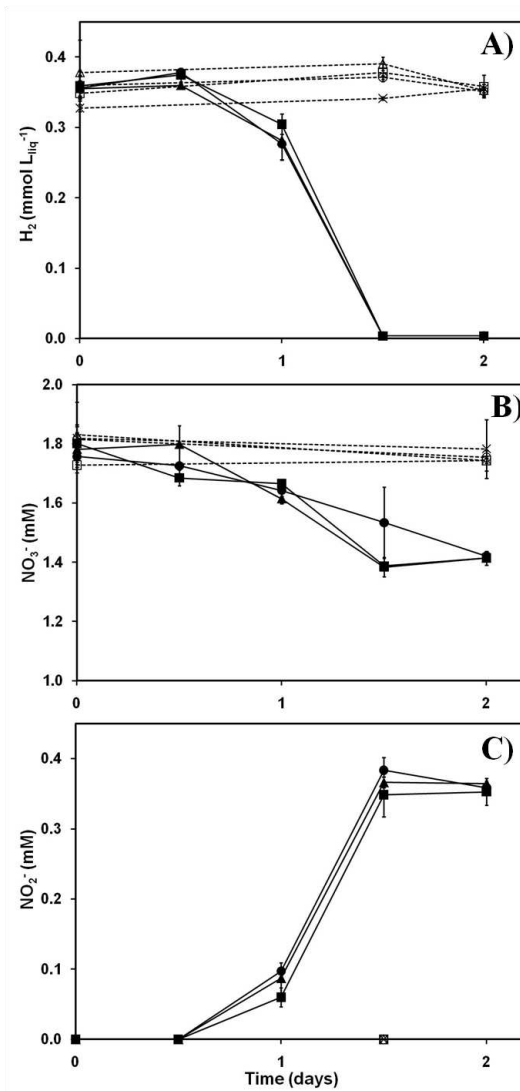


Fig. 3.3 H_2 oxidation linked to NO_3^- reduction. Treatments with 0.35 mmol H_2/L_{liq} and 1.5 mM of NO_3^- . Legends: panel A, H_2 oxidation biological treatments supplied with NO_3^- and H_2 , EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) supplied with H_2 but no NO_3^- , EC1 (□), EC3 (○) and MC (Δ); non inoculated control (x); panel B, decrease of NO_3^- concentration; panel C, NO_2^- formation. Full treatments supplied with NO_3^- and H_2 and inoculated with EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) with only H_2 and no NO_3^- : EC1 (□), EC3 (○) and MC (Δ); non inoculated control (x).

3.4.5. As^{III} oxidation after pre-incubation of cultures with alternative electron donors and acceptors

The ability of the pure cultures to oxidize As^{III} with NO₃⁻ was evaluated after being previously incubated with H₂/NO₃⁻ or H₂/O₂ (Fig. 3.4A and 3.4B, respectively). In both cases, As^{III} oxidation linked to NO₃⁻ reduction was confirmed following the pre-incubation. Complete As^{III} oxidation took place in less than 1 d in both experiments. NO₃⁻ consumption was also confirmed in the full treatments with inoculum and As^{III}. These results indicate that the use of alternative electron acceptors and donors did not suppress the capacity of the bacteria to oxidize As^{III}. The pre-incubation with H₂/NO₃⁻ or H₂/O₂ likely increased the cell concentration in the assays, which may have been the reason for a faster oxidation of As^{III} compared to earlier experiments in which As^{III} grown cells were used as inoculum.

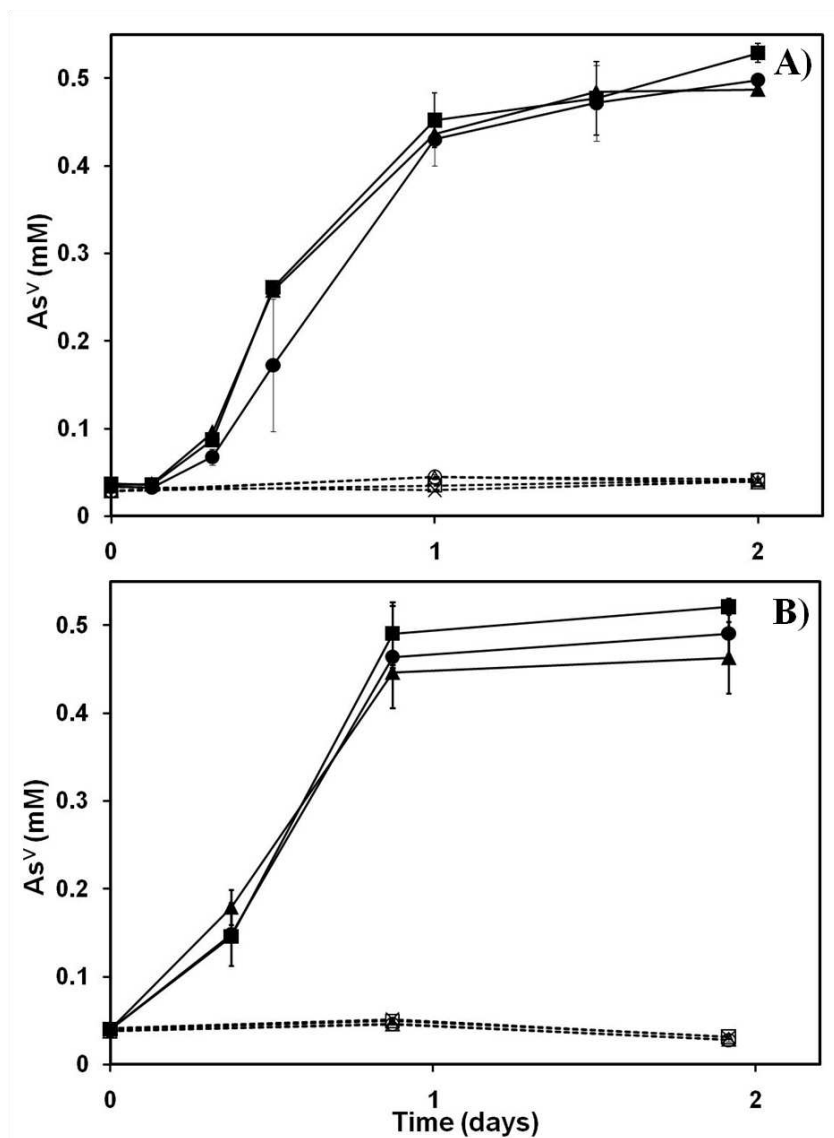


Fig. 3.4 Ability of the pure cultures to oxidize As^{III} under denitrifying conditions after being incubated with: panel A, 0.3 mM NO₃⁻ and 0.4 mmol H₂/L_{liq} or panel B, 0.45 mmol H₂/L_{liq} and 0.3 mmol O₂/L_{liq}. Treatment with 0.5 mM of As^{III} and 0.3 mM of NO₃⁻: EC1 (■), EC3 (●) and MC (▲). Controls (dashed lines) with only 0.5 mM of As^{III} and no NO₃⁻: EC1 (□), EC3 (○) and MC (Δ). Non inoculated controls (x).

The difference in cell concentration when the cultures are incubated either with $\text{NO}_3^-/\text{As}^{\text{III}}$ or H_2/O_2 was estimated by quantifying the DNA at the beginning and at the end of the experiments. These two experiments were the two extreme scenarios, the lowest cell yield ($\text{NO}_3^-/\text{As}^{\text{III}}$) and the highest cell yield (H_2/O_2). In both cases, DNA production was shown to be clearly linked to the use of As^{III} or H_2 as a growth substrate (Fig A.1 and Fig A.2 in Appendix A). There was no increase in DNA concentration in non-inoculated controls, nor in controls lacking either the electron acceptor or electron donor. The concentration of DNA was approximately five times greater at the end of the experiment when the pure cultures were incubated with H_2/O_2 compared with $\text{NO}_3^-/\text{As}^{\text{III}}$. This outcome also confirms the hypothesis that the cell yield was significantly increased with pre-incubation with the better growth substrate, H_2 .

3.4.6. Functional gene PCR and cloning

Functional gene PCR targeting *aroA* and *arrA* genes, *nosZ* and RuBisCO genes were performed on all three As^{III} -oxidizing, NO_3^- -reducing pure cultures. Various primer sets were used to detect the existence of *aroA* or *arrA* genes. No PCR products were observed for any of the primer sets for *aroA*, *nosZ* which are genes responsible for As^{III} oxidation (Inskeep et al. 2007) and nitrous oxide reductase (Scala and Kerkhof 1998), respectively. PCR products were found for primer set #1 of *arrA*, a gene encoding for dissimilatory As^{V} reductase (Saltikov and Newman 2003) but is related in structure to

As^{III} oxidizing enzymes believed to catalyze the reverse reaction of the dissimilatory reductase (Richey et al. 2009). However, the sequence of the amplicons did not correspond to *arrA*. PCR products were obtained from a primer set targeting the large unit, *cbbL*, of ribulose-1,5-biphosphate carboxylase oxygenase genes (RuBisCO genes, an indicator of autotrophy) in all the three pure cultures (Fig. A.3 in Appendix A). The amplicons were 800 bp in agreement with the expected size (Elsaied and Naganuma 2001). The amplicons of two of the strains, EC1 and EC3, were also checked by cloning and sequencing. The sequences were deposited in GenBank with accession numbers JN008173 and JN008174, respectively. The EC1 and EC3 derived sequences corresponded 95% and 80% similarity to the RuBisCO large unit *cbbL* genes of *Nitrosomonas* sp. ENI-11 [GenBank accession number AB061373] (Hirota et al. 2002) and an uncultured organism [GenBank accession number AB505078] (Kojima et al. 2009), respectively.

3.5. Discussion

The three As^{III}-oxidizing bacteria presented in this study, EC1, EC3 and MC, were found to be flexible in their ability to use different electron acceptors and electron donors. The ability to oxidize As^{III} in anoxic nitrate-reducing as well as aerobic conditions is unique and has not been observed before by a single strain. The strains therefore have the ability to adapt to changing redox conditions in the environment.

Functional gene PCR was used to determine which genes might be involved in arsenic metabolism. As^{III} oxidase (Aro) and As^{V} respiratory reductase (Arr) are two As metabolizing enzymes within the dimethyl sulfoxide (DMSO) reductase family (Silver and Phung 2005) that are involved in growth linked metabolism of As^{III} and As^{V} , respectively. However this approach provided no definitive evidence for the presence of *aroA* nor *arrA* genes in any of the three pure cultures.

As^{III} was not the only substrate that can be used as electron donor by the isolated strains. H_2 and acetate were readily oxidized under NO_3^- -reducing conditions. Also H_2 was shown to be utilized as electron-donor with O_2 . The flexibility to utilize different substrates in order to support growth seems to be a common characteristic of many As^{III} oxidizing isolates. The facultative, As^{III} oxidizing chemoautotroph *Alkalimnicola ehrlichii* strain MLHE-1, belonging to the γ -Proteobacteria group, was able to grow with As^{III} , H_2 , sulfide, thiosulfate and acetate while reducing NO_3^- to NO_2^- . Aerobic oxidation was also possible with H_2 and acetate (Hoeft et al. 2007). Two autotrophic As^{III} oxidizing denitrifying bacteria, *Azoarcus* strain DAO1 and *Sinorhizobium* strain DAO10 were also capable of using H_2 , acetate, glucose, lactate and other organic substrates as electron donors (Rhine et al. 2006). Three newly isolated aerobic As^{III} oxidizing strains *Ancylobacter* strain OL-1, *Thiobacillus* strain S-1 and *Hydrogenophaga* strain CL-3 can also oxidize different sulfur species (thiosulfate, elemental sulfur and sulfide) to sulfate (Garcia-Dominguez et al. 2008). Ammonium was also found to be an electron donor for

strains S-1 and CL-3 and the latter can also oxidize NO_2^- to NO_3^- under aerobic conditions.

None of the three bacterial strains characterized in this study were able to completely denitrify NO_3^- to N_2 . Instead, NO_3^- was partially reduced to NO_2^- , which accumulated as a product at the end of the experiments. The enrichment cultures, EC1 and EC3, from which *Azoarcus* sp. strains EC1 and EC3 were isolated, respectively, were shown to produce N_2 gas as the final product of denitrification linked to As^{III} oxidation (Sun et al. 2009). Also, strain DAO1 isolated by Rhine *et al.* (Rhine et al. 2006), closely related to *Azoarcus* (96% 16S rRNA gene sequence similarity to strains EC1 and EC3) was able to perform complete denitrification. The dilution to extinction technique used to isolate the pure cultures may have favored subpopulations lacking the full set of denitrifying genes, in particular the *nosZ* gene. The functional PCR assay confirmed the absence of *nosZ* genes which corresponds to their inability to completely denitrify of NO_3^- to N_2 .

The presence of RuBisCO genes, a key enzyme of CO_2 fixation for cell growth (Miziorko and Lorimer 1983) in all of the strains studied here, provides DNA-based evidence of autotrophic nature of pure cultures. Two amplicon sequences were confirmed to be related to RuBisCO large unit *cbbL* genes. There are several examples of autotrophic anoxic As^{III} -oxidizing bacteria reported before. *Azoarcus* sp. DAO1,

Sinorhizobium sp. DAO10 and *Alkalimnicola ehrlichii* sp. MLHE1 were shown to contain RuBisCO genes (Hoeft et al. 2007, Rhine et al. 2006). Likewise, many aerobic As^{III}-oxidizing bacteria also contain RuBisCO genes such as *Ancylobacter* strain OL-1, *Thiobacillus* strain S-1 and *Hydrogenophaga* strain CL-3 (Garcia-Dominguez et al. 2008).

The most well studied aerobic As^{III}-oxidizing strains have been isolated from the As bearing sediments of a gold mine in Australia (Santini et al. 2000, Santini et al. 2002). Earlier anoxic As^{III}-oxidizing isolates have either been isolated from an alkaline lake, with high levels of As, in California (Oremland et al. 2002, Oremland et al. 2005, Oremland et al. 2004) or were isolated from As contaminated soil and sediments (Rhine et al. 2006). The three As^{III}-oxidizing bacteria presented in this study (EC1, EC3 and MC) were isolated from anoxic As^{III}-oxidizing EC and a mixed culture originating from pristine, non-contaminated environments (Sun et al. 2009).

The ability of As^{III}-oxidizing strains to survive in environments in which As is present in negligible concentrations is most likely related to their flexibility in using different electron donors. The isolates are able to survive with environmentally relevant electron donors such as H₂ and acetate, which can be expected in anaerobic conditions where organic matter is decomposing. Therefore, anoxic As^{III} oxidizing bacteria like

those described in this study are probably more widespread in the environment than previously thought due to the high level of flexibility in the use of substrates.

CHAPTER IV:
MICROBIAL MOBILIZATION OF ARSENIC FROM
ORPIMENT AND ARSENOPYRITE
WITH OXYGEN OR NITRATE AS ELECTRON ACCEPTOR UNDER
CIRCUMNEUTRAL PH CONDITIONS

4.1. Abstract

Arsenic (As) is a metalloid of extreme concern due to its high toxicity and widespread occurrence in groundwater. As-sulfide minerals (ASM) are known to serve as a natural source of arsenic in the environment. This study investigated the abiotic and biological dissolution of two ASM, orpiment (As_2S_3) and arsenopyrite (FeAsS), at circumneutral pH and 30°C , in aerobic- and or anoxic conditions where nitrate was amended as electron acceptor. The experiments were conducted in microcosms where the minerals were incubated at 30°C under circumneutral pH conditions. The percentage of As released from the minerals ranged from zero when FeAsS was biologically incubated to 87% for $\text{As}_2\text{S}_{3(s)}$ under anoxic abiotic conditions. $\text{As}_2\text{S}_{3(s)}$ was readily dissolved under aerobic conditions at higher rates than FeAsS . In anoxic conditions, the biological As mobilization from $\text{As}_2\text{S}_{3(s)}$ was in the same rate as for aerobic conditions, whereas the

release of As from FeAsS was enhanced in anoxic conditions, even though nitrate was not used as electron acceptor for the oxidation of FeAsS. The presence of inocula provided as sludge served as a sink for As, therefore abiotic conditions favored the As mobilization, although the dissolution of the ASM was greater in biological conditions. Thus, As mobilization from ASM can be controlled by altering the environmental conditions such as the redox conditions or by stimulating microbial activity.

4.2. Introduction

Arsenic (As) awareness has increased over the past years due to its high toxicity and its widespread occurrence in nature. Arsenic is a toxic metalloid that is present in various concentrations in the groundwater all over the world (ATSDR 2007). It is known for its carcinogenic effects, which led the U.S. Environmental Protection Agency (EPA) to set a maximum contaminant level (MCL) of 10 µg/L of As in drinking water in 2006 (US-EPA 2001). Improved understanding of the biogeochemical cycle of As, as well as its interaction with the biogeochemical cycle of other elements (most notably sulfur and iron), is critical to devise strategies to prevent As dissolution into groundwater or promote As immobilization. Several efforts are being carried out to understand the factors that contribute to As (im)mobilization in the environment.

Arsenic-containing sulfide minerals (ASM), such as orpiment (As_2S_3) and arsenopyrite (FeAsS) are known to contribute to high background As groundwater concentrations (Welch et al. 2000). There are several factors that can affect the As mobilization from ASM, among them redox conditions, pH and the presence of microorganisms are the main ones to consider. As_2S_3 and FeAsS are reduced minerals which will undergo oxidation when exposed to oxidizing conditions (Lengke et al. 2009).

Variations in the pH of the aquifer will affect the dissolution of ASM. The dissolution of As_2S_3 and FeAsS has been studied in a wide range of pH from highly acidic to strongly alkaline values. While dissolution of As_2S_3 has been proven to increase with increasing pH (Lengke and Tempel 2001, 2002, Lengke 2002, Mamedov and Mikhailov 1997), studies on the pH effect on FeAsS dissolution have shown little to no dependence on the pH (Asta et al. 2010, Walker et al. 2006, Yu et al. 2007), with the exception of the results provided by McKibben et al. (2008) where the dissolution of FeAsS increased when increasing the pH, measured by iron (Fe) mobilization instead of As like in the previous studies.

The dissolution of FeAsS in the presence of alternative oxidants other than oxygen (O_2) has been analyzed in few studies using either Fe or nitrate (NO_3^-). Fe, either added as FeCl_3 or $\text{Fe}_2(\text{SO}_4)_3$, contributed to a higher dissolution of FeAsS (Yu and Zhu 2002, Yu et al. 2007, Yu et al. 2004), whereas nitrate (NO_3^-) has not show any effect on

the dissolution rate of FeAsS (McKibben et al. 2008). The dissolution of As₂S₃ has been characterized in reducing conditions (Mamedov and Mikhailov 1997) incubated with sulfide (H₂S) but without an alternative electron acceptor, which has shown an increase in the dissolution rate of As₂S₃, most likely due to the formation of As-S aqueous species, thioarsenites (Suess and Planer-Friedrich 2012, Wilkin et al. 2003) .

The effect of microbial activity has been studied in acidic pH, demonstrated that the presence of some microorganisms can enhance the mobilization of As from ASM (e.g., *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*) (Corkhill et al. 2008, Ehrlich 1963, 1964, Jiang et al. 2008, Tuovinen et al. 1994), however there is evidence that some microorganism (sulfur-oxidizing bacteria) can decrease the dissolution rate of some ASM (e.g., *Thiobacillus caldus*) even when contributing to the oxidation of H₂S to sulfate (SO₄²⁻) (McGuire et al. 2001). Despite the fact of the important role that microorganisms appear to play in the dissolution of ASM, no work has been conducted in evaluating the effect of microbial activity at other pH range than acidic in the mobilization of As from ASM. Furthermore, the characterization of the biological dissolution of ASM has been focused in aerobic conditions, regardless of the role alternative electron acceptors such as NO₃⁻ are known to play in the microbial dissolution of other sulfide minerals (pyrite) in the environment (Haaijer et al. 2007, Torrento et al. 2010, Torrento et al. 2011)

To sum up, the mobilization of As from ASM is influenced by different factors therefore is complicated to predict which will be the fate of ASM in natural environments. First, it is clear that As mobilization can be enhanced in alkaline and oxidative environments, but As is also released in neutral and reductive conditions. Second, while aerobic dissolution of ASM has been widely study, little research has been conducted about the effect of alternative electron acceptors in abiotic conditions, and none has been done in biological conditions with an electron acceptor other than O₂. Last, microbial activity may enhanced also the mobilization of As, but its effect is not completely clear. For these reasons, this study assesses the mobilization of As from As₂S₃ and FeAsS under aerobic or anoxic conditions, using NO₃⁻ as electron acceptor. The effect that aerobic and anaerobic biofilms could have in the mobilization of As has also been tested and compared to the same experiments in abiotic conditions.

4.3. Materials and methods

4.3.1. Materials

High purity commercial powdered As₂S₃ was purchased from ESPI Metals (Ashland, OR, USA). FeAsS was obtained from a general mineral supplier. Minerals were conserved in an inert atmosphere of N₂ gas to avoid oxidation and deterioration of

the samples. All the chemicals were analytical grade and the solutions were prepared using ultrapure water (Milli-Q system; Millipore).

4.3.2. Source of microorganisms

Aerobic biomass was a return activated sludge (“RA” sludge) obtained from a local wastewater treatment plant (Ina Road, Tucson, Arizona, USA) containing 0.139 g volatile suspended solids (VSS) per gram wet wt. Three different anaerobic granular sludges were obtained from different full scale upflow anaerobic sludge bioreactors (UASB) : (i) bioreactor treating recycled paper wastewater at Eerbeek, The Netherlands), (“EB” sludge”, 0.135 g VSS/g wet wt); (ii) UASB treating sugar beet distillery effluent at Bergen op Zoom, The Netherlands (“ND” sludge, 0.065 g VSS/g wet wt); (iii) A sulfide oxidizing bacteria enrichment culture was obtained from a UASB reactor (V= 0.25 L) initially inoculated with Eerbeek sludge (“ES” sludge). The reactor was fed with basal mineral medium supplemented with 20 mM thiosulfate (as $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$) and 37.5 mM nitrate (as KNO_3). After a stabilization period of 65 d, all the thiosulfate was successfully oxidized to SO_4^{2-} . After more than 170 days of operation, the reactor was stopped and the enriched biomass was stored anaerobically in the same basal mineral medium at 4°C.

4.3.3. Mineral dissolution experiments

The stability of amorphous As_2S_3 and FeAsS was evaluated under aerobic and anoxic conditions. Batch experiments were performed in duplicate using in glass serum bottles (160 mL) supplied with basal medium (120 mL) and either of As_2S_3 (104.2 g/L, 1 mM As) or FeAsS (75 g/L, 0.5 mM As). Assays were inoculated with 0.5 g VSS/L of biomass. Subsequently, the flasks were sealed with rubber septa and aluminum crimp caps. In the aerobic experiments, O_2 was provided at a concentration of 2.7 mmol $\text{O}_2/\text{L}_{\text{liq}}$, which was added by flushing the treatments with $\text{O}_2/\text{He}/\text{CO}_2$ (20:60:20, v/v). In the anoxic experiments, nitrate was used as the terminal electron acceptor and it was amended as KNO_3 at a concentration of 1.4 mM. Anoxic assays were flushed with N_2/CO_2 (80:20, v/v) to exclude oxygen. Non-inoculated abiotic controls, and controls with no electron acceptor or with no mineral were run in parallel. Assays were incubated at 30°C in an orbital shaker at 115 rpm.

The standard basal mineral medium (pH 7.1-7.2) was prepared using ultrapure water and contained (mg/L): K_2HPO_4 (638); KH_2PO_4 (1,700); NH_4Cl (595); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (166); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (21.3); NaHCO_3 (4,250), and 2 mL/L of a trace element solution containing (mg/L): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (2,000); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (2,000); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (500); $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (90); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (30); ZnCl_2 (50); H_3BO_3 (50); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4$

H₂O (50); Na₂SeO₃·5 H₂O (100); NiCl₂·6H₂O (50); EDTA (1,000); resazurin (200); HCl 36% (1 mL). The system bicarbonate:CO₂ was used as buffer system.

4.3.4. Mineral characterization

FeAsS was ground and pulverized and further analyze using a CAMECA SX50 electron microprobe at the University of Arizona. All analyses were performed at 15 hV, 20 nA, and a peak count time of 40 seconds. Natural FeAsS was used as the standard for Fe, As, and S. A WDS scan of all spectrometers at 15 kV, 200 nA, for 8 min indicated that the arsenopyrite in the sample might contain a very small amount of Ni at the level of 10s of ppm. Backscattered electron imaging revealed the presence of a few small (approx. 5 µm), widely separated inclusions of a lead sulfide mineral containing minor amounts of Sb, Ag, and Fe.

4.3.5. E_h-pH diagrams

E_h-pH diagrams were used to understand the stability of As₂S₃ and FeAsS under the experimental conditions previously described as a function of the pH and the redox potential (E_h). The As₂S₃ diagram was prepared for an As concentration of 0.5 mM and S concentration of 0.75 mM, while for the FeAsS experiments the concentrations of As, S and Fe were 0.5 mM. The thermodynamic data was obtained from MinTEQ and NBS

databases and the diagrams were built using the W32-Stabcal modeling software (Huang and Young 1996).

4.3.6. Analytical methods

Liquid samples were taken from sealed anaerobic serum flasks by piercing the stoppers using syringes with 16-gauge needles. All samples were centrifuged (10 min, 14,000 g) immediately after sampling and stored in polypropylene vials at 4°C. Arsenate (As^{V}), SO_4^{2-} , NO_3^- and nitrite (NO_2^-) were analyzed by suppressed conductivity ion chromatography (IC) using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 analytical column (4 x 250 mm) and a AG16 guard column (4 x 40 mm). Total As in the samples was determined by adding the same volume than the sample of an oxidant solution containing 1.1 g/L KIO_3 and 4.7 g/L HCl to oxidize any possible As compound in solution to As^{V} . The samples were incubated for an hour to allow the reaction to occur and then the total As was measured by using the IC. The As^{III} concentration was calculated by subtracting the As^{V} concentration from the total As concentration. Measurements of pH, E_h and VSS were conducted according to standard methods (APHA 1999).

Table 4.1. Summary of the relevant reactions for the dissolution of orpiment and arsenopyrite in aerobic or denitrifying conditions

Minerals oxidation:		
$\text{As}_2\text{S}_{3(s)} + 18\text{H}_2\text{O} \rightarrow 2\text{AsO}_3^{3-} + 3\text{SO}_4^{2-} + 36\text{H}^+ + 24\text{e}^-$		
$\text{FeAsS}_{(s)} + 10\text{H}_2\text{O} \rightarrow \text{AsO}_3^{3-} + \text{SO}_4^{2-} + \text{Fe}(\text{OH})_3 + 17\text{H}^+ + 12\text{e}^-$		
Arsenite oxidation:		
$\text{AsO}_3^{3-} + \text{H}_2\text{O} \rightarrow \text{AsO}_4^{3-} + 2\text{H}^+ + 2\text{e}^-$		
Reduction reactions:		
$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$		
$\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$		
$2\text{NO}_2^- + 8\text{H}^+ + 6\text{e}^- \rightarrow \text{N}_2 + 4\text{H}_2\text{O}$		
$2\text{NO}_3^- + 12\text{H}^+ + 10\text{e}^- \rightarrow \text{N}_2 + 6\text{H}_2\text{O}$		
Combined relationships:		
<i>Aerobic conditions:</i>		
$\text{As}_2\text{S}_{3(s)} + 6\text{O}_2 + 6\text{H}_2\text{O} \rightarrow 2\text{AsO}_3^{3-} + 3\text{SO}_4^{2-} + 12\text{H}^+$	Eq. 4.1	
$\text{FeAsS}_{(s)} + 3\text{O}_2 + 4\text{H}_2\text{O} \rightarrow \text{AsO}_3^{3-} + \text{SO}_4^{2-} + \text{Fe}(\text{OH})_3 + 5\text{H}^+$	Eq. 4.2	
$\text{AsO}_3^{3-} + \text{O}_2 \rightarrow \text{AsO}_4^{3-}$	Eq. 4.3	
<i>Denitrifying conditions:</i>		
$\text{As}_2\text{S}_{3(s)} + 12\text{NO}_3^- + 6\text{H}_2\text{O} \rightarrow 2\text{AsO}_3^{3-} + 3\text{SO}_4^{2-} + 12\text{NO}_2^- + 12\text{H}^+$	Eq. 4.4	
$\text{As}_2\text{S}_{3(s)} + 8\text{NO}_2^- + 2\text{H}_2\text{O} \rightarrow 2\text{AsO}_3^{3-} + 3\text{SO}_4^{2-} + 4\text{N}_2 + 4\text{H}^+$	Eq. 4.5	
$\text{As}_2\text{S}_{3(s)} + 4.8\text{NO}_3^- + 3.6\text{H}_2\text{O} \rightarrow 2\text{AsO}_3^{3-} + 3\text{SO}_4^{2-} + 2.4\text{N}_2 + 7.2\text{H}^+$	Eq. 4.6	
$\text{FeAsS}_{(s)} + 6\text{NO}_3^- + 4\text{H}_2\text{O} \rightarrow \text{AsO}_3^{3-} + \text{SO}_4^{2-} + \text{Fe}(\text{OH})_3 + 6\text{NO}_2^- + 5\text{H}^+$	Eq. 4.7	
$\text{FeAsS}_{(s)} + 4\text{NO}_2^- + 2\text{H}_2\text{O} \rightarrow \text{AsO}_3^{3-} + \text{SO}_4^{2-} + \text{Fe}(\text{OH})_3 + 2\text{N}_2 + \text{H}^+$	Eq. 4.8	
$\text{FeAsS}_{(s)} + 2.4\text{NO}_3^- + 2.8\text{H}_2\text{O} \rightarrow \text{AsO}_3^{3-} + \text{SO}_4^{2-} + \text{Fe}(\text{OH})_3 + 1.2\text{N}_2 + 2.6\text{H}^+$	Eq. 4.9	
$\text{AsO}_3^{3-} + \text{NO}_3^- \rightarrow \text{AsO}_4^{3-} + \text{NO}_2^-$	Eq. 4.10	
$3\text{AsO}_3^{3-} + 2\text{NO}_2^- + 2\text{H}^+ \rightarrow 3\text{AsO}_4^{3-} + \text{N}_2 + \text{H}_2\text{O}$	Eq. 4.11	
$5\text{AsO}_3^{3-} + 2\text{NO}_3^- + 2\text{H}^+ \rightarrow 5\text{AsO}_4^{3-} + \text{N}_2 + \text{H}_2\text{O}$	Eq. 4.12	

4.4. Results

This study investigated the mobilization of As from two common arsenic sulfide minerals, As_2S_3 and FeAsS , under circum-neutral conditions ($\text{pH} = 7.18 \pm 0.19$). The contribution of abiotic and microbial processes to As dissolution was evaluated in batch experiments maintained under oxic- and NO_3^- -reducing conditions. ASM dissolution was monitored by analyzing the products expected to accumulate in the liquid medium: As^{III} , As^{V} , and SO_4^{2-} . In addition to these species, NO_3^- as well as NO_2^- , an intermediate product of NO_3^- reduction, were monitored in NO_3^- -reducing assays.

4.4.1. Dissolution of ASM under aerobic conditions

4.4.1.1. As_2S_3 dissolution

The oxidation and dissolution of As_2S_3 under aerobic conditions was analyzed in the presence or absence of microbial inoculum (Fig.4.1 and Table 4.2). The total As concentration released into solution after 111 d of incubation was slightly higher in the abiotic treatment ($11.6 \pm 1.9\%$ of the total As supplied) compared to the microbial treatment using RA as inocula ($8.6 \pm 1.0\%$) (Fig. 4.1C). Arsenic release in the inoculated control lacking O_2 corresponded to a dissolution of $6.5 \pm 0.9\%$ of the total As supplied (Fig. 4.1C). Arsenic was not detected in the control with no mineral addition. During the

first 65 d of incubation, most of the As mobilized was in its reduced form, As^{III} , corresponding to an average of $83.1 \pm 3.6\%$ of the total As in solution in the abiotic treatment, $46.9 \pm 17.1\%$ in the inoculated treatment supplied with O_2 and $96.2 \pm 5.4\%$ in the inoculated control lacking O_2 (Figs. 4.1A and 4.1B). After day 65, As^{III} was gradually oxidized to As^{V} and on day 111 As^{V} was the major species in solution, accounting for $85.7 \pm 15.5\%$ in the abiotic treatment, $109.1 \pm 1.4\%$ in the inoculated treatment with O_2 , and $83.1 \pm 2.6\%$ in the inoculated control with no O_2 .

Moderate release of SO_4^{2-} into solution was observed in all the assays containing orpiment. SO_4^{2-} increased quickly during the first 19 d of incubation in all the treatments containing As_2S_3 (Fig. 4.1D) In the abiotic treatment, the SO_4^{2-} released accounted for up to $17.1 \pm 1.5\%$ of the total S supplied as ASM; whereas the highest SO_4^{2-} concentration determined in the microbial assays was $32.4 \pm 1.9\%$ and $36.1 \pm 5.5\%$ in the presence and absence of O_2 , respectively. The amount of SO_4^{2-} determined in the inoculated treatments was up to 5.6-fold higher compared to the stoichiometric level expected for the amount of As released, according with Eq. 4.1 described in Table 4.1, whereas in the abiotic treatments it was just 1.5 times higher.

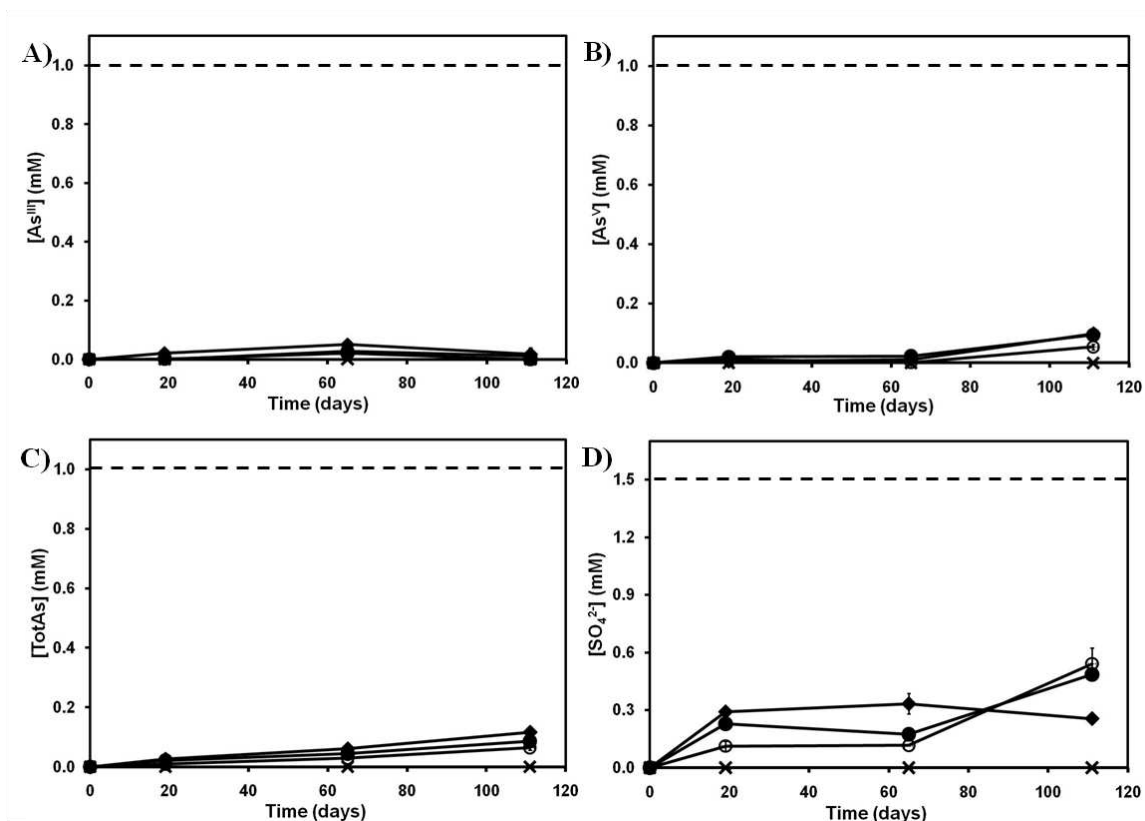


Fig. 4.1. Dissolution of orpiment (1 mM as As) under aerobic conditions (20% O₂ in headspace) in batch assays with or without aerobic RAS sludge. Legends: Dissolved concentration of: As^{III} (A); As^V (B); total As (C); and SO₄²⁻ (D). Complete microbial treatment (●); non-inoculated abiotic control (◆); inoculated control lacking O₂ (○); inoculated control lacking orpiment (x, dashed line). The horizontal dashed lines indicate the maximum concentrations of As and SO₄²⁻ that would be released if orpiment would be completely dissolved, 1.0 mM and 1.5 mM, respectively.

Table 4.2. Summary of the experimental results obtained for the incubation of orpiment at neutral pH under aerobic or anoxic conditions (NO_3^- as electron acceptor).

Treatment description	Time (d)	$[\text{As}^{\text{V}}]_{\text{final}}$ (mM)	$[\text{TotAs}]_{\text{final}}$ (mM)	% TotAs mobilized	$[\text{SO}_4^{2-}]_{\text{final}}$ (mM)	$[\text{NO}_3^-]_{\text{initial}}$ (mM)	$[\text{NO}_3^-]_{\text{final}}$ (mM)	$[\text{NO}_2^-]_{\text{final}}$ (mM)
Aerobic								
Abiotic	111	0.098±0.002	0.116±0.013	11.62±1.91	0.256±0.023	n.a. [#]	n.a.	n.a.
RA	111	0.094±0.011	0.086±0.009	8.65±0.96	0.486±0.029	n.a.	n.a.	n.a.
RA no O_2	111	0.054±0.009	0.065±0.009	6.47±0.89	0.541±0.083	n.a.	n.a.	n.a.
RA no mineral	111	n.d. [*]	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.
Anoxic								
Abiotic	176	0.824±0.013	0.870±0.044	87.08±4.46	0.761±0.171	5.807±0.058	5.253±0.171	0.017±0.024
ND	62	0.022±0.002	0.027±0.003	2.69±0.39	n.d.	5.876±0.074	5.123±0.082	n.d.
ND no NO_3^-	62	0.006±0.005	0.006±0.001	0.64±0.13	n.d.	n.d.	n.d.	n.d.
ND no mineral	62	n.d.	n.d.	n.d.	n.d.	5.893±0.041	5.058±0.033	n.d.
EB	62	0.062±0.014	0.069±0.016	6.87±1.58	n.d.	5.850±0.040	5.580±0.021	n.d.
EB no NO_3^-	62	0.017±0.001	0.049±0.002	4.89±0.21	n.d.	n.d.	n.d.	n.d.
EB no mineral	62	n.d.	n.d.	n.d.	n.d.	5.784±0.015	5.637±0.053	n.d.
ES	176	0.108±0.083	0.119±0.066	11.96±6.65	1.162±0.018	5.658±0.094	2.805±0.452	n.d.
ES no NO_3^-	176	0.139±0.025	0.201±0.009	20.08±0.90	1.210±0.152	n.d.	n.d.	n.d.
ES no mineral	176	n.d.	n.d.	n.d.	0.088±0.001	5.821±0.035	3.740±0.002	2.998±0.067

^a The total concentration of As and S expected for the total dissolution of orpiment was 1 mM and 1.5 mM, respectively.. The % of the total As mobilized was calculated based on the theoretical amount of As if all of it was mobilized from the mineral. The concentration of O_2 in the aerobic experiment was 20%.

[#]n.a. No applicable

^{*} n.d. No detectable

4.4.1.2. FeAsS dissolution

Figs. 4.2A to 4.2C show the release of As^{V} , As^{III} , and total As determined in the aerobic bioassay with arsenopyrite as a function of time. Arsenic was not detected in the inoculated treatments at the end of the experiment (0%). In contrast, a moderate release of As was observed in the abiotic treatment reaching $9.6 \pm 2.5\%$ of the total As after 111 d of incubation (Fig. 4.2C). The dissolved As was present as As^{III} , and no further oxidation to As^{V} was detected.

Like occurred with As, SO_4^{2-} was only detected in the abiotic treatment where the concentration of SO_4^{2-} released accounted for $118.0 \pm 2.2\%$, as if the entire mineral was dissolved, which was 24 times greater than the theoretical expected for the amount of As measured according to Eq. 4.2 (Table 4.1), thus the process dominating the weathering of FeAsS under these conditions might be an incongruent oxidation leading to the formation of other As precipitates.

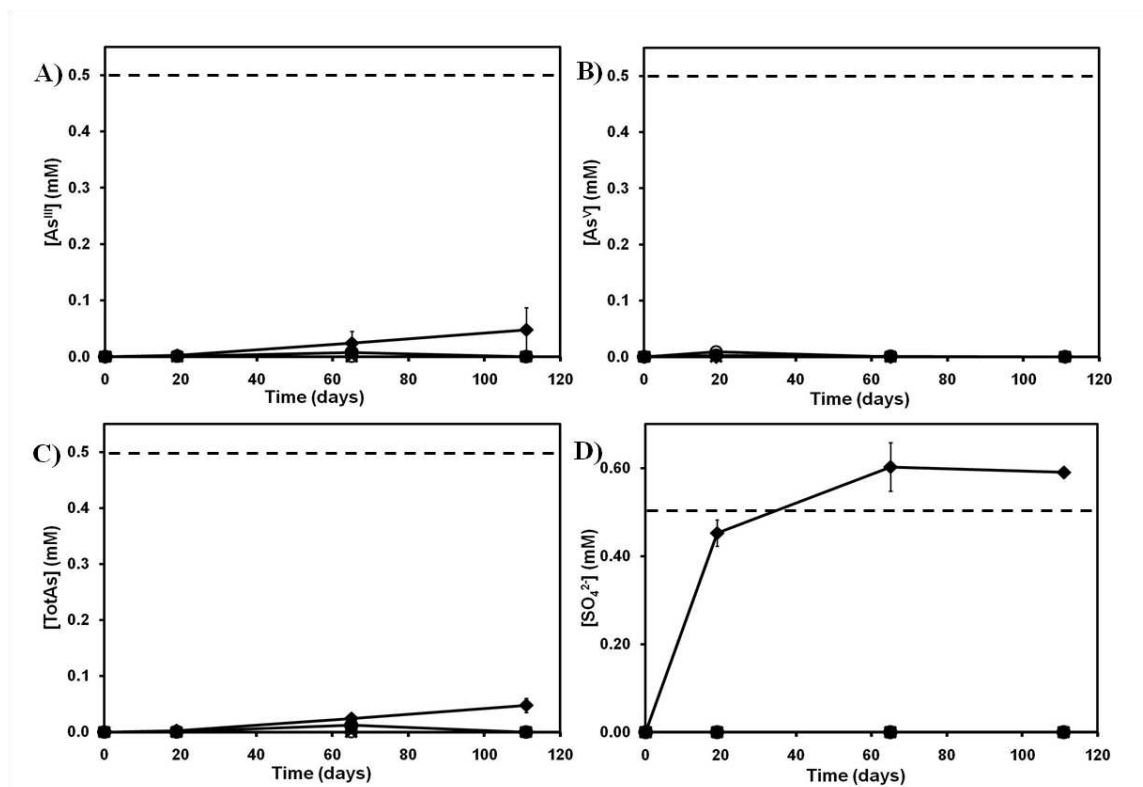


Fig. 4.2. Dissolution of arsenopyrite (0.5 mM as As) under aerobic conditions (20% O₂ in headspace) in batch assays with or without aerobic RAS. Legends: Dissolved concentration of: As^{III} (A); As^V (B); total As (C); and SO₄²⁻ (D). Complete microbial treatment (●); non-inoculated control (◆); inoculated control lacking O₂ (○); and inoculated control lacking arsenopyrite (x, dashed line). The horizontal dashed lines indicate the maximum concentrations of As and SO₄²⁻ that would be released if arsenopyrite would be completely dissolved, 0.50 mM for both compounds.

Table 4.3. Summary of the experimental results obtained for the incubation of arsenopyrite at neutral pH under aerobic or anoxic conditions (NO_3^- as electron acceptor).

Treatment description	Time (d)	$[\text{As}^{\text{V}}]_{\text{final}}$ (mM)	$[\text{TotAs}]_{\text{final}}$ (mM)	% TotAs mobilized	$[\text{SO}_4^{2-}]_{\text{final}}$ (mM)	$[\text{NO}_3^-]_{\text{initial}}$ (mM)	$[\text{NO}_3^-]_{\text{final}}$ (mM)	$[\text{NO}_2^-]_{\text{final}}$ (mM)
Aerobic								
Abiotic	111	n.d.*	0.05±0.01	9.57±2.52	n.d.	n.a. [#]	n.a.	n.a.
RA	111	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.
RA no O₂	111	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.
RA no mineral	111	n.d.	n.d.	n.d.	n.d.	n.a.	n.a.	n.a.
Anoxic								
Abiotic	176	n.d.	0.166±0.056	33.31±11.36	n.d.	2.797±0.054	1.905±0.315	n.d.
ND	62	0.051±0.001	0.118±0.010	23.56±2.08	0.459±0.070	2.831±0.029	0.833±0.035	n.d.
ND no NO₃⁻	62	n.d.	0.089±0.017	17.73±3.33	0.390±0.010	n.d.	n.d.	n.d.
ND no mineral	62	n.d.	n.d.	n.d.	n.d.	2.848±0.028	0.760±0.287	n.d.
EB	62	n.d.	0.008±0.001	1.67±0.20	n.d.	2.934±0.043	2.766±0.103	n.d.
EB no NO₃⁻	62	0.003±0.001	0.006±0.001	1.22±0.02	n.d.	2.893±0.036	2.801±0.072	n.d.
EB no mineral	62	n.d.	n.d.	n.d.	n.d.	2.931±0.067	2.884±0.099	n.d.
ES	176	n.d.	0.021±0.003	4.12±0.59	0.621±0.040	2.884±0.001	0.881±0.069	n.d.
ES no NO₃⁻	176	n.d.	n.d.	n.d.	0.472±0.112	n.d.	n.d.	n.d.
ES no mineral	176	n.d.	n.d.	n.d.	0.079±0.012	2.434±0.193	2.127±0.002	n.d.

^a The total concentration of As and S expected for the total dissolution of arsenopyrite was 0.5 mM. The % of the total As mobilized was calculated based on the theoretical amount of As if all of it was mobilized from the mineral. The concentration of O₂ in the aerobic experiment was 20%.

[#] n.a. No applicable

* n.d. No detectable

4.4.2. Dissolution of ASM under anoxic conditions

4.4.2.1. As₂S₃ dissolution

The dissolution of As₂S₃ in the presence of NO₃⁻ was characterized in assays inoculated with three different methanogenic sludges, namely, ND-, EB- and ES sludges, and a summary of the results obtained in these experiments is presented in Table 4.2. Fig. 4.3 shows the results obtained for the experiment with ES (greater mineral dissolution). The highest As released was observed in the abiotic control (Fig. 4.3C) where a steady increase in the concentration of dissolved As was observed after 37 d of incubation, resulting in the As solubilization of 87.1±4.5% of the total As supplied at the end of the experiment (176 d). As^V was the main specie detected in the abiotic treatment through the experiment accounting for 90.3±6.4% of the total As released (Figs. 4.3A, 4.3B and 4.3C). Considerably lower concentrations of soluble As were observed in the inoculated assays with or without NO₃⁻ where the As dissolved at the end of the experiment amounted to 12.0±6.7% and 20.1±0.9% of the total As, respectively (Fig. 4.3C). The speciation of As in the biological treatments showed a lower percentage of the As as As^V than in the abiotic treatment, 68.6±2.7% in the complete treatment with NO₃⁻, and 76.0±9.3% in the control lacking NO₃ (Figs. 4.3A and 4.3B).

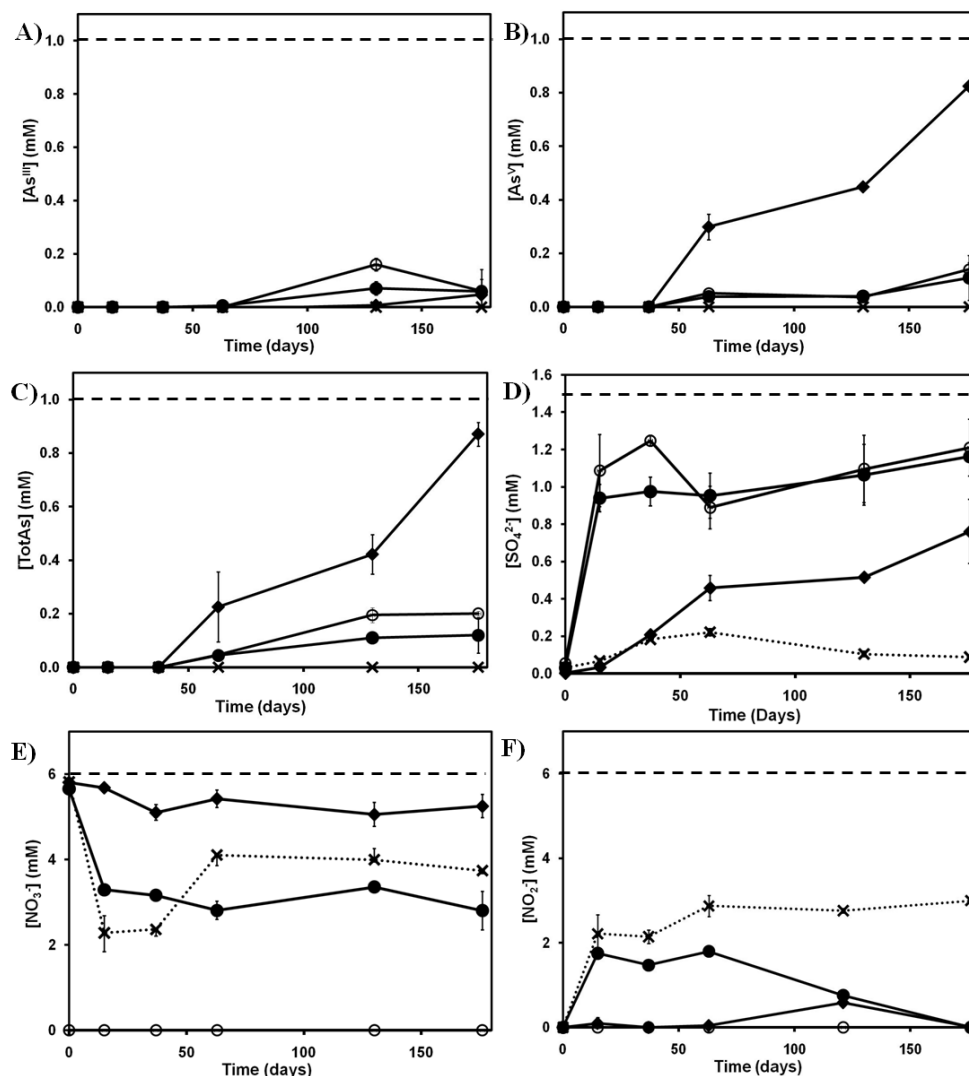


Fig. 4.3. Dissolution of orpiment (1 mM as As) under nitrate-reducing conditions in batch assays with or without sulfide-oxidizing Enrichment sludge. Legends: Dissolved concentration of: As^{III} (A); As^V (B); total As (C); SO₄²⁻ (D); NO₃⁻ (E); and, NO₂⁻ (F). Complete microbial assay (●); abiotic assays (◆); inoculated control lacking NO₃⁻ (○); inoculated control lacking orpiment (x, dashed line). The horizontal dashed lines indicate the maximum concentration of As and SO₄²⁻ that would be released if orpiment would be completely dissolved, 1.0 mM and 1.5 mM, respectively, or the maximum concentration of NO₃⁻ and NO₂⁻ that could be present in solution (6 mM).

Fig. 4.3D shows the concentration of SO_4^{2-} determined in the experiments with ES sludge as a function of time. The accumulation of SO_4^{2-} in the abiotic treatment followed a similar trend that for total As, with a gradual increase in SO_4^{2-} concentration throughout the experiment that led to a total SO_4^{2-} released of $50.7 \pm 11.4\%$ of the total S expected if the As_2S_3 added was completely dissolved. This amount of SO_4^{2-} was 1.7 times less than the stoichiometric expected for the amount of As detected (Eq. 4.4 to 4.6 in Table 4.1). On the other hand, the concentration of SO_4^{2-} in the biological treatments started accumulating earlier than As in the experiment. $77.5 \pm 1.2\%$ and $80.7 \pm 10.2\%$ of the total S amended was accounted as SO_4^{2-} in the solution by day 176, for the inoculated treatments with or without NO_3^- , respectively. In the biological treatments, the amount of SO_4^{2-} detected was up to 6.5 times higher than the calculated for the measured As dissolution. Little amount of SO_4^{2-} was also detected in the control with no mineral, which could be explained by a contribution from the ES.

The concentration of NO_3^- in the abiotic assay decreased just from 5.8 ± 0.1 mM to 5.2 ± 0.2 mM over the course of the experiment (Fig. 4.3E). The concentration of NO_2^- was always close to zero but at day 121 when 0.6 ± 0.1 mM were detected. 2.4 mM of NO_3^- should be necessary to oxidize As_2S_3 to obtain 0.9 mM of As in solution (Eq. 4.10 in Table 4.1), therefore the dissolution of As_2S_3 in abiotic conditions was not due to the presence of the NO_3^- (Fig. 4.3F). In the biological treatments, NO_3^- seemed not to affect the amount of As freed in the solution, however it affected the speciation of As during the

experiment. As it can be observed in Fig. 4.3A and 4.3B, if NO_3^- was present in the experiment, at day 130, $62.6 \pm 13.2\%$ of the As was as As^{III} , while if no NO_3^- was added the percentage of the reduced form increased to $81.7 \pm 0.2\%$. NO_3^- concentration decreased from 5.7 ± 0.1 mM to 3.3 ± 0.1 mM in 15 d in the complete treatment, and it stayed stable until the end of experiment (Fig. 4.3E). 1.7 ± 0.1 mM of NO_2^- was formed as a result of the reduction of NO_3^- (Eq. 4.10 in Table 4.1), which was further utilized and over by the end of the experiment. In the control with no mineral, the reduction of NO_3^- from 5.8 ± 0.0 to 3.7 ± 0.0 mM produced 2.9 ± 0.1 mM of NO_2^- that was maintained constant the rest of the experiment.

As_2S_3 dissolution in experiments performed with the other two methanogenic inocula evaluated, ND- and EB sludge, was almost negligible with both sludges (Table 4.2). The amount of As released into solution after 62 d of incubation with ND as inocula corresponded to the mobilization of just $2.7 \pm 0.4\%$ for the complete treatment with NO_3^- , and $0.6 \pm 0.1\%$ in the control without NO_3^- , of the total As available in the mineral. Similarly, the dissolution of $\text{As}_2\text{S}_{3(\text{s})}$ when incubated with EB sludge mobilized just $6.9 \pm 1.6\%$ and $4.9 \pm 0.21\%$ in the treatments with and without NO_3^- , respectively. SO_4^{2-} was not detected over the duration of both experiments. NO_3^- was not reduced in these experiments; therefore it was not used as electron acceptor to oxidize As_2S_3 .

4.4.2.2. FeAsS dissolution

The dissolution of FeAsS under anoxic conditions using NO_3^- as alternative electron acceptor has been studied in abiotic conditions and in experiments inoculated with three different methanogenic sludges (ND, EB and ES sludge as described for the As_2S_3 experiments). A summary of the results obtained for the abiotic experiment and the biological experiment are presented in Table 4.3. Fig. 4.4 shows the mobilization of FeAsS when incubated in abiotic conditions and with the ND sludge (greatest mineral dissolution) using NO_3^- as electron acceptor. The dissolution of FeAsS in abiotic conditions mobilized $33.3 \pm 11.4\%$ of the total As in the mineral 176 d of incubation, all of it was as As^{III} , as it can be appreciated in Figs. 4.3A to 4.3C. Fig. 4.3D shows that SO_4^{2-} was never detected over the course of the experiment, since SO_4^{2-} was not obtained from the dissolution of FeAsS but As was, other S compounds might probably be in the solution. As observed in Fig. 4.3E, NO_3^- decreased from 2.8 ± 0.0 mM to 1.9 ± 0.3 mM, which was double the amount needed for the oxidation of FeAsS to As^{III} (Eq. 4.9 in Table 4.1). NO_2^- was not the product of NO_3^- reduction (Fig. 4.3F). This suggests that NO_3^- is being used as electron acceptor for other processes taking place in the experiments besides the oxidation of FeAsS.

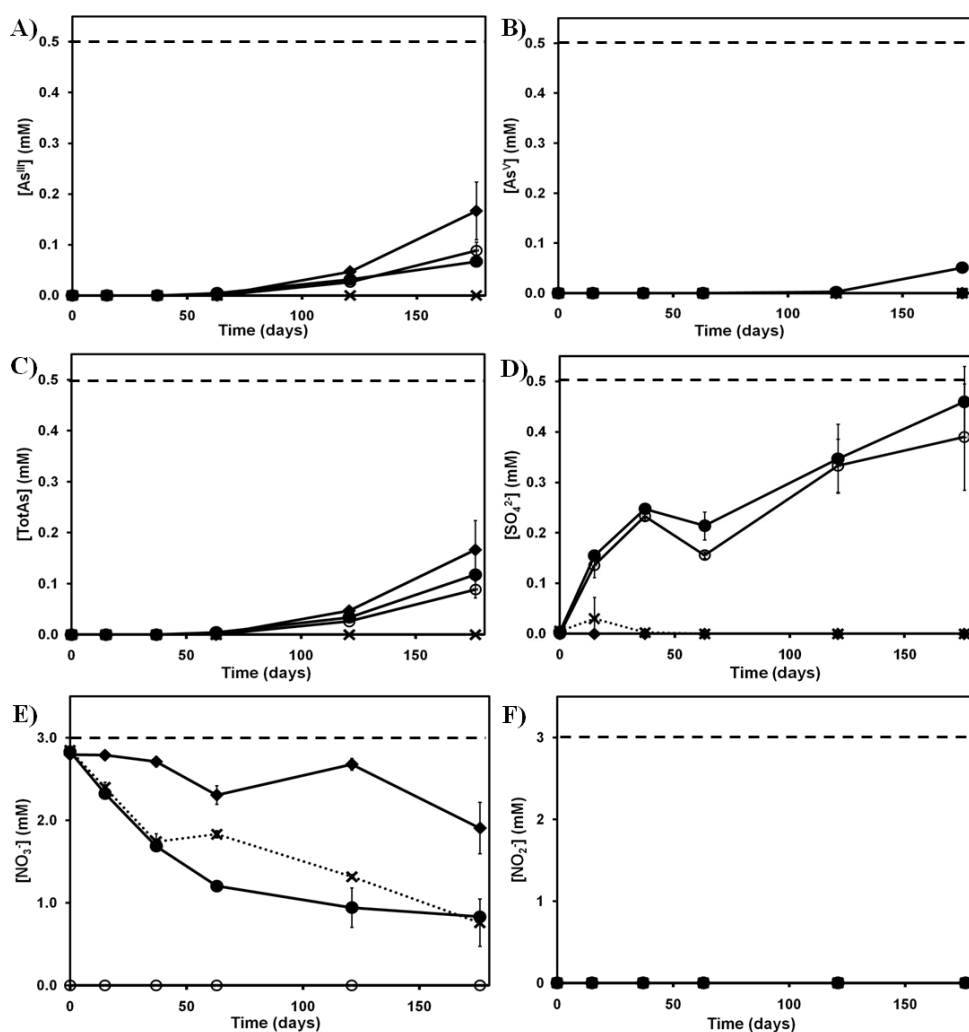


Fig. 4.4. Dissolution of arsenopyrite (0.5 mM as As) under nitrate-reducing conditions in batch assays with and without microbial inoculum (Nedlco methanogenic sludge). Legends: Dissolved concentration of: As^{III} (A); As^V (B); total As (C); SO_4^{2-} (D); NO_3^- (E); and, NO_2^- (F). Inoculated assay (●); abiotic assay (◆); inoculated control lacking NO_3^- (○); inoculated control lacking arsenopyrite (x, dashed line). The horizontal dashed line indicates the maximum concentration of As and SO_4^{2-} that would be released if arsenopyrite would be completely dissolved (0.5 mM), or the maximum concentration of NO_3^- and NO_2^- that could be present in solution (3 mM).

The biological mobilization of As from FeAsS was only relevant when incubated with ND sludge. As shown in Fig. 4.3C, the total concentration of As released in solution over the 176 d of incubation was $23.6 \pm 2.1\%$ in the treatment with NO_3^- , and $17.7 \pm 3.3\%$ when NO_3^- was not amended to the experiment. If NO_3^- was present, the speciation of the As in solution was $100.0 \pm 0.0\%$ as As^{III} at day 63 but after 110 more days of incubation the percentage of As^{III} decreased to $56.5 \pm 3.3\%$ and the rest as As^{V} ; while in the absence of NO_3^- all the As was in its reduced form, As^{III} (Figs. 4.3A and 4.3B), indicating the oxidation of As^{III} by NO_3^- as expected according to Eq. 4.12 (Table 4.1).

Fig. 4.3D shows how SO_4^{2-} concentration increased all over the course of the experiment until day 176 when $91.9 \pm 14.1\%$ and $78.0 \pm 21.1\%$ of the total S present in the mineral was measured as SO_4^{2-} , for the experiment with NO_3^- and without NO_3^- , respectively. The total amount of SO_4^{2-} was again higher than the expected for the dissolved As (Eq. 4.7 to 4.9, Table 4.1), 3.9 times greater when the mineral was incubated with NO_3^- and 4.4 times in the absence of NO_3^- . The concentration of NO_3^- decreased at a similar rate whether or not the mineral was present in solution, as shown in Fig. 4.3E. NO_2^- was not detected at any time in the experiment. The same consumption of NO_3^- in the complete treatment and in the control could be explained by an endogenous consumption by the sludge.

FeAsS biological dissolution with the other sludges, EB and ES, was almost negligible. Just $1.7 \pm 0.2\%$ of the total As in the mineral was mobilized when FeAsS was incubated with EB and $4.1 \pm 0.6\%$ when incubated with ES sludge, in both cases As was present as As^{III} . No accumulation of SO_4^{2-} or NO_3^- reduction was observed when the mineral was incubated with EB. However, in the experiment with the ES sludge, SO_4^{2-} was accumulated in dissolution, close to the expected concentration if the entire mineral becomes dissolved; $124.3 \pm 8.0\%$ and $94.4 \pm 22.4\%$ mM of the total S from the mineral were in solution in the treatments with and without NO_3^- , respectively. SO_4^{2-} was also detected in the control treatment with no mineral, in a concentration of 0.1 ± 0.0 mM, due to a possible contribution from the ES sludge, as it also occurred in the As_2S_3 dissolution experiment with the same inocula. NO_3^- concentration decreased from 2.9 ± 0.0 mM to 0.9 ± 0.1 in the complete treatment and it was initially converted to NO_2^- , which was further reduced and it was not detected in the solution (Eq. 4.7 and 4.8, Table 4.1). In the control with no mineral, NO_3^- concentration just decreased to 2.4 ± 0.2 mM to 2.1 ± 0.0 mM, therefore NO_3^- is probably being used to oxidize FeAsS. Although, little As was in the solution, the fact that SO_4^{2-} is being accumulated and NO_3^- is reduced, suggests that FeAsS is being dissolved when incubated with ES sludge, but As is probably being entrapped in other processes on the medium, such as adsorption on the sludge.

4.4.3. E_h -pH diagrams

E_h -pH diagrams were built to evaluate the thermodynamic stability of As_2S_3 and $FeAsS$ as a function of the pH and the E_h . The measured E_h was 0.40 ± 0.05 V under aerobic conditions and 0.20 ± 0.05 V under anoxic conditions. Fig. 4.5A shows the stability area of As_2S_3 . In a solution with 0.5 mM of As and 0.75 mM of S, the formation of thioarsenical species ($AsS(OH)HS^-$) which will promote the dissolution of As_2S_3 , at the experimental pH (7.2) and at E_h range between -0.3 to -0.1 V. At E_h higher than -0.1 V, As_2S_3 would dissolved leading to the accumulation of soluble As species and SO_4^{2-} in the solution. Fig. 4.5B shows the stability of $FeAsS$ for As species in a solution containing 0.5 mM of As, S and Fe. $FeAsS$ is thermodynamically stable in the entire reductive E_h until -0.5 V, where other minerals will start precipitating. Therefore, if the E_h increases to values higher than -0.5 V, $FeAsS$ would initially undergo an incongruent dissolution with the formation of other solid phases such as realgar (AsS), As_2S_3 and FeS_2 until reaching an E_h higher than -0.1 V where the new minerals formed would start dissolving freeing As, S and Fe species into the solution.

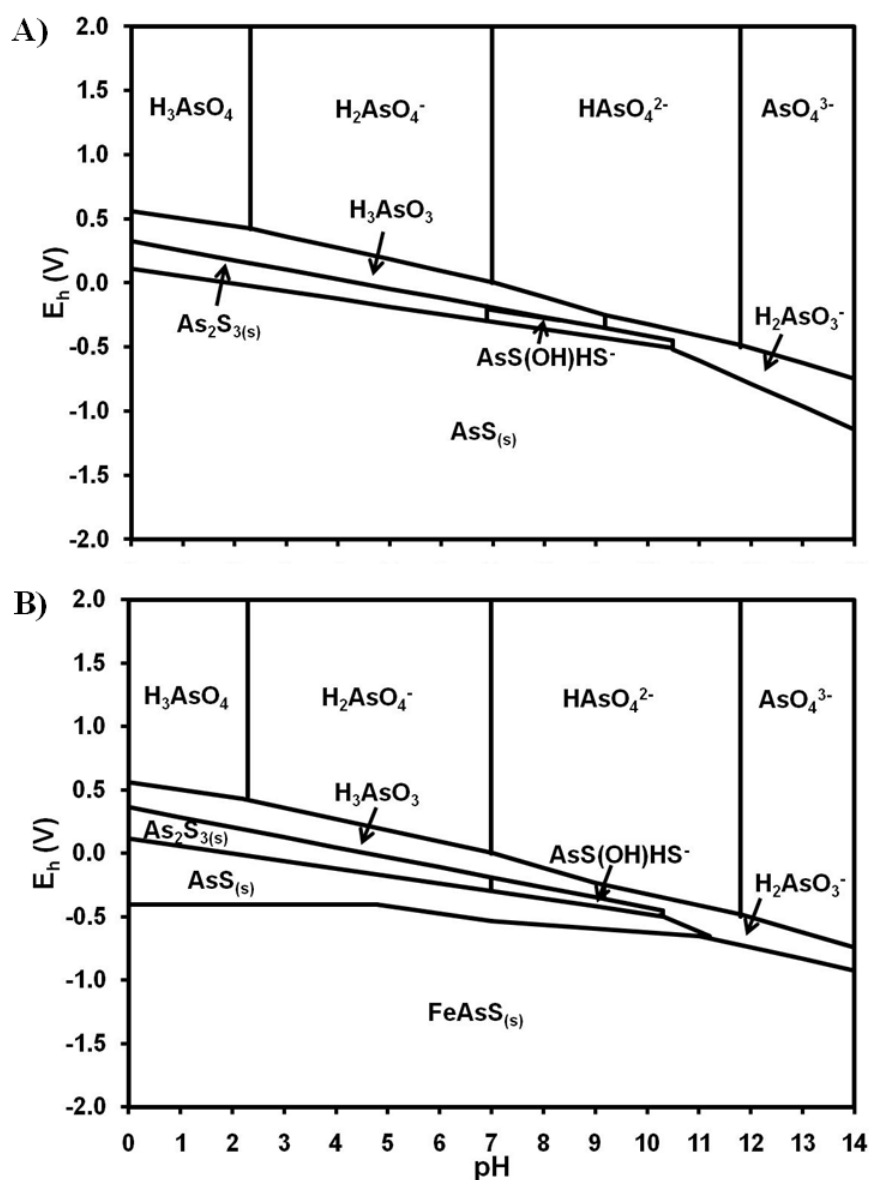


Fig. 4.5. E_h -pH diagrams for As minerals at 25°C and 1 atm pressure showing the stability areas for solid phases at the conditions described dissolution experiments. Diagram (A) shows the stability areas for the As_2S_3 in a solution with 0.5 mM of As and 0.75 mM of S; and Diagram (B) shows the stability areas for the FeAsS in a solution containing 0.5 mM of As, S and Fe.

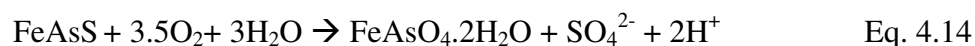
4.5. Discussion

4.5.1. Role of the inocula in the mobilization of As from the minerals

The mobilization of As from $\text{As}_2\text{S}_{3(s)}$ and $\text{FeAsS}_{(s)}$ has been shown to depend greatly on the conditions the minerals are incubated. The maximum mobilization of As was observed in the abiotic treatments, whereas the presence of the inocula seemed to play a role in preventing the mobilization of As. If SO_4^{2-} concentration is considered, the higher rates of SO_4^{2-} to As were obtained in the inoculated treatments, this suggests that mineral dissolution was higher in the biological treatments and As is being immobilized by other processes. One possibility to explain the lower As mobilization in the biological treatments could be that the sludge matrixes are acting as a sorption material for As. Chowdhury and Mulligan (2011) demonstrated that As^{V} is readily adsorbed on anaerobic sludge; however, As sorption by activated sludge was not relevant in the studies done by Goldstone et al. (1990) and Andrianisa et al. (2006). Another possibility could be that the microorganisms stimulated the formation of secondary As-bearing throughout incongruent dissolution of ASM. As_2S_3 can be transform to claudetite (As_2O_3) under aerobic conditions as described by Foley and Ayuso (2008):



Similarly, FeAsS can be aerobically oxidized to scorodite (FeAsO₄·2H₂O) according to the following reaction:



Thus, the presence of inocula seems to prevent the mobilization of As from the ASM, either by acting as a sorption material for As or by stimulating the oxidation of ASM to secondary As-bearing minerals.

4.5.2. Dissolution of As₂S₃ and FeAsS in aerobic conditions

The mobilization of As from As₂S₃ in aerobic conditions occurred as described by Lazaro et al. (1997). As^{III} was the initial product of the As₂S₃ dissolution and then As^{III} was oxidized to As^V in all the treatments, even in the control lacking O₂, although the amount of As released was lower in the absence of O₂, confirming the dependence of As₂S₃ dissolution with the availability of O₂. These results are in agreement with the findings of Lengke when studying the oxidation of amorphous As₂S₃ in neutral to alkaline pH (Lengke and Tempel 2001). The amount of SO₄²⁻ released in the abiotic treatment was close to the theoretical calculated by the stoichiometric of Eq 4.1 (Table 4.1) confirming the congruent dissolution of As₂S₃ in aerobic abiotic conditions. The higher ratio SO₄²⁻ to As concentration in the biological treatments can be explained by the

incongruent dissolution of As_2S_3 to As_2O_3 (Eq. 4.13). Since the concentration of SO_4^{2-} in the presence of RA double the concentration of SO_4^{2-} in the abiotic treatment, the dissolution of As_2S_3 was enhanced by biotic processes.

The dissolution of FeAsS produced As^{III} which was not oxidized to As^{V} in the duration of the experiment in the abiotic treatment. SO_4^{2-} concentration was 6.8 times higher than the concentration of As, which cannot be explained by Eq. 4.2 (Table 4.1). Instead, the incongruent dissolution of FeAsS leading to the formation of $\text{FeAsO}_4 \cdot 2\text{H}_2\text{O}$ might be the reaction that occurred (McKibben et al. 2008, Murciego et al. 2011, Rimstidt et al. 1994), or a combination of both (Eq. 4.14).

Whereas no As was detected in the inoculated treatments, the concentration of SO_4^{2-} in the biological treatments demonstrated dissolution of FeAsS . Again, the concentration of SO_4^{2-} was higher in the presence of RA than in the abiotic experiment, therefore, the presence of the sludge seems to stimulate the dissolution of the mineral.

4.5.3. Dissolution of As_2S_3 and FeAsS in anoxic conditions

McKibben et al. (2008) concluded that NO_3^- does not oxidize FeAsS in acidic conditions. In this study, the As mobilization from As_2S_3 in abiotic conditions with NO_3^- was higher than in the rest of the treatments and it never reached the stationary state as

the concentration of total As keep increasing even at the end of the experiment. As^{V} dominated the speciation of As, as it accounted for almost 95% of the total As. SO_4^{2-} concentration was lower than the expected for this case. A slower release of S from the mineral has been reported for realgar (AsS) (Douglass et al. 1992, Lengke and Tempel 2003) and arsenopyrite (Nesbitt et al. 1995) due to the different bond strength of S and As in the mineral and different oxidation rates. The reduction of NO_3^- was not enough to explain the dissolution of As_2S_3 , but the oxidation of As^{III} to As^{V} , which could probably be the factor that favor the high As dissolution rate of As_2S_3 in abiotic conditions.

The incubation of As_2S_3 with the anaerobic sludges decreased the released of As. The soluble As was initially present as As^{III} and then oxidize to As^{V} , similarly to the dissolution of As_2S_3 in aerobic conditions. The amount of As released was independent of whether or not NO_3^- was present, but NO_3^- controlled the speciation of As in solution. With the exception of the experiment with ES sludge where the concentration of SO_4^{2-} at the end of the experiment corresponds with a mineral dissolution at the same rate than for the abiotic conditions, the SO_4^{2-} concentration in the experiments with ND and EB was almost negligible. The different in behavior between the different sludges can be explained by the fact that ES has been enriched with S-oxidizing bacteria in a denitrifying reactor. The lower SO_4^{2-} concentration can also possible be due to the formation of thioarsenate species in dissolution (Suess and Planer-Friedrich 2012), and since total As is obtained by oxidizing the sample with an oxidant solution, As concentration would still

be relevant. Otherwise, it seems that NO_3^- cannot be successfully used as electron acceptor to promote As mobilization from As_2S_3 , even though several other studies have found that sulfide minerals can be used as electron donors for denitrification (Haaijer et al. 2007, Torrento et al. 2010, Torrento et al. 2011).

The dissolution of FeAsS released As^{III} which controlled the speciation of total As, as it occurred in aerobic conditions. The amount of As mobilized in the abiotic treatment was higher than when incubated with ND or ES, and no As was detected when the sludge was EB. As it occurred for As_2S_3 dissolution, SO_4^{2-} concentration in the abiotic treatment was barely detected over the experiment. SO_4^{2-} concentration in the biological treatments with ND and ES was similar to the expected if the entire mineral was dissolved, for that reason it is possible to affirm that the dissolution of FeAsS is enhanced by the microbial activity. The mobilization of As and SO_4^{2-} occurred at the same level with or without NO_3^- , thus NO_3^- was not responsible for the oxidation of FeAsS.

4.6. Conclusions

The As mobilization from As_2S_3 and FeAsS was limited in the biological treatments due to adsorption processes of As on the organic matter of the sludge and the formation of secondary As-bearing minerals, as it will occur in a natural system in the environment, where different substrates can act as sinks for As immobilization. The

aerobic oxidation of As_2S_3 leads to higher As concentrations than the aerobic oxidation of FeAsS , whereas in anoxic conditions, the dissolution of FeAsS leads to higher As concentrations, even with NO_3^- which was not used as electron acceptor for the oxidation of the minerals. Future work to study the role of NO_3^- in the oxidation of ASM involving mineral surface characterization is necessary in order to understand the role of NO_3^- in ASM oxidation.

CHAPTER V:
BIOMINERALIZATION OF ARSENATE TO ARSENIC SULFIDES IS
GREATLY ENHANCED AT MILDLY ACIDIC CONDITIONS

5.1. Abstract

Arsenic (As) is an important water contaminant due to its high toxicity and widespread occurrence. Arsenic-sulfide minerals (ASM) are formed during microbial reduction of arsenate (As^{V}) and sulfate (SO_4^{2-}). The objective of this research is to study the effect of the pH on the removal of As due to the formation of ASM in an iron-poor system. A series of batch experiments was used to study the reduction of SO_4^{2-} and As^{V} by an anaerobic biofilm mixed culture in a range of pH conditions (6.1-7.2), using ethanol as the electron donor. Total soluble concentrations and speciation of S and As were monitored. Solid phase speciation of arsenic was characterized by x-ray adsorption spectroscopy (XAS). A marked decrease of the total aqueous concentrations of As and S was observed in the inoculated treatments amended with ethanol, but not in the non-inoculated controls, indicating that the As-removal was biologically mediated. The pH dramatically affected the extent and rate of As removal, as well as the stoichiometric composition of the precipitate. The amount of As removed was 2-fold higher and the rate

of the As removal was up to 17-fold greater at pH 6.1 than at pH 7.2. Stoichiometric analysis and XAS results confirmed the precipitate was composed of a mixture of orpiment and realgar, and the proportion of orpiment in the sample increased with increasing pH. The results taken as a whole suggest that ASM formation is greatly enhanced at mildly acidic pH conditions.

5.2. Introduction

Arsenic (As) contamination of natural waters is a major health and environmental concern. The United States Environmental Protection Agency (US-EPA) have set the As standard in drinking water at 10 ppb (US-EPA 2001). The concentration of As in groundwater and drinking water exceeds this limit in many locations across the world (Murcott 2012). Elevated As concentrations generally occur due to As mobilization from high As-content rocks and sediments driven by changes under the biogeochemical conditions of the aquifer (Welch et al. 2000), therefore a better understanding of the biogeochemistry of As is necessary to predict and control As mobilization and to remediate As contaminated waters.

Arsenopyrite (FeAsS), realgar (AsS) and orpiment (As_2S_3) are naturally formed As-bearing sulfide minerals (ASM) (O'day et al. 2004) which are known to be a source of As contamination due to weathering processes that dissolve the mineral and release the

retained As into the environment (Welch et al. 2000). However, the formation of ASM can be harnessed to promote the immobilization of As. The biogeochemical cycle of As is dominated by the microbial transformations between the two main inorganic species of As, arsenate (As^{V} , H_2AsO_4^- and HAsO_4^{2-} in circumneutral environments) and arsenite (As^{III} , H_3AsO_3) (van Lis et al. 2013). In oxidizing environments, As^{V} is the predominant species, and the accumulation of As is limited by sorption processes of As on iron (Fe) oxides and oxyhydroxides surfaces (Jonsson and Sherman 2008); in reducing environments, As^{V} can be microbially reduced to As^{III} (van Lis et al. 2013). While As^{III} is also adsorbed onto Fe oxides and oxyhydroxides, its sorption strength with Fe surface complexation is weaker than As^{V} (Jonsson and Sherman 2008). In environments where Fe is lacking and sulfur (S) is present, the solubility of As is potentially controlled by the precipitation of As in ASM (O'Day et al., 2004). The predominant species of S are sulfide (H_2S) and sulfate (SO_4^{2-}), the most reduced and oxidized species, respectively. Microorganisms oxidize or reduce S depending on the redox conditions present in the aquifer (Tang et al. 2009). The microbial reduction of As^{V} and SO_4^{2-} can cause the biomineralization of As and ASM will be formed (Newman et al. 1997a).

Recent evidence demonstrates the biological nature of the formation of ASM. Rittle (1995) first proved the precipitation of As^{III} was due to biological SO_4^{2-} reduction. In 1997, Newman (1997a) discovered a new bacterial strain *Desulfotomaculum auripigmentum* sp. OREX-4 which was able to precipitate As_2S_3 through the

heterotrophic reduction of As^{V} and SO_4^{2-} . The biological precipitation of AsS by a thermophilic bacterium *Caloramator* strain YeAs (Ledbetter et al. 2007) and by a hyperthermophilic archaea *Pyrobaculum arsenaticum* sp. PZ6 (Huber et al. 2000); and, the formation of AsS nanotubes by *Shewanella* strains (Lee et al. 2007) have reinforced the evidence of ASM biogenesis. Furthermore, Demergasso (2007) has demonstrated the biological origin of As_2S_3 in Andean sediments by analyzing the sulfur isotope ratios ($^{34}\text{S}/^{32}\text{S}$) in chemically and biologically formed ASM, and comparing it with the minerals found in the sediments. In addition, Saunders (2008) evaluated the effect of SO_4^{2-} and electron donor addition on the As mobility in As contaminated groundwater, which resulted in a decrease of the dissolved As in the aquifer, most likely due to the formation of FeAsS.

Several lab-scale experiments, conducted in microcosm or bioreactors, have been performed to study the biological precipitation of ASM at circumneutral or acidic pH. Most of these experiments studied the precipitation of ASM in Fe-containing systems (Kirk et al. 2010, Onstott et al. 2011). Fe-sulfide minerals, such as pyrite (FeS_2) or mackinawite (FeS) have lower solubility than the ASM, therefore they would precipitate first removing Fe and S from solution (Kirk et al. 2010, O'day et al. 2004). In high SO_4^{2-} waters, Fe would become limited and the system would essentially behave as an Fe-poor environment, stressing the importance of understanding the formation of ASM in the absence of Fe.

Table 5.1 Summary of the important reaction to consider in the microcosm studies

Ethanol acetogenesis:		
$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+$		Eq. 5.1
Acetoclastic methanogenesis:		
$\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$		Eq. 5.2
Hydrogenotrophic methanogenesis:		
$4 \text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$		Eq. 5.3
Sulfate reduction coupled to H₂ oxidation:		
$\text{SO}_4^{2-} + 4 \text{H}_2 + 2 \text{H}^+ \rightarrow \text{H}_2\text{S} + 4 \text{H}_2\text{O}$		Eq. 5.4
Arsenate reduction coupled to H₂ oxidation:		
$\text{H}_2\text{AsO}_4^- + \text{H}_2 + \text{H}^+ \rightarrow \text{H}_3\text{AsO}_3 + 2 \text{H}_2\text{O}$		Eq. 5.5
Mineralization:		
$x\text{H}_3\text{AsO}_3 + y\text{HS}^- + (3x-y) \text{H}^+ \rightarrow \text{As}_x\text{S}_{y(s)} + 3x\text{H}_2\text{O}$		Eq. 5.6
$x = y = 1$, realgar (α -AsS) formation		
$x = 2$; $y = 3$, orpiment (As_2S_3) formation		

The objective of this study was to determine the effect of the transition from slightly acidic to alkaline pH on the rate, extent and type of biological ASM formation in Fe-poor environments. In order to attain this objective, a series of batch experiments, with pH conditions ranging from 6.1 to 7.2, were performed using an anaerobic biofilm mixed culture as inocula with only trace levels of Fe. The batch experiments were amended with As^{V} and SO_4^{2-} , and ethanol was used as electron donor. The main reactions occurring in the microcosms are summarized in Table 5.1. The precipitation of ASM was

evaluated by measuring the total As and S concentration and speciation in solution. Likewise the solid phase was characterized by different spectroscopic techniques.

5.3. Materials and methods

5.3.1. Source of microorganisms

An anaerobic granular biofilm was obtained from full scale upflow anaerobic sludge bioreactor (UASB) from a beer brewery wastewater treatment plant Mahou (Guadalajara, Spain) (0.042 ± 0.002 g volatile suspended solids (VSS)/g wet wt). The sludge was examined for As content, and As level was below detectable limits (digestion of sludge using aqua regia and further analysis in the ICP-OES, see section 5.3.6. Analytical Methods).

5.3.2. Medium composition

The basal medium was prepared using ultra pure water (Milli-Q system; Millipore) and contained (mg/L): K_2HPO_4 (600); $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (899); NH_4Cl (280); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (83); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (10); yeast extract (20), and 1 mL/L of a trace element solution that was added to the medium to provide a final concentration of ($\mu\text{g/L}$): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (2,000); $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$ (2,000); $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$ (500); $\text{AlCl}_3 \cdot 6 \text{H}_2\text{O}$ (90);

CuCl₂·2H₂O (30); ZnCl₂ (50); H₃BO₃ (50); (NH₄)₆Mo₇O₂₄·4 H₂O (50); Na₂SeO₃·5 H₂O (100); NiCl₂·6H₂O (50); EDTA (1,000); resazurin (200); HCl 36% (1 µL). 0.75 mM of SO₄²⁻ was added as Na₂SO₄ and 0.5 mM of As^V Na₂HAsO₄·7H₂O. The electron donor used was ethanol to a final concentration of 12 mM by adding 283 µL/L. The experiments were flushed with N₂/CO₂ (80:20) to ensure anaerobic conditions. NaHCO₃ was used to control the pH of the solution from 6.1 (0.4 g/L NaHCO₃), 6.5 (1 g/L NaHCO₃), 6.85 (2 g/L NaHCO₃) and 7.2 (4 g/L NaHCO₃). 1.5 g VSS/L of sludge was added to the treatment, after being sieved and clean with Milli-Q water to remove any soluble contaminant.

5.3.3. *Experimental incubations*

The biomineralization of ASM was evaluated in batch mode in 160 mL serum bottles containing 120 mL of the liquid medium. The liquid phase was flushed with N₂/CO₂ (80:20) for 10 min, then the 34 µL of ethanol were added to the proper treatments and quickly sealed with rubber septa and aluminum crimp seal. The headspace was flushed for 5 min. needle in-needle out with N₂/CO₂ (80:20). The treatments were run in triplicate with one bottle dedicated to pH measurements and solid phase analysis. Proper controls were set up in parallel to ensure the fidelity of the results. These controls were: (i) non-inoculated with As^V and ethanol, SO₄²⁻ and ethanol, or both, As^V and SO₄²⁻, and ethanol; (ii) inocula with just one of the electron acceptors and the ethanol; (iii) inocula

with no electron acceptor; and, (iv) inocula with one electron acceptor but no ethanol. Non-inoculated controls were prepared under sterile conditions and the medium was autoclaved at 121°C for 10 min. In the non-inoculated controls, ethanol was added after autoclaving to avoid degradation. The assays were incubated at 30°C in the dark, and in an elliptical shaker (115 rpm).

5.3.4. Pourbaix diagrams

Pourbaix diagrams (E_h -pH diagrams) were used to understand the formation and stability of ASM for the experimental conditions (0.5 mM As^V, 0.25 mM SO₄²⁻). The thermodynamic data was obtained from Visual MinTEQA2 and National Bureau of Standards (NBS) databases and the diagrams were built using the W32-Stabcal modeling software.

5.3.5. As removal rate calculation

The As removal rate was obtained by calculating the slope for the percentage of As removal over time during the experiment. It is defined by the following equation:

$$\% \text{ As Removal Rate} = \left[\frac{\Delta(\% \text{ As Removal})}{\Delta(t)} \right]_0^t \quad \text{Eq. 5.7}$$

The As removal rate was calculated for the period of increasing As removal until the steady state is reached.

5.3.6. Analytical methods

Liquid samples were taken from sealed serum flasks by piercing the stoppers using sterile syringes with 16-gauge needles. All samples were centrifuged (10 min, 14,000 g) after sampling and stored in polypropylene vials. As^{V} and SO_4^{2-} were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 analytical column (4 x 250 mm) and AG16 guard column (4 mm x 40 mm). The injection eluent (KOH) was 30 mM for 10 min. Total As concentration was measured by using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system model Optima 2100 DV from Perkin-Elmer TM (Shelton, CT, USA) monitored at wavelength 193.7 nm. H_2S was determined using the methylene blue method described by Truper (1964) and measured using an UV-visible spectrophotometer (Agilent 8453, Palo Alto, CA, USA). The measurement of H_2S provides the amount as H_2S in the liquid phase only. The total concentration of H_2S was calculated by considering the speciation of H_2S at the measured pH using the dissociation constants and the partition of H_2S between the liquid medium and the headspace at the incubation temperature.

Headspace samples in the batch experiments were taken with a pressure lock gas tight syringe (1710RN, 100 μ l (22s/2"/2), Hamilton Company). Ethanol, acetate and CH₄ were monitored in an Agilent Technologies 7890A gas chromatography system with a Restek Stabilwax®-DA Column (30 m x 0.35 mm, ID 0.25 μ m) with flame ionization detector, and He used as a carried gas.

Solid samples were taken under anaerobic conditions inside the anaerobic chamber (COY Laboratory Products Inc., Grass Lake, MI), to avoid any oxidation of the mineral. The solid samples were obtained by homogenizing and concentrating by centrifugation the solid phase contained in 50 mL to 1.5 mL. The solid phase was cleaned by centrifuging and replacing the supernatant with O₂ free Milli-Q water obtained by adding 100 mL of Milli-Q water to a 160 mL serum bottle, and flushing it following the same procedure than for the experimental incubations. Solid phase was characterized using a Scanning Electron Microscopy (SEM) combined with energy dispersive spectroscopy (EDS), and K-edge X-ray absorption spectra (XAS) with X-ray absorption near-edge structure (XANES) and extended x-ray absorption fine-structure (EXAFS) according to the methodology described in Appendix B.

Measurements of pH, E_h and VSS were conducted according to standard methods (APHA 1999).

5.4. Results

5.4.1. As and S biological transformations

The biological transformation of As and S and the precipitation of ASM was evaluated at three different pH conditions (6.1, 6.5 and 7.2) using ethanol as electron donor. Fig. 5.1 shows the evolution of As^{V} (A), total As (B), SO_4^{2-} (C) and total H_2S (soluble + volatile) (D), over the incubation time of the experiment at pH 6.1. Both As^{V} and SO_4^{2-} reduction were required for the formation of ASM to occur. When SO_4^{2-} was not amended in the treatment, As^{V} became reduced but the total As concentration in solution was not affected. Similarly, when As^{V} was not added to the treatment, SO_4^{2-} concentration decreased with a stoichiometric increment in H_2S concentration. But, when As^{V} and SO_4^{2-} were incubated together, the total As and S concentrations decreased, and 100% of the total As was removed in only 9 d. Therefore, both the reduction of As^{V} and SO_4^{2-} must occur for the formation of ASM, as evidenced by the loss of total aqueous As and S.

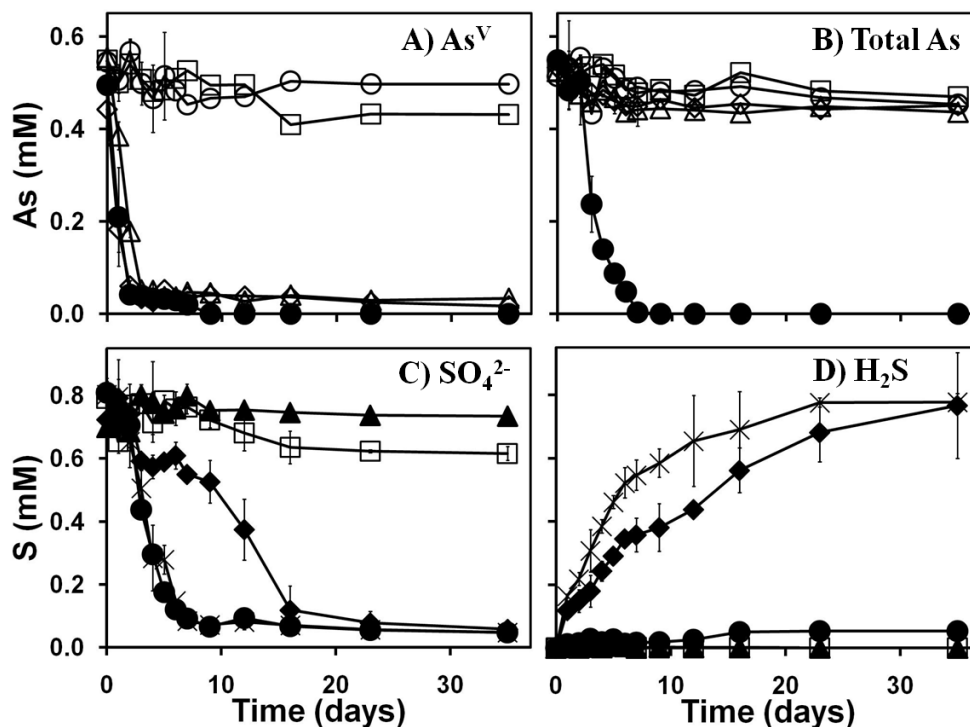


Fig. 5.1. Precipitation of ASM through the biological mediated reduction of 0.5 mM of As^{V} and 0.75 mM of SO_4^{2-} using 12 mM of ethanol as the electron donor at pH 6.1. Dissolved As^{V} concentration of (A); total As concentration (B); SO_4^{2-} concentration (C); and total H_2S as the sum of $\text{H}_2\text{S}_{(\text{g})}$ and all the aqueous species (mmol/L_{liq} or mM) (D). The complete treatment containing inoculum, As^{V} , SO_4^{2-} and ethanol (●); Inoculum, SO_4^{2-} and ethanol (x), Inoculum As^{V} and ethanol (◇), inoculum and SO_4^{2-} (◆), inoculum and As^{V} (Δ), inoculum and ethanol (+); Sterile controls with As^{V} and ethanol (○), the sterile control with SO_4^{2-} and ethanol (▲) and the combined reduction sterile control with SO_4^{2-} and As^{V} with ethanol (□). Treatments with value zero over the time course of the experiment are not shown: treatments lacking As in panels A and B, and treatments lacking S in panels C and D.

The importance of the electron donor was evaluated in controls lacking ethanol. The addition of the electron donor was essential for ASM formation by promoting the reduction of SO_4^{2-} , which was limited in the controls lacking ethanol. Compared to the full treatment, the rate of SO_4^{2-} reduction in the absence of ethanol was 4.1-fold lower during the critical time period (days 1 and 9) when arsenic was being removed in the full treatment. In contrast, the rate of As^{V} reduction in the treatments without ethanol was as fast as in the ethanol-amended treatments. The addition of the electron donor greatly boosted the SO_4^{2-} reduction rate, enabling the formation of ASM.

The reduction of As^{V} and SO_4^{2-} in the non-inoculated controls was not noteworthy in comparison with the inoculated treatments. Total As and total S decreased by $10.7 \pm 2.6\%$ and $21.6 \pm 2.9\%$ respectively in non-inoculated treatments including both As^{V} and SO_4^{2-} . What little removal that did occur took place at the start and thereafter the concentrations were stable. The lack of important changes in the non-inoculated controls indicates that abiotic reactions are relatively unimportant compared with the biological reactions, stressing the significance of the biological transformations of As and S under the studied conditions.

The amount of total As and S removed in the treatments can be calculated by applying a mass balance in the system. The ratio of S loss to As loss ($S_{\text{loss}}/As_{\text{loss}}$) was used to predict mineral phase precipitation based on the expectation that an S/As of 1.5

and 1.0 corresponds to As_2S_3 and AsS , respectively. Fig. 5.2 compares the mass balances for S and As between different inoculated treatments after 35 d incubation at pH 6.1. In the absence of As^{V} , all the SO_4^{2-} reduced was recovered as H_2S , but if As^{V} was amended to the treatment, 0.71 mM of S as H_2S was missing from the experiment. Similarly, the total As concentration hardly decreased (15.3% of the total As) without SO_4^{2-} but the decrease was substantial (100% of the total As) if SO_4^{2-} was present in the treatment. The resulting $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ ratio corresponded to 1.29 in the treatment amended with As^{V} and SO_4^{2-} . These results suggest the formation of a mixture of AsS and As_2S_3 .

The formation of ASM was confirmed by visual observation of a yellow precipitate just in the inoculated assays containing SO_4^{2-} and As^{V} . The formation of the mineral could be appreciated with the naked eye after 5 d of incubation. The amount of precipitate increased and the difference in the color of the medium between the complete inoculated treatment and the control missing As^{V} was very intense at day 12 (Fig. B.1 in Appendix B).

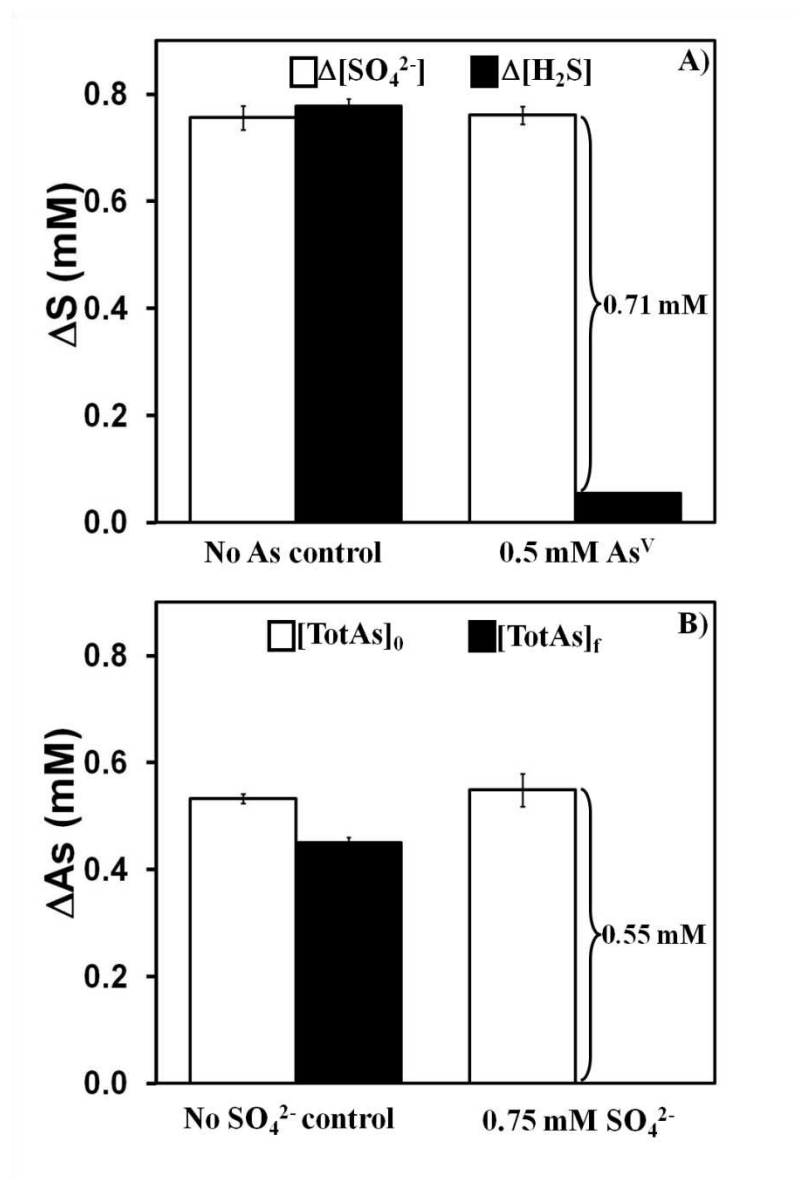


Fig. 5.2. S and As concentration loss between day 0 and the end of the experiment (day 35) for the treatment at pH 6.1. Panel (A) shows the S loss for the inoculated control with no As^V addition and for the complete treatment; the initial SO_4^{2-} concentration is represented in the open column and the formation of H_2S in the filled column. Panel (B) illustrates the total As loss in the inoculated control lacking SO_4^{2-} and in the complete treatment; initial total As concentration is represented with the open column and the final total As concentration with the filled column.

5.4.2. Role of pH on the precipitation of ASM and the removal of As.

Table 5.2 Experimental results at different times for the precipitation of As-S mineral treatments at different pH (6.1, 6.45 and 7.1)

Experiment	t_{exp} (d)	pH_t	$[\text{TotAs}]_t$ (mM)	$[\text{SO}_4^{2-}]_t$ (mM)	$[\text{H}_2\text{S}]_t$ (mM)	$S_{\text{loss}}/As_{\text{loss}}^{\dagger}$
pH = 6.1	0	6.17	0.55±0.01	0.81±0.03	0.00±0.00	--
	6	5.93	0.05±0.02	0.12±0.01	0.01±0.00	1.34
	9	5.97	0.00±0.00	0.07±0.01	0.02±0.01	1.32
	12	5.90	0.00±0.00	0.09±0.01	0.03±0.01	1.25
	35	5.96	0.00±0.00	0.05±0.00	0.05±0.00	1.29
pH = 6.45	0	6.44	0.51±0.00	0.78±0.02	0.00±0.00	--
	6	6.49	0.25±0.03	0.35±0.02	0.05±0.01	1.47
	9	6.49	0.10±0.00	0.20±0.02	0.04±0.01	1.31
	12	6.47	0.07±0.02	0.17±0.04	0.04±0.01	1.31
	34	6.49	0.03±0.00	0.14±0.01	0.03±0.00	1.27
pH = 7.1	0	7.13	0.51±0.01	1.09±0.05	0.00±0.00	--
	6	7.15	0.39±0.01	0.87±0.05	0.08±0.01	1.27
	9	7.15	0.27±0.03	0.57±0.01	0.11±0.00	1.80
	12	7.19	0.24±0.00	0.38±0.03	0.26±0.02	1.72
	33	7.15	0.12±0.00	0.38±0.00	0.25±0.04	1.19

[†] S and As losses are defined as the difference between the total initial concentration and the concentration at time t:

$$S_{\text{loss}} = (\text{SO}_4^{2-} + \text{H}_2\text{S})_0 - (\text{SO}_4^{2-} + \text{H}_2\text{S})_t$$

$$As_{\text{loss}} = (\text{TotAs})_0 - (\text{TotAs})_t$$

Two additional experiments were performed at pH 6.5 and 7.2. Similar as the results obtained for pH 6.1, As and S removal from solution was only significant in inoculated treatments containing both As^{V} and SO_4^{2-} . However, the extent and rate of As and S removal as well as the $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ ratios varied depending on the pH. Table 5.2 provides the total As, SO_4^{2-} , H_2S , pH and $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ ratio at five different times over the experiment for the three pH conditions, for the inoculated treatment with As^{V} and SO_4^{2-} amended with ethanol. The total loss of soluble As and S decreased as the pH conditions of the assay increased, which corresponded to more of the biogenic H_2S from SO_4^{2-} reduction being recovered in the medium (especially at pH 7.2). The ratio $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ was 1.25 to 1.47 for the treatments at pH 6.1 and 6.5, but higher ratios were observed on days 9 and 12 at pH 7.2 (Table 5.2). These results suggest a pH dependence of the As removal and ASM formation.

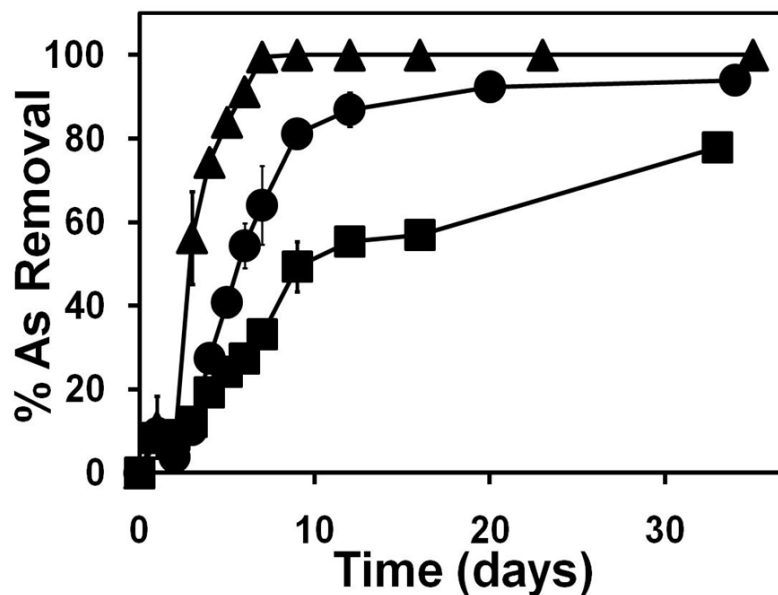


Fig. 5.3. Total As removal over the course of the experiments shown as the As removal percentage at the three investigated pH 6.1 (▲), 6.5 (●) and 7.1 (■).

The rate and extent of As removal was greatly impacted by the pH. Fig. 5.3 compares the percentage of As removal as a function of time for the three pH treatments. The percentage of As removed over the entire experiment was $93.9 \pm 0.6\%$ and $77.9 \pm 0.8\%$ at pH 6.5 and 7.2, respectively. The relationship between the extent and rate of As removal as a function of the pH is shown in Fig. 5.4. An inversely proportional dependency between the As removal and As removal rates was observed with pH. The

percentage of As removed after 9 days was 2-fold higher at pH 6.1 than at pH 7.2. The rate of As removal was 3.4-fold higher at pH 6.1 than at pH 7.2 over the first 9 d of the experiment, and then it increased to 17-fold higher after H₂S started to accumulate at pH 7.2. The data fit with a linear equation over the pH range with a high correlation (R-squared values higher than 0.94). The results indicate a sharp pH-dependency in the near neutral range, with large rate enhancements at mildly acidic conditions.

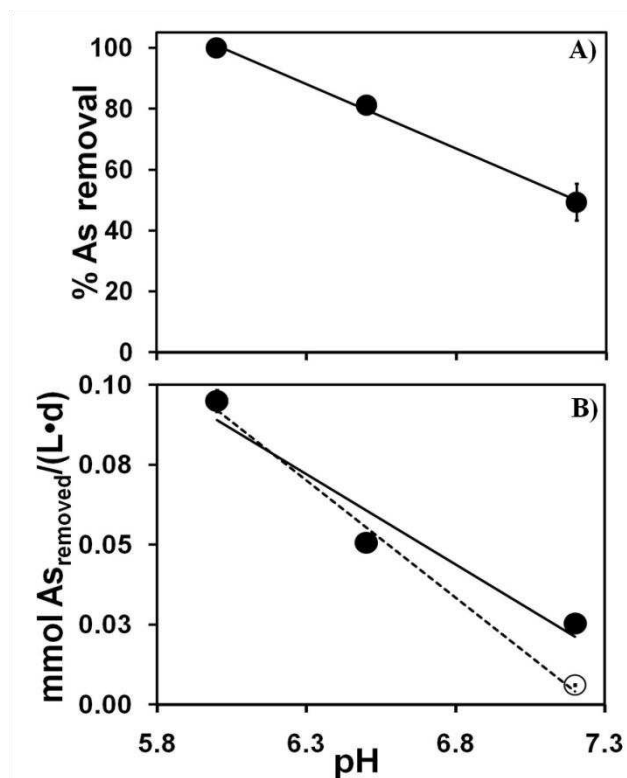


Fig. 5.4. Relationship between the total As removal and the pH. Panel (A) illustrates the trend and linear regression line for As removal as a function of the pH at day 9. The linear regression equation was obtained and the relationship between the As removal and pH can be represented by the linear equation $\%As_{\text{removal}} = A - B \cdot \text{pH}$. The constants and the R-squared are: $A = 355.3\% \text{ As}$, $B = 42.4\% \text{ As}$, $R^2 = 0.9973$. Panel (B) shows the total As removal rate ($\text{mmol As}/(\text{L} \cdot \text{d})$) as a function of the pH. The total As removal rate was calculated using the slope for the first 6 d at pH 6.1, and for the first 9 d at pH 6.5. At pH 7.2, two different As removal rates were observed, high rate, from day 0 to 9 (●), and a lower rate, from day 9 until the end of the experiment (○). The rate of As removal is related to the pH by linear regression equations considering the higher rate at pH 7.2 (continuous line) and the lower rate at pH 7.2 (dashed line). The constants and the R-squared are: higher rate at pH 7.2, $A = 0.4286 \text{ mmolAs}/(\text{L} \cdot \text{d})$, $B = 0.0566 \text{ mmolAs}/(\text{L} \cdot \text{d})$, and $R^2 = 0.9374$; lower rate at pH 7.2, $A = 0.5324 \text{ mmolAs}/(\text{L} \cdot \text{d})$, $B = 0.0734 \text{ mmolAs}/(\text{L} \cdot \text{d})$, and $R^2 = 0.9911$.

An independent set of experiments was performed with an older sample of the anaerobic biofilm at different pHs. The same relationship was observed between As removal extent and rate as a function of the pH (results are shown in the Appendix B, Figs. B.2 and B.3). The percentage of As removed was higher at the lower pH over a long term incubation. As removal and As removal rate were inversely proportionally dependent on the pH and the data also had a near perfect a linear equation, with a negative slope and a high correlation. The reproducibility of the results with a different sample of the anaerobic biofilm serves to validate the dependency of biogenic ASM formation on pH.

5.4.3. Mineral characterization

Solid samples from the three treatments were analyzed using SEM-EDS. Small particles of ASM were present as aggregates and on the surface of the bacteria. Fig. 5.5 provides an SEM image and EDS analysis for two different points, on the surface of a bacterium (Point 1) and on a mineral aggregate (Point 2). The micrograph shows different bacteria surrounded by minerals. The EDS analysis demonstrates that the minerals are composed of As and S. These results confirm the close association between bacteria and mineral formation, supporting a microbial role in the formation of ASM. The solid mineral samples were further characterized using XAS.

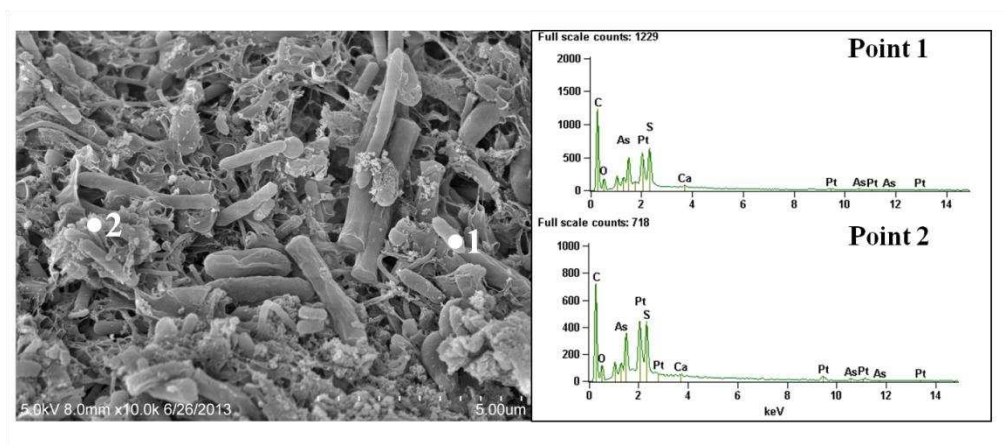


Fig. 5.5. SEM-EDS analysis of the precipitate from the complete treatment at pH 6.1 containing 0.5 mM As^{V} , 0.75 mM SO_4^{2-} and 14 mM ethanol after 21 days of incubation.

XAS enabled the identification of As coordinative environment in precipitates formed at the two pH extremes of the conditions evaluated in the experiments (pH 6.1 and 7.2). Fig. 5.6A shows the XANES spectra for the two analyzed samples along with those of reference AsS and As_2S_3 . The main XANES peak absorbance for As_2S_3 is shifted to slightly higher energy relative to that for AsS. However, this shift is within the resolution at the As edge and should not be used as a sole diagnostic for As coordinated in an As_2S_3 versus AsS structure. Fig. 5.6B shows the EXAFS spectrum of the two analyzed samples and the reference minerals. The solid formed at pH 6.1 (S2) has high similarity with AsS, but it lacks the valleys of the AsS spectra, suggesting the presence of As_2S_3 in the sample. The spectrum of the mineral formed at pH 7.1 (S3) seems to fall in between the two mineral references. Linear combination fitting (LCF) of As K-edge

EXAFS data suggests that the mineral formed at any pH was a mixture of AsS with As_2S_3 . The fractional contribution of each As coordination environment in the sample, as estimated by LCF of the EXAFS spectra, indicates that the mineral formed at pH 6.1 is best represented by a mixture 59% AsS and 38% As_2S_3 (sum $\neq$ 100% because fits not normalized to unity). At pH 7.2, the mineral composition was 52% AsS and 50% As_2S_3 . The occurrence of AsS and As_2S_3 was fully confirmed by the XAS characterization.

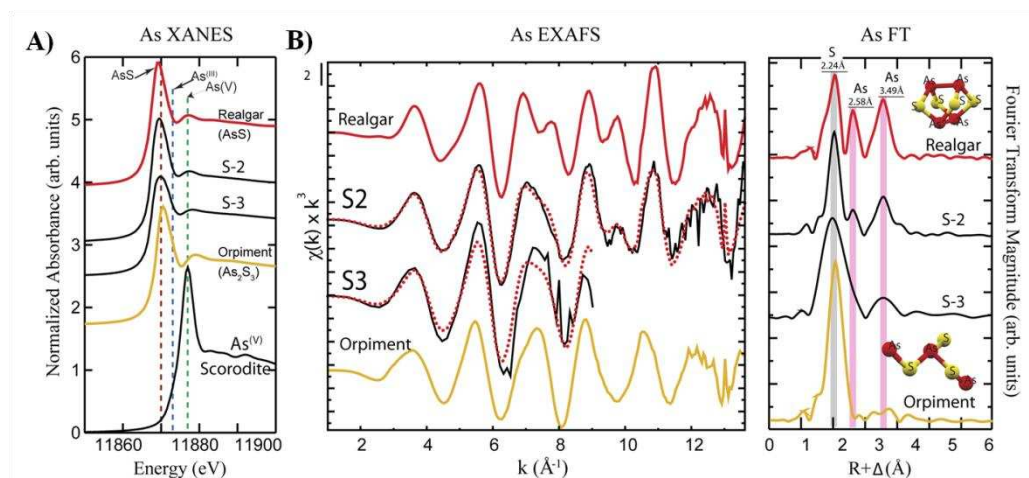


Fig. 5.6. Arsenic K- α x-ray absorption spectra of the solids precipitated in the experiments at pH 6.1 (S-2) and 7.2 (S-3). Panel (A) shows the XANES spectra for As-S mineral compared with standards of realgar (AsS; red line) and orpiment (As_2S_3 ; yellow line), with dashed lines indicating the diagnostic energy position (± 1 eV) of As species; 11870 = arsenic sulfides, 11872 = As^{III} , 11875 = As^{V} . Panel (B) shows the EXAFS spectra and the Fourier transforms for the experimental data (black lines) and linear combination fits (dotted lines) for the As-S mineral and the standards used in the fits. Vertical bars in the FT panel indicate As-backscatter distances.

5.4.4. Ethanol as the electron donor source and the production of acetate and CH_4

In order to monitor the electron-donating process, the conversion of ethanol to acetate and CH_4 was measured. The degradation pathway of ethanol to CH_4 by the microbial consortium in the anaerobic biofilm can be evaluated by studying the treatment lacking As^V and SO_4^{2-} addition. Ethanol is transformed to acetate and hydrogen (H_2) by acetogenic bacteria (Eq. 5.1). Both acetate and H_2 are used by methanogens (Eq. 5.2 and 5.3) to produce CH_4 . As can be appreciated in Fig. 5.7, in the treatment missing SO_4^{2-} and As^V , ethanol concentration decreased quickly after just one day of incubation, accompanied by a small initial accumulation of acetate and subsequently the formation of CH_4 . CH_4 production increased rapidly until reaching a concentration of 8.7 ± 0.4 mmol/L_{liq}. Thereafter, CH_4 kept increasing for the rest of the experiment at a lower rate. By the end of the experiment, the production of CH_4 was 13.9 ± 0.7 mmol/L_{liq}. These results illustrate the rapid transformation of ethanol to acetate and subsequently to CH_4 . The addition of As^V and SO_4^{2-} to the treatments can potentially impact the utilization of ethanol since electron equivalents (e^- eq) could be used for their reduction. The H_2S and As^{III} formed from the reduction could potentially inhibit the activity of the methanogens.

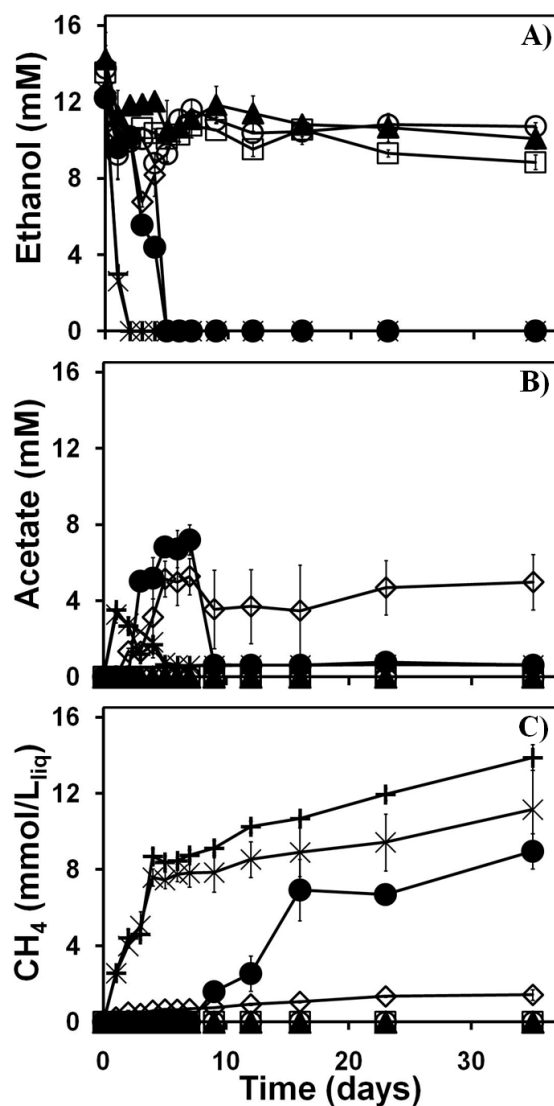


Fig. 5.7. Conversion of ethanol 14 mM in the experiment conducted at pH 6.1. Panels show the ethanol concentration (A); acetate concentration (B); and CH₄ production (C). The complete treatment containing inoculum, As^V, SO₄²⁻ and ethanol (●); Inoculum, SO₄²⁻ and ethanol (x), Inoculum As^V and ethanol (◇), inoculum and SO₄²⁻ (♦), inoculum and As^V (Δ), inoculum and ethanol (+); Sterile controls with As^V and ethanol (○), the sterile control with SO₄²⁻ and ethanol (▲) and the sterile control with SO₄²⁻, As^V and ethanol (□). Treatments lacking ethanol addition (with zero values over the time course of the experiment) are not shown.

Ethanol utilization rate was the same in the presence or absence of SO_4^{2-} , indicating that the addition of SO_4^{2-} and its reduction to H_2S did not affect the metabolic activity of acetogens. In addition, the pattern of acetate accumulation and subsequent consumption as well as the profile of CH_4 production was similar in both cases. The CH_4 production was however slightly lower in the presence of SO_4^{2-} . The difference between the CH_4 produced was 1.1 mmol/L_{liq}, since 3.2 mmol/L_{liq} of H_2 would be required to reduce the supplied 0.8 mM of SO_4^{2-} to H_2S , 0.8 mmol/L_{liq} less CH_4 would have been expected in the treatment with SO_4^{2-} . This analysis supports the expectation that H_2 from ethanol conversion was utilized as the electron donor for SO_4^{2-} reduction.

The inhibitory impact of As^{V} to the acetogenic and methanogenic activity was also evaluated. The presence of As greatly reduced the rate of ethanol conversion, and it inhibited the methanogenic activity. Ethanol concentration decreased at a much lower rate in the presence compared to the absence of As. In the presence of As, the acetate concentration increased until day 5, when it reached 5.1 ± 0.9 mM; thereafter, the concentration was stable until the end of the experiment. The accumulated acetate was clearly not being used as a substrate by the methanogens to produce CH_4 . CH_4 formed slowly throughout the course of the experiment, and the production rate was approximately 10-fold less than in the treatment with no As. These results demonstrates that the presence of As can delay the utilization of ethanol by acetogenic bacteria, and it greatly inhibit the acetoclastic methanogenic activity.

In treatments receiving both SO_4^{2-} and As^{V} , the formation of ASM reversed the methanogenic inhibition by As. The inhibition reversal did not occur immediately but instead corresponded to the moment in time when full precipitation of ASM minerals occurred on day 8 (Fig. 5.7). Consequently during the first 8 days, the full treatment (receiving both As^{V} and SO_4^{2-}) behaved the same as the treatment with just As^{V} addition. There was a delay in the ethanol utilization, with an initial accumulation of acetate and no CH_4 production in both cases (Fig. 5.7). On day 9, after the entire total soluble As was removed (Fig. 5.1) due to ASM precipitation, the inhibition reversed. The accumulated acetate decreased to low levels and the CH_4 production all of sudden commenced, reaching a final production of $8.9 \pm 0.9 \text{ mmol/L}_{\text{liq}}$ (Fig. 5.7). Therefore, the removal of As by the biogenic formation of ASM rendered the As non-bioavailable and thus the As was no longer capable of causing microbial toxicity.

5.5. Discussion

The results taken as a whole demonstrate that the biological reduction of As^{V} and SO_4^{2-} by an anaerobic mixed culture biofilm leads to the formation of ASM in Fe-poor environments, leading to the immobilization of As to non-bioavailable forms. The biomineralization of the ASM depended strongly on the pH conditions in the near neutral range. The amount and rate of As removal were highly enhanced at mildly acidic conditions. Ethanol was readily used as an electron donor source to stimulate the reduction of As^{V} and SO_4^{2-} . The presence of soluble As was found to completely inhibit the activity of the methanogens in the biofilm inoculum; however, the insolubilization of As by biogenic ASM formation reversed the inhibition.

5.5.1. Microbial reduction of As^{V} and SO_4^{2-} promotes the bioprecipitation of ASM

The biogenic formation of ASM can be attained by the combined reduction of As^{V} and SO_4^{2-} . A mixed culture biofilm from a methanogenic environment, which was not previously exposed to high As levels, readily reduced As^{V} . As^{V} and SO_4^{2-} can be biologically reduced by a pure or by a mixed culture. Several SO_4^{2-} -reducing bacteria have been reported as As^{V} -reducing bacteria (Macy et al. 2000). But only five strains of three bacterial genera, *Desulfotomaculum* (Newman et al. 1997a), *Caloramator* (Ledbetter et al. 2007) and *Shewanella* (Lee et al. 2007) have been reported to precipitate

As₂S₃, AsS and As-S nanotubes, respectively; and, a hyperthermophilic archaea genus, *Pyrobaculum* (Huber et al. 2000) can precipitate AsS. However, the presence of As^V and SO₄²⁻-reducers in a mixed culture has been proven to promote the precipitation of ASM in natural environments (Demergasso et al. 2007, Saunders et al. 2008) as well as in a laboratory scale bioreactor (Battaglia-Brunet et al. 2012). In this study, the anaerobic mixed culture biofilm reduced As^V and SO₄²⁻ when both were amended into the same treatment, causing biogenesis of ASM which effectively immobilized the soluble As. The natural co-occurrence between As^V and SO₄²⁻-reducing bacteria can explain the ability of anaerobic microorganisms to promote the bioprecipitation of ASM.

The addition of ethanol as exogenous electron donor was not a requirement to achieve As^V reduction. In the treatments lacking ethanol, there are two sources of e^- eq to support the reduction of As^V, the endogenous decay of the mixed culture biofilm and the degradation of the yeast extract amended to support the growth of the biofilm. The potential of methanogenic sludge to reduce As^V without the addition of an electron donor has been reported before (Sierra-Alvarez et al. 2005). Furthermore, the contribution of the endogenous substrate decay in a comparable methanogenic biofilm corresponded to 16-21 e^- meq/g VSS, available due to the hydrolysis of biomass in the sludge over 30 d (Tapia-Rodriguez et al. 2010). The initial rate of endogenous decay was found to be 0.4 to 1.1 e^- meq/g VSS.d. According to these results, the biofilm can donate 24-31 e^- meq/L electron donor at a rate of 0.6-1.7 e^- meq/L.d with the 1.5 gVSS/L used in the

experiments. In addition to the e^- eq donated by the endogenous substrate decay, the degradation of the yeast extract (20 mg/L) could provide up to 3.1 e^- meq/L. Therefore, the amount of e^- eq released by the decay of the biofilm and the yeast extract would be more than ample to support the reduction of 0.5 mM of As^{V} (1 e^- meq/L).

The addition of an exogenous electron donor greatly enhanced SO_4^{2-} reduction. This coincided with a previous study where ethanol was found to be an effective electron donor promoting enhanced SO_4^{2-} reduction in an anaerobic granular sludge biofilm beyond the endogenous rate (Liu et al. 2010). The reduction of 0.8 mM of SO_4^{2-} to H_2S requires 6.4 e^- meq/L, which are available from the endogenous substrate decay and the degradation of yeast extract; however, an initial competition between SO_4^{2-} -reducing bacteria and methanogens delayed the reduction of SO_4^{2-} . The competition for e^- eq between SO_4^{2-} -reducing bacteria and methanogens has been reported in several studies in the past. SO_4^{2-} reducers will outcompete methanogens for the electron donor utilization, since they have a higher substrate affinity for H_2 , but an initial competition would occur due to lower initial numbers of SO_4^{2-} reducers than methanogens in a methanogenic sludge (Elferink et al. 1994).

5.5.2. Slightly increasing the pH decreased the amount of As removal and percentage of AsS in the mineral

Small variations in the pH affected the removal of As from the system. The extent and rate of As and S removal from aqueous solution were highest at the lower pH conditions corresponding to mildly acidic pH values. When the pH increased, less H₂S was removed due to ASM formation. High H₂S concentrations at neutral pH are known to favor the formation of thioarsenite species (Wilkin et al. 2003), limiting the elimination of soluble As by biomineralization. Newman (1997a) studied the chemical precipitation of As₂S₃ at different pH values and different H₂S concentrations. As₂S₃ was readily precipitated at pH lower than 7 but not at higher pH values when the H₂S concentration was 0.1 mM. Increasing the H₂S concentration to 1 mM caused the minimum pH required for As₂S₃ precipitation to decrease to 6.6.

ASM formation is impacted by the stoichiometry of the available As^{III} and H₂S, which will be dictated by the reduction of As^V and SO₄²⁻. Microorganisms gain more energy from the dissimilatory reduction of As^V compared to SO₄²⁻ reduction, thus As^V reduction is expected to proceed first. A bioenergetic analysis of the redox pair shows that As^V/As^{III} has higher standard reduction potential (60 mV) than SO₄²⁻/H₂S (-220 mV) (Hoeft et al. 2004). In this study, As^V was reduced first prior to SO₄²⁻ reduction, but it is not clear if it was due to an energetic advantage or the fact that there was a 2 d lag phase

before SO_4^{2-} reduction started, both in the presence and in the absence of As^{V} . Hence, As^{III} was already formed before H_2S started to accumulate, favoring the biomineralization of $\text{As}^{\text{III}}\text{-H}_2\text{S}$ and removing H_2S from the medium. This conclusion is in agreement with the observations made by Newman (1997a). *D. auripigmentum* first reduced As^{V} and then SO_4^{2-} , allowing the precipitation of As_2S_3 , while another tested bacterium, *Desulfobulbus propionicus*, that quickly reduced SO_4^{2-} before reducing As^{V} , was not able to promote the formation of ASM. The concomitant reduction of As^{V} and thiosulfate ($\text{S}_2\text{O}_3^{2-}$) by *Shewanella* strain HN-41 also promoted the precipitation of As-S nanotubes (Lee et al. 2007). Therefore, biological activity can enhance the precipitation of ASM by controlling the rate of As^{III} and H_2S formation in a favorable stoichiometric ratio.

The pH changes also affected the mineralogy of the precipitate. The $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ ratio and XAS analysis showed an increase in As_2S_3 proportion over AsS at the higher pH values. The stoichiometric calculations from the $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ ratios indicates 70% AsS and 30% As_2S_3 at pH 6.1 which are in relatively good agreement with XAS characterization results of the solid phase. However the ratios during days 9 and 12 at pH 7.2 indicate 100% As_2S_3 which differs from the solid characterization results. A plausible explanation is that both the stoichiometric ratio and the spectral data obtained for pH 7.2 have a higher associated error compared to data obtained at pH 6.1. Nevertheless, both the solid characterization and the stoichiometric ratio analysis correctly predict an increase in As_2S_3 percentage with increasing pH. The relationship between the mineral

phase proportion and the pH has not been studied before. The difference in behavior with pH can be explained by thermodynamic relationships.

The prediction of ASM species in a solution was evaluated by creating Pourbaix diagrams. Fig. 5.8A shows the Pourbaix diagram for an As concentration of 0.5 mM and S concentration of 0.25 mM (the maximum concentration of H₂S at equilibrium in the pH 7.2 experiment). At the studied pH and E_h range (E_h = -200±50 mV, measured), As₂S₃ and AsS are the minerals expected to precipitate for pH values close to 6 within the range of E_h in the treatments. With increasing pH, the formation of AsS is limited to more reducing conditions, and As₂S₃ is the more likely precipitate up to a pH of 7.0, thereafter, thioarsenites species become predominant, limiting the precipitation of ASM. The thermodynamic stability areas for As₂S₃ and AsS predicted in this study is similar to the Pourbaix diagrams constructed by Lu and Zhu (2011) for a system containing 1 mM of S and As. Fig. 5.8B was built to show the formation of thioarsenites as a function of the pH and H₂S concentration. As the concentration of H₂S increases, the minimum pH at which thioarsenites could be formed decreases. The same trend was predicted by Wilkin (2003) when studying the solubility of As in the presence of S. In conclusion, for the experimental E_h range, the formation of As₂S₃ and AsS is expected over the mildly acidic range of pH; at circumneutral and higher pH values, the formation of thioarsenite species becomes dominant and limits the precipitation of ASM, however, for any precipitation that does occur it would be predominantly in the form of As₂S₃.

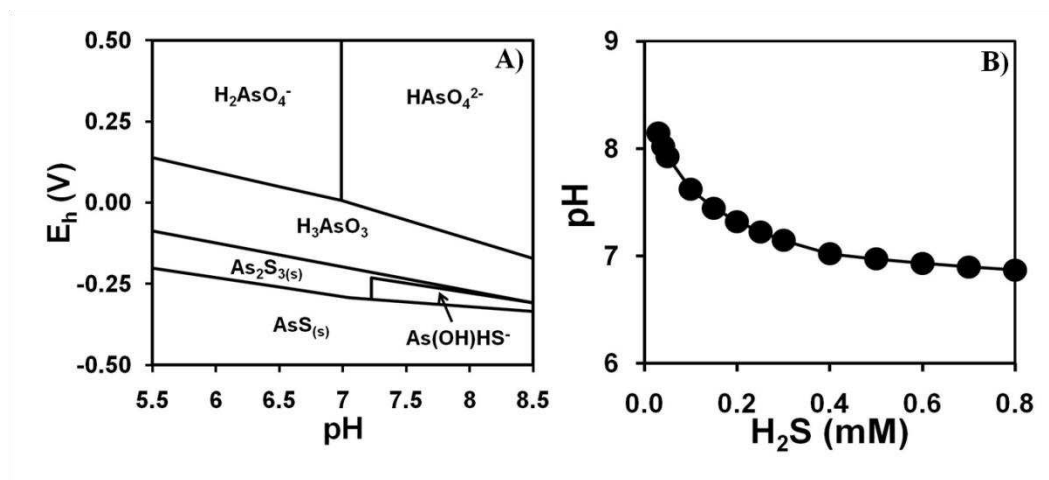


Fig. 5.8. Predicted stable mineral and aqueous phases at equilibrium, including thioarsenite species. Panel A: Pourbaix diagram for As minerals at 25°C and 1 atm showing the stability fields for solid phases at the conditions the orpiment and realgar, in a solution with 0.5 mM of As and 0.25 mM of S. Panel B: Minimum pH enabling formation of thioarsenite species as a function of the H_2S equilibrium concentration, ranging from 0 to 0.75 mM (maximum H_2S production due to SO_4^{2-} reduction)

5.5.3. As toxicity effect on the methanogenic activity

Soluble As was highly toxic to the methanogenic archaea community. The soluble As^{III} formed from the reduction of As^{V} caused a severe inhibition in the methanogenic activity, as demonstrated by the accumulation of acetate and the extremely low CH_4 production. However, the inhibition was largely attenuated by the removal of As throughout the precipitation of ASM. The high toxicity of As^{III} in methanogenic consortium has been established by Sierra-Alvarez (2004). Very low As^{III} concentrations are enough to greatly inhibit the methanogenic activity, the 80% inhibitory concentrations were 23.5 μM and 79.2 μM , for the acetoclastic and hydrogenotrophic methanogenesis, respectively. The As^{III} concentration in this study was 500 μM . The high concentration of As^{III} (produced by the bio-reduction of As^{V}) greatly inhibited the metabolic activity of the methanogenic community.

5.6. Conclusions

- This study demonstrates that the biological reduction of As^{V} and SO_4^{2-} by a mixed microbial culture in a methanogenic biofilm can be harnessed to precipitate ASM.
- Small changes in the pH over the circumneutral range will greatly affect the biomineralization of As to ASM.
- The extent and rate of As removal is highly influenced by the pH, with the highest rates achieved at mildly acidic conditions.
- The pH would also impact the mineralogical composition of the ASM, with an increase in orpiment compared to realgar at neutral pH.
- Arsenic biomineralization can potentially be used to promote the immobilization of As groundwaters by stimulating the As^{V} and SO_4^{2-} reducing bacteria.

CHAPTER VI:
ARSENIC BIOREMEDIATION BY FORMATION OF
ARSENIC SULFIDE MINERALS IN A CONTINUOUS-FLOW
ANAEROBIC BIOREACTOR

6.1. Abstract

Arsenic (As) has been identified at relatively high concentrations in groundwater in certain locations around the world which is a concern since it is a highly toxic compound, widely known for its carcinogenic effects. The interaction of the As and sulfur (S) biogeochemical cycles can be harnessed to promote the immobilization of As. Concurrent microbial reduction of arsenate (As^{V}) and sulfate (SO_4^{2-}) can result in the formation of insoluble arsenic sulfide minerals (ASM). The objective of this research was to study the biomineralization of As in a minimal iron environment for the bioremediation of As-contaminated groundwater using simultaneous As^{V} and SO_4^{2-} reduction. A continuously operated bioreactor, inoculated with an anaerobic sludge was maintained at circumneutral pH (6.25-6.50) and fed with As^{V} and SO_4^{2-} , utilizing ethanol as an electron donor for over 250 d. A second bioreactor running under the same conditions but lacking SO_4^{2-} was operated as a control to study the fate of As removal (without S). The reactor fed with both SO_4^{2-} and As^{V} removed on the average 91.2% of

the total soluble As at volumetric rates up to 2.9 mg As/(L•h), while less than 5% removal was observed in the control bioreactor. Soluble S was also removed by the bioreactor, with a molar ratio of S to As removal of 1.2, suggesting the formation of a mixture of orpiment (As_2S_3) and realgar (AsS). The biomineralization of As in the bioreactor was also evident from the visual formation of a yellow precipitate in the bioreactor. The precipitate was confirmed as a mixture of As_2S_3 and AsS minerals using K-edge X-Ray absorption spectroscopy. The methanogenic community on the reactors was impacted by addition of As. The biogenic ASM was shown to inhibit the acetoclastic methanogens causing an accumulation of acetate. In the control bioreactor, the methanogens were initially highly sensitive to arsenite (formed from As^{V} reduction) but quickly adapted to its toxicity. These results taken as a whole indicate that a bioremediation process relying on the addition of a simple, low-cost electron donor offers potential to promote the removal of As from groundwater by precipitation of ASM.

6.2. Introduction

Arsenic (As) is a highly toxic, ubiquitous metalloid found globally in groundwater. The maximum As concentration considered safe in drinking water by the World Health Organization (WHO) is 10 $\mu\text{g/L}$ (WHO 1993), which corresponds with the maximum contaminant level (MCL) for As in the USA enforced by the Environmental Protection Agency (EPA) (US-EPA 2001). The exposure to As in drinking waters poses a

risk to millions of people around the world in locations where As levels are well above the acceptable limit (Mandal and Suzuki 2002, Murcott 2012).

High As concentrations are often related with As-bearing parent rock materials which, either by natural or anthropogenic causes, will become exposed or weathered resulting in the mobilization of As (Smedley and Kinniburgh 2002, Welch et al. 2000). Due to its chalcophile nature, As is commonly present in sulfide rock deposits, as part of As-sulfide minerals (ASM). The most common ASMs are orpiment (As_2S_3), realgar (AsS) and arsenopyrite (FeAsS), which are found in association with some valuable metals, such as gold, silver and copper (Williams 2001). Mining operations that extract the ore from these rock deposits will produce acid rock drainage (ARD) characterized by high acidity and an elevated concentration of As and other toxic metals, metalloids, iron (Fe) and sulfate (SO_4^{2-}) (Sahoo et al. 2013). The ARD can be treated, promoting the biological reduction of SO_4^{2-} to sulfide (H_2S), which will result in the precipitation of low solubility metal sulfides (Kaksonen and Puhakka 2007). Similar to this process, As could be removed from contaminated water by stimulating the biological reduction of SO_4^{2-} and the precipitation of ASM.

The interrelationship between the As and sulfur (S) biogeochemical cycles can be harnessed to promote the precipitation of ASM, and therefore, the removal of As from contaminated waters. Biological precipitation has been proven to be the source of natural

formed ASM. Firstly, an isotopic study made by Demergasso et al. (2007) demonstrated that the ratio $^{34}\text{S}/^{32}\text{S}$ of laboratory biologically formed ASM corresponded with the ratio of ASM in Andean sediments. Secondly, Saunders et al. (2008) promoted the removal of As in two alluvial aquifers via the addition of SO_4^{2-} and electron donors which is consistent with formation of ASM.

The role of ASM as an As sink is complicated in many studies by the presence of Fe. Most lab-scale bioreactors and microcosms studies have focused on studying the formation of iron-bearing ASM that result when Fe is also present in the system (Altun et al. 2014, Keimowitz et al. 2007, Kirk et al. 2010, Omeregic et al. 2013, Onstott et al. 2011). However, the formation of ASM in systems lacking Fe will be relevant in SO_4^{2-} rich waters (O'day et al. 2004). Battaglia-Brunet et al. (2012) studied the precipitation of ASM in an Fe-free bioreactor under acidic conditions (pH 5). Since the solubility of ASM is low under acidic conditions (pH < 7) and increases drastically around pH 7 due to the formation of thioarsenite species (Lengke et al. 2009, Wilkin et al. 2003), it is necessary to understand the process of ASM formation in the near neutral pH range.

The oxidized species of As, arsenate (As^{V} , H_2AsO_4^- and HAsO_4^{2-} , in circumneutral pH), and S (SO_4^{2-}), will be microbially reduced to arsenite (As^{III} , H_3AsO_3) and H_2S , respectively (Tang et al. 2009, van Lis et al. 2013), under reducing conditions and in the presence of an electron donor that supports the microbial reduction processes.

Ethanol is a commonly used electron donor in SO_4^{2-} -reducing operations due to its low cost and availability (Kaksonen et al. 2003). Under anaerobic conditions, ethanol will be degraded by acetogenic bacteria producing acetate and hydrogen (H_2), which can also be used by methanogenic archaea to produce methane (CH_4). Ethanol transformation pathways can be summarized by the following reactions (Rodriguez-Freire et al. 2014):

- Ethanol acetogenesis:



- Acetoclastic methanogenesis:



- Hydrogenotrophic methanogenesis:



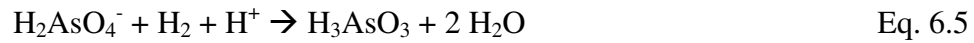
H_2 can also be used as the electron-donating substrate by many SO_4^{2-} (Muyzer and Stams 2008) and As^{V} reducing bacteria (Slyemi and Bonnefoy 2012). Acetate utilization by SO_4^{2-} reducers is limited by competition with the methanogens (Omil et al.

1998). Once in the reduced state, As^{III} (Eq. 6.4) and H_2S (Eq. 6.5) could undergo the mineralization reaction (Eq. 6.6) and form an ASM (Rodriguez-Freire et al. 2014):

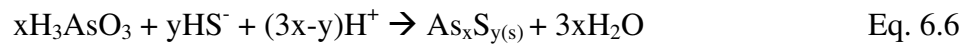
- SO_4^{2-} reduction:



- As^{V} reduction:



- Mineralization:



where; $x = y$, if AsS is being formed; or, $y = 1.5x$, if As_2S_3 is the mineral formed.

The main objective of this study was to evaluate the bioremediation of As in a continuously operated bioreactor resulting from the precipitation of ASM under mildly acidic pH conditions (6.25-6.50) in a minimal Fe environment. An anaerobic bioreactor, inoculated with a methanogenic biofilm, was used to promote the reduction of SO_4^{2-} and As^{V} , using ethanol as the electron donor. A second bioreactor without SO_4^{2-} addition was

run in parallel as a control (in order to ascertain the role of S in the immobilization of As and the toxicity of As in the methanogenic sludge). The performance of the reactors was evaluated by the speciation of As and S in the liquid medium, while the solid phase was characterized by spectroscopic methods.

6.3. Materials and methods

6.3.1. Chemicals and source of anaerobic inocula

High purity commercial powdered (diameter < 200 nm) $\text{As}_2\text{S}_{3(s)}$ was purchased from Acros Organics (Thermo Fisher Scientific, Pittsburgh, PA, USA). The mineral was conserved in an inert atmosphere of N_2 gas to avoid oxidation and deterioration of the samples. All the chemicals were analytical grade and the solutions were prepared using ultrapure water (Milli-Q system; Millipore).

The two bioreactors were inoculated using an anaerobic granular biofilm obtained from an upflow anaerobic sludge blanket treating wastewater from a beer brewery (Mahou, Guadalajara, Spain). The volatile suspended solids (VSS) composition of the sludge was 0.042 ± 0.002 g VSS/g wet wt. The sludge was maintained in anaerobic conditions, with a nitrogen gas (N_2) atmosphere and at 4 °C.

6.3.2. Mineral medium composition

The bioreactors were fed with mineral medium prepared using ultra pure water (Milli-Q system; Millipore). The composition of the mineral medium was as follows (mg/L): K_2HPO_4 (1200); KH_2PO_4 (1568); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (166); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (20); NH_4Cl (560); yeast extract (40). 2 mL/L of a trace element solution that was added to the medium to provide a final concentration of ($\mu\text{g/L}$): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (4,000); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (4,000); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1000); $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (180); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (60); ZnCl_2 (100); H_3BO_3 (100); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (100); $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ (200); $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (100); EDTA (2,000); resazurin (400); HCl 36% (2 μL). The mineral medium was flushed using N_2/CO_2 (80:20) to ensure anaerobic conditions. The bicarbonate (NaHCO_3): CO_2 system was used to control the pH of the solution in the reactors varying from 6.5 (1 g/L NaHCO_3) to 6.25 (0.5 g/L NaHCO_3).

6.3.3. Anaerobic bioreactors set-up

Two laboratory-scale upflow bioreactors were operated in parallel as shown in Fig C.1 of the Appendix C. Reactor 1 (622 mL) was used as the treatment reactor to study the SO_4^{2-} reduction activity and the formation of ASM; and reactor 2 (616 mL) was used as the control reactor to study the methanogenic and the As^{V} reduction activity. Both reactors were inoculated with 13.3 g VSS/L. The medium was fed to the reactors at a flow rate that corresponded with a hydraulic retention time (HRT) of 24 h. The reactors were kept in an incubator at 30 °C. The influent of the reactors was stored in a refrigerator at 4 °C. An anaerobic atmosphere in the influent container was maintained using a gas bag (SKC-West Inc, Fullerton, CA) containing N_2/CO_2 (80:20).

There were three different operation stages in each reactor, stage I, II and III:

Stage I was intended to study the sulfate reduction activity (reactor 1) and the methanogenic activity (reactor 2) of the inocula using ethanol as electron donor at pH 6.5. 1.59 mM of ethanol was added to the medium in both reactors; and, 1 mM of SO_4^{2-} as Na_2SO_4 was amended in reactor 1.

During *Stage II and Stage III*, 1 mM of As^{V} (as $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$) was added to both reactors. In reactor 1, the SO_4^{2-} load to the reactors was increased from 1 to 1.5 mM

to provide the stoichiometric amount required for the precipitation of As_2S_3 . In both reactors the concentration of ethanol was increased from 1.59 to 4.9 mM to ensure an excess of electron donor to the system. The average pH of the influent was 6.5 during stage II of operation. In stage III, the concentration of As^{V} , SO_4^{2-} and ethanol was maintained the same but the pH was decreased to 6.25.

The actual values of pH and HRT during reactor operation are presented in the Appendix C (Fig. C.2). A summary of operating condition in the different stages is provided in Table 6.1.

Table 6.1 Description of the conditions of operation in the reactors^(a).

Stage	Period (d)	Reactor 1					Reactor 2			
		SO ₄ ²⁻ (mM)	As ^V (mM)	Ethanol (mM)	pH ^(b)	HRT (h)	As ^V (mM)	Ethanol (mM)	pH	HRT (h)
<u>I</u> ^(c) :	0-83	0.83±0.12	----	1.00±0.29	6.55±0.08	24.39±0.54	----	1.11±0.28	6.54±0.07	24.68±0.52
<u>II</u> ^(d) :	84-156	1.35±0.15	0.99±0.06	4.55±0.18	6.47±0.07	24.08±0.64	1.01±0.08	4.53±0.14	6.47±0.05	24.40±0.67
<u>III</u> ^(e) :	157-259	1.59±0.10	1.04±0.05	4.32±0.26	6.28±0.04	24.56±0.47	1.02±0.05	4.30±0.35	6.28±0.04	24.12±0.59

^(a) The data shown represents the average of the experimental values for the influents of the reactors, over the period of operation with the error as the standard deviation of all the results.

^(b) The actual values for the pH and HRT for the time course of operation in the reactor can be found in the Supplementary Information, Fig. 1S.

^(c) Lacking As^V. Study of the sulfate reduction and methanogenic activity in the reactors in the absence of As^V.

^(d) Addition of As^V to the reactors. Study of the biomineralization and As effect on methanogens.

^(e) Decrease of the operational pH from 6.50 to 6.25 keeping the same conditions as stage II.

Liquid samples from the influent and effluent were taken from the sampling ports. pH, Oxidation-Reduction Potential (ORP) and H₂S were measured immediately. As^V, total soluble As, additional As species, SO₄²⁻, ethanol and acetate samples were centrifuged (10 min, 14,000 g) and stored in polypropylene vials at -20 °C until analysis. Methane (CH₄) gas production was monitored by measuring the volume of liquid displaced in an inverted bottle containing 2% NaOH (to scrub CO₂) connected to the gas effluent. Solid samples were taken periodically from the reactor for mineral characterization. The solid samples were washed with ultra-pure water to remove any soluble fraction of As, flushed with N₂ gas and frozen at -4°C.

6.3.4. Mass balance of As in the reactors

At the end of the stage III, the bioreactors were sealed and transferred to an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI). Once inside, the reactors were emptied to a sieve to separate the liquid and the solid phases. The liquid phase was collected in a serum bottle. The solid phase was homogenized and samples were taken for acid extraction and for solid characterization. Samples from the liquid media were also collected for extraction. The acid extraction was performed on triplicate using 50 mg of solid phase in 4 mL of aqua regia (HCl:HNO₃, 3:1). These samples were analyzed for total As content.

6.3.5. Batch experiments: Soluble arsenic and ASM toxicity to the methanogenic activity

The toxicity of As^{III} in the methanogenic sludge was tested using the sludge without previous exposure to As and the sludge from reactor 2 after being in contact with 1 mM of As for more than 160 d. The methanogenic activity was evaluated for acetate (acetoclastic) or H₂ (hydrogenotrophic) as electron donor. The batch experiments were carried out in 50 mL serum bottles. The mineral medium (pH 6.5) of the batch experiments contained (mg/L): NH₄Cl (224), NaHCO₃ (3,200), K₂HPO₄ (200), CaCl₂•2H₂O (8), MgCl₂•6H₂O (80), MgSO₄•7H₂O (80); yeast extract (80) and 0.8 mL/L of trace element solution for a final concentration of (μg/L): FeCl₃•4H₂O (160); CoCl₂•6 H₂O (160); MnCl₂•4 H₂O (4); AlCl₃•6 H₂O (7.2); CuCl₂•2H₂O (2.4); ZnCl₂ (4); H₃BO₃ (50); (NH₄)₆Mo₇O₂₄•4 H₂O (4); Na₂SeO₃•5 H₂O (8); NiCl₂•6H₂O (4); EDTA (80); resazurin (16); HCl 36% (0.16 μL). Additionally, the proper concentration of As^{III} was added from a stock solution prepared using NaAsO₂. The final concentration of VSS in the assays was 0.8 g VSS/L. In the experiments to evaluate the toxicity of As^{III} in the acetoclastic methanogenic, the mineral medium also contained 25mM of acetate (as CH₃COONa). The inocula was incubated with 10 mL of 1.25-fold concentrated mineral medium were added to the serum bottles and flushed with N₂/CO₂ (80:20) for 5 min, Then, the bottles were closed using butyl rubber septa and aluminum seals and the headspaces were flushed for an additional 5 min with N₂/CO₂, needle in needle out. In the

experiments evaluating the hydrogenotrophic methanogenesis, H_2 was supplied as overpressure using H_2/CO_2 (80:20) for a final partial pressure inside the bottles of 0.1 atm of H_2 . All the bottles were pre-incubated overnight at 30 °C in an orbital shaker rotating at 120 rpm.

Following the pre-incubation, a specific volume of a stock solution of As^{III} was added to the bottles to obtain at least 5 different concentrations of As^{III} in each experiment. The volume of liquid added was completed (if needed) to 2.5 mL with ultra-pure water, for a total volume of liquid in the treatments of 12.5 mL (head space volume of 37.5 mL). Controls with no As^{III} were run in quadruplicates, and the rest of the treatments were run in duplicates. The bottles were then flushed with N_2/CO_2 (80:20), and H_2/CO_2 (80:20) was added as overpressure for the hydrogenotrophic methanogenic activity test.

The toxicity of the ASM to the original methanogenic sludge was evaluated using a commercial As_2S_3 and using the biogenic ASM precipitated in reactor 1. In this study, the toxicity was only studied under acetoclastic conditions. The batch experiments were performed in the same fashion as the experiments for the toxicity of soluble As, with little modifications. 25 mL culture tubes were used for the incubation. 10 mL of the mineral medium (same composition as previously described) were added to 237 mg of anaerobic biofilm, for a final concentration of 1 g VSS/L. The tubes were flushed with N_2/CO_2 and

closed as explained before. After an overnight pre-incubation, the tubes were opened to add the proper amount of As₂S₃ or biogenic ASM was added to the tubes to obtain 4 different concentrations. After the addition of the mineral, the tubes were flushed and closed again. The treatments were incubated at 30°C in an orbital shaker rotating at 120 rpm.

CH₄ production was monitored three or four times a day until the maximum methane production was reached. Liquid samples were collected at the beginning and at the end of the experiment to detect any change in As speciation. H₂ gas was measured at the beginning of the experiment to determine the exact amount of H₂ added in the treatments. The actual concentration of VSS in each treatment was analyzed at the end of the experiment.

The concentration of CH₄ produced in the treatments was calculated as a function of the amount of VSS of the treatment and not the volume of the liquid. The normalized methanogenic activity (NMA) was calculated as the ratio of maximum methane production rates in each specific treatment (for each As^{III} concentration) and the control (no As^{III}), as shown in equation 6.7:

$$\text{NMA}(\%) = \left(\frac{\text{Maximum CH}_4 \text{ production rate at tested As}^{\text{III}} \text{ concentration}}{\text{Maximum CH}_4 \text{ production rate of the control}} \right) \times 100 \quad \text{Eq. 6.7}$$

The IC₅₀ concentration was also calculated and it is defined as the concentration of As^{III} that causes 50% of inhibition in the normalized methanogenic activity compared with the control.

6.3.6. Analytical techniques

Total As concentration was measured by using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system model Optima 2100 DV from Perkin–Elmer TM (Shelton, CT, USA) monitored at wavelength 193.7 nm. As^V and SO₄²⁻ were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 analytical column (4 x 250 mm) and AG16 guard column (4 mm x 40 mm). The injection eluent (KOH) was 30 mM for 10 min. Few samples were sent for As speciation to detect As^{III}, As^V and the organics methylarsonic acid (MMA^V) and dimethylarsonious acid (DMA^V) was performed using a high pressure liquid chromatography system (Agilent 1100 HPLC, Agilent Technologies, Inc.) with a reverse-phase C18 column (Gemini 5u C18 110A, 150x4.60mm, Phenomenex, Torrance, CA) and guard cartridge. The mobile phase (pH 5.85) contained 4.7mM tetrabutylammonium hydroxide, 2mM malonic acid and 4% (v/v) methanol at a flow rate of 1.2 ml/min. The column temperature was maintained at 50C and samples are kept at 4C in a thermally controlled autosampler. Following the HPLC, the As species were detected using an ICP-mass spectrometer (ICP-MS) (Agilent 7500ce) with a

Conikal nebulizer (Glass Expansion). The operating parameters were as follows: Rf power 1500 watts, plasma gas flow 15 L/min, carrier flow ~0.9L/min, 0.15 L/min makeup, and arsenic was measured at 75 m/z. Low detection limits for the measured species are: 0.03 ppb As^{III}, 0.17 ppb As^V, 0.11 ppb DMA^V, 0.15 ppb MMA^V, 0.05 ppb total As.

As^V and total As concentrations were measured once every other day. The concentration of As^{III} can be calculated as the difference between total As and As^V in reactor 2. In order to validate the previous assumption, seven set of samples, evenly distributed over the operation time of the reactors, were sent to an external lab for As speciation. The concentration of organoarsenicals, methylarsonic acid (MMA^V) and dimethylarsonious acid (DMA^V) was below the detection limit in all the samples (Table C.1 in Appendix C). As^{III} concentration was below the detection limit in the effluent of reactor 1 (Table C.1 in Appendix C); and, it corresponded with the difference between the total As and As^V concentration in the effluent of reactor 2. Therefore, the concentration of As^{III} in the effluent of reactor 2 can be calculated as:

$$[\text{As}^{\text{III}}]_{\text{effluent}} = [\text{TotAs}]_{\text{effluent}} - [\text{As}^{\text{V}}]_{\text{effluent}} \quad \text{Eq. 6.8}$$

H₂S was determined using the methylene blue method described by (Truper 1964) and measured using an UV-visible spectrophotometer (Agilent 8453, Palo Alto, CA,

USA). The measurement of H₂S provides the amount as H₂S in the liquid phase only. The total amount of dissolved sulfide (DS) was calculating by considering the H₂S speciation at the measured pH using the dissociation constant for H₂S. Consequently,

$$DS = [H_2S]_{aq} + [HS^-] + [S^{2-}] \quad \text{Eq. 6.9}$$

Headspace samples in the batch experiments were taken with a pressure lock gas tight syringe (1710RN, 100 µl (22s/2"/2), Hamilton Company). H₂, ethanol, acetate and CH₄ were detected in an Agilent Technologies 7890A gas chromatography system. H₂ was detected using a CarboxenTM 1010 Plot Fused Capillary Column (30 m x 0.53 mm) with a thermal conductivity detector and N₂ used as the carrier gas. Ethanol, acetate and CH₄ were monitored using a Restek Stabilwax®-DA Column (30 m x 0.35 mm, ID 0.25 um) with flame ionization detector, and He used as a carried gas.

Solid phase was characterized using a Scanning Electron Microscopy (SEM) combined with energy dispersive spectroscopy (EDS), and K-edge X-ray absorption spectra (XAS) with X-ray absorption near-edge structure (XANES) and extended x-ray absorption fine-structure (EXAFS) according to the methodology previously described in the Appendix C.

Measurements of pH, ORP and VSS were conducted according to standard methods (APHA 1999).

6.3.7. S and As loss in the reactors

The ratio of S to As loss ($S_{\text{loss}}/As_{\text{loss}}$) in the reactor has been calculated to predict the stoichiometric relation between S and As in the precipitate. S_{loss} is defined as the average removal of S over time in the reactor and it is calculated as follow:

$$S_{\text{loss}} = (\text{SO}_4^{2-} + \text{DS})_{\text{influent}} - (\text{SO}_4^{2-} + \text{DS})_{\text{effluent}} \quad \text{Eq. 6.10}$$

As_{loss} is defined as the average removal of As over time in the reactor and it is calculated as follow:

$$As_{\text{loss}} = (\text{TotAs})_{\text{influent}} - (\text{TotAs})_{\text{effluent}} \quad \text{Eq. 6.11}$$

6.4. Results

6.4.1. SO_4^{2-} and As^{V} reducing activity in reactor 1

The SO_4^{2-} reducing activity of the sludge using ethanol as electron donor was evaluated during the time course of the operation in reactor 1. Fig. 6.1A shows the reduction of SO_4^{2-} and Fig. 6.1B shows the DS production over the time course of operation of the reactor. SO_4^{2-} was reduced by the anaerobic sludge during all reactor stages, but the recovery of SO_4^{2-} as DS was greatly affected by the introduction of As in the reactor at the end of stage I. During stage I, $91.8 \pm 6.7\%$ of the SO_4^{2-} added in the influent was reduced to DS. The concentration of DS leaving in the effluent of the reactor corresponded to the amount of SO_4^{2-} removed. At the start of stage II, feeding As^{V} in reactor 1 commenced and the concentration of SO_4^{2-} was increased. The reduction of SO_4^{2-} reached a new steady state after 9 d, averaging $94.3 \pm 3.4\%$ for the rest of the experiment. However, the amount of DS discharged with the effluent decreased and as a consequence the S_{loss} increased to an average of 1.16 ± 0.15 mM and the total S volumetric removal rate was 1.5 ± 0.2 mg S/L.h for the remainder of reactor 1 operation (stages II and III). The pH change from 6.5 (stage II) to 6.25 (stage III) resulted in an average increase of 10.7% in the total S_{loss} and 14.3% in the S removal rate. In conclusion, the addition of As^{V} did not affect the SO_4^{2-} reduction but it promoted the immobilization of S in the reactor, which was slightly enhanced by the decrease in the pH from 6.5 to 6.25.

The fate of As^{V} once introduced in the reactor followed a similar trend as the SO_4^{2-} . The concentration of As^{V} and total soluble As in the influent and effluent of the reactor are shown in Fig. 6.1C and 6.1D, respectively. As^{V} was readily eliminated by the anaerobic biofilm and total soluble As was effectively removed during the entire operation of the reactor. Steady state was quickly achieved and the removal efficiencies averaged $93.2 \pm 4.3\%$ and $91.2 \pm 8.3\%$ for the rest of the reactor operation for As^{V} and total As, respectively. Overall, the total As volumetric removal rate in the reactor at the steady state was $2.9 \pm 0.4 \text{ mg As}/(\text{L} \cdot \text{h})$. Neither As^{III} , MMA^{V} or DMA^{V} were detected during the speciation analysis, as shown in Table C.1 in Appendix C. The change of the pH from stage II to III did not impact the removal of As^{V} significantly. These results confirm the hypothesis that As can be immobilized over the long-term during the operation of a SO_4^{2-} reducing reactor.

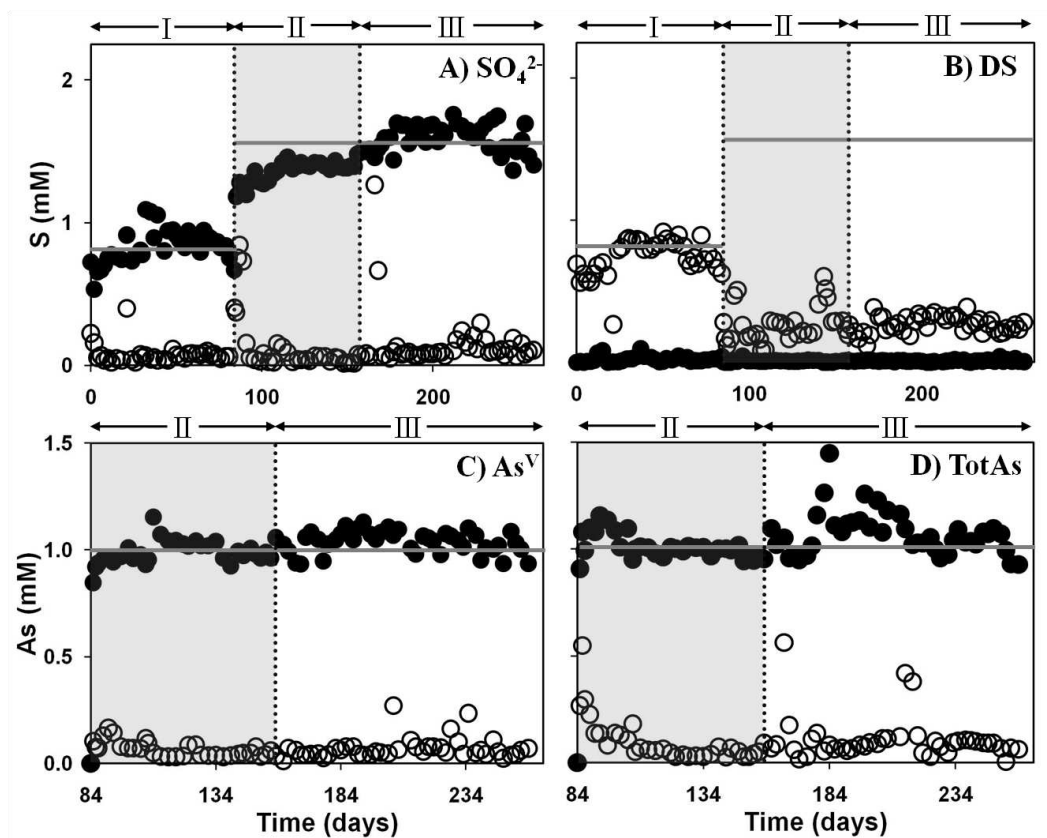


Fig. 6.1. S and As speciation in reactor 1 in the influent (●) and in the effluent (○). (A) SO_4^{2-} concentration; (B) DS concentration; (C) As^{V} concentration; (D) total As concentration. The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation, right after the addition of As^{V} . The horizontal grey lines represent the theoretical concentration of the specie in the effluent if not transformation/immobilization would take place. Measured As^{III} concentration in the effluent was below the detectable limit (data not shown).

6.4.2. Biomineralization of As as a sulfide mineral in reactor 1

S_{loss} in the reactor was simultaneous with the removal of As, indicating the formation of ASM. The precipitation of ASM was indicated visually by observation of a yellow precipitate which was evident starting 4 d after introducing As into the reactor, and increased in volume with the operation time (Fig. C.3 in the Appendix C).

Table 6.2 As and S removal in reactor 1 and stoichiometric relationship between the S and As loss.

	As_{loss} (mM)	S_{loss} (mM)	$S_{\text{loss}}/As_{\text{loss}}$	%As _{removal}
Stage I	----	0.03±0.13	----	----
Stage II	0.94±0.11	1.08±0.11	1.15±0.14	93.56±3.99
Stage III	0.97±0.13	1.21±0.14	1.25±0.18	89.86±9.82
Overall (Stages II & III)	0.96±0.10	1.16±0.15	1.22±0.18	91.21±8.25

The stoichiometric relationship between the total As and S losses in the reactor provides information on the minerals formed (Table 6.2). A ratio of S to As loss ($S_{\text{loss}}/As_{\text{loss}}$), of 1.5 and 1.0 would be expected for As_2S_3 and 1.0 for AsS; respectively. The actual values of the ratio over the time course of the reactor after the addition of As are shown in Fig. C.4, in Appendix C. During stage II, the average ratio $S_{\text{loss}}/As_{\text{loss}}$ was 1.15±0.14 (30% As_2S_3 and 70% AsS), but increased by 8.7% during stage III (1.25±0.18,

50% As_2S_3 and 50% AsS). Overall, the average ratio ($\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$) for the entire operation of the reactor was 1.22 ± 0.18 , which would correspond to a mixture of 45% As_2S_3 and 55% AsS . Therefore the stoichiometric data suggests the precipitate is composed of a mixture of As_2S_3 and AsS .

6.4.3. ASM characterization

Solid samples from the reactor were analyzed using XAS. Two different samples were collected, sample 1 for the stage II of operation, 60 d after the addition of As, and sample 2 for stage III, the last day of operation in the reactor, 176 d after starting the addition of As. Fig. 6.2A shows the Fourier transform and the EXAFS spectra for the two samples analyzed in the As coordinative environment. The sample 1 spectrum shows an amplitude and As-S distance similar to the reference mineral, As_2S_3 . The sample 2 spectrum shows more similarity with AsS , since the amplitude is similar to the reference mineral AsS , but the As-S distance is still like the reference mineral As_2S_3 . Using the EXAFS non-linear least squares fits, which gives near neighbor atoms and bond distances, and the stoichiometry of the atoms in the fits at the crystallographic distances, the composition of the precipitated was obtained. Sample 1 composition was 70% As_2S_3 and 30% AsS , and sample 2 was 40% As_2S_3 and 60% AsS . The As-As distance seemed to be slightly deformed in both samples, most likely due to an amorphous character of the

mineral, and the modeled structures of the minerals are shown in Fig. 6.2B. In conclusion, the mineral fraction seems to be formed by a mixture of As_2S_3 and AsS .

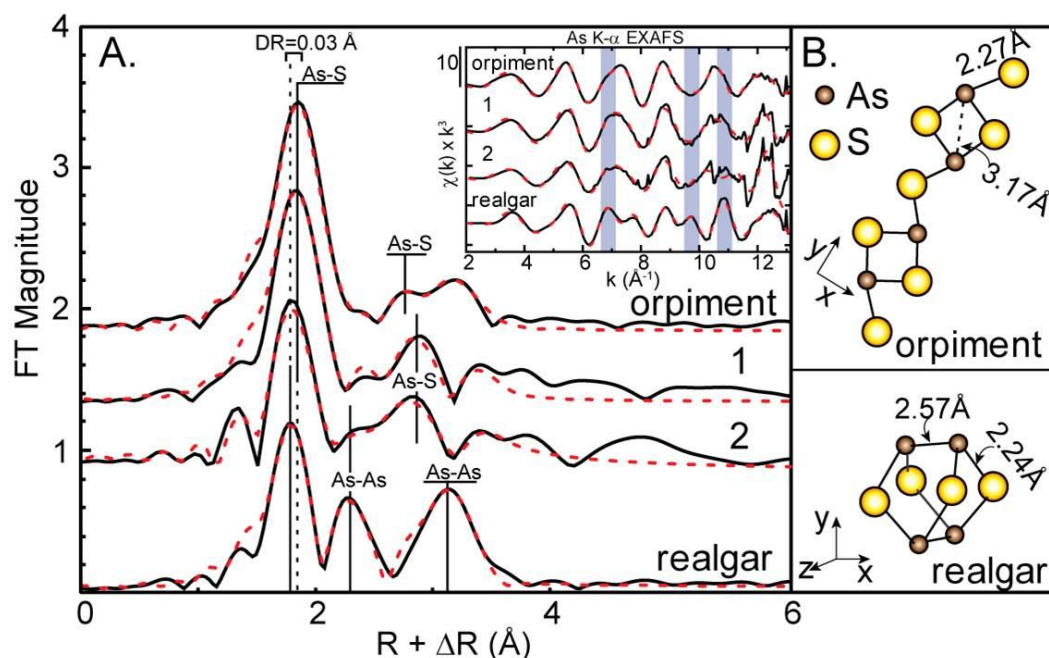


Fig. 6.2. As K- α x-ray absorption spectra of the solids precipitated in the experiments 60 d(sample 1) and 176 d (2). (A) shows the Fourier transforms and the EXAFS spectra (small window) for the experimental data (black lines) and linear combination fits (dotted lines) for the As-S mineral and the standards used in the fits, vertical bars indicate As-backscatter distances. Shaded areas in the EXAFS spectra indicate the contribution from both standard minerals to the sample spectra. (B) shows the modeled structure of the minerals with the estimated distance between the atoms As-As.

In order to fully characterize the mineral, the solid phase was also analyzed using SEM-EDS. These results are presented in the Appendix C, Fig. C.5. The mineral precipitated formed 20 μm aggregates with the biofilm in the reactor. A closer look to one of the aggregate shows particles of minerals of less than 0.1 μm surrounding or coating the different bacteria. The EDS was performed in two different points, on the surface of a mineral (point 1) and of a bacterium (point 2). The EDS confirmed the presence of an ASM. The counts for As and S were similar for both points indicating the bulk extracellular precipitate had a similar elemental composition as the mineral precipitates associated with the bacteria.

6.4.4. Arsenic mass balance in reactor 1

A mass balance analyzing the cumulative removal of As over the 176 d of operation (stages II and III) and the amount of As precipitated in the reactor was determined at the end of the experiment. The total cumulative amount of As immobilized from the influent was 7.59 g As, which corresponded to a concentration of 915.8 mg As/g VSS of sludge. The As extracted from the solid phase was 898.1 ± 2.4 mg As/g VSS, which corresponds to a 98.1% recovery of the total As immobilized in the sludge bed of that lost during the operation of the reactor. These results demonstrate a great capacity of the anaerobic sludge to retain the ASM precipitated in the reactor.

6.4.5. As^V reducing activity in reactor 2 lacking SO₄²⁻ addition

Reactor 2 was operated with no SO₄²⁻ addition to study the fate of As in the bioreactor under methanogenic conditions. As^V was readily reduced to As^{III} but there was no As immobilization in the reactor. Fig. 6.3 shows the time course for the concentration of As^V and total As in the influent and effluent of reactor 2. In less than 2 d after start adding addition, 98.52% of the As^V was being reduced to As^{III}. MMA^V and DMA^V concentrations in the effluent were below detection limits for the samples analyzed for speciation. The average reduction of As^V to As^{III} was 91.6±6.5% over the entire time of operation. The change of pH from stage II to III did not affect the reduction of As^V. The As^{III} produced in the reactor did not undergo any other process and it exited the reactor in the effluent. On the average during stages II and III, 0.91±0.12 mM of As^{III} was leaving reactor 2. Overall, the data indicates that microorganisms in methanogenic sludge readily reduced As^V using ethanol as the electron donor source. The As^V reducing capacity was not affected by the pH change (stage III) or by the the long time operation (176 d) of the reactor.

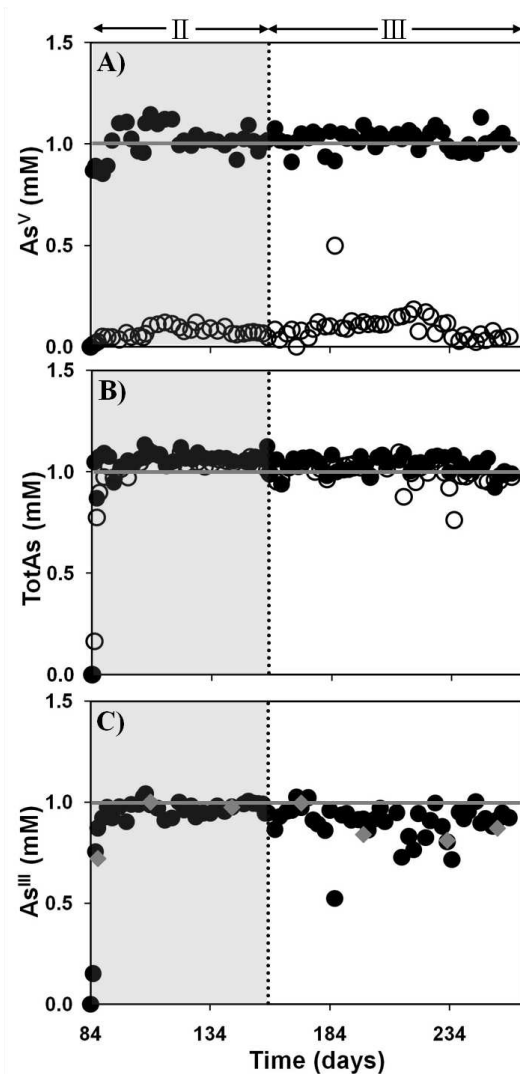


Fig. 6.3. Arsenic speciation in reactor 2 influent (●) and effluent (○). (A) As^{V} concentration; (B) total As concentration; (C) As^{III} concentration calculated as the difference between total As and As^{V} concentrations in the effluent. Measured As^{III} concentrations are shown with (◆). The vertical dashed line indicates the separation between stages II and III in the reactor. The shaded area designates the stage II of operation, right after the addition of As^{V} . The horizontal grey lines represent the theoretical concentration of the specie in the effluent if not transformation/immobilization would take place.

6.4.6. Comparison between reactor 1 and reactor 2

A comparison between the removal of As between the two reactors stresses the importance of the interaction between the As and S biogeochemical cycles. Fig. 6.4 depicts the % of As removal in reactor 1 and in reactor 2 over the time of operation in the reactors. In reactor 1, where the concomitant reduction of SO_4^{2-} and As^{V} occurred, the removal of As was over 90% for most of the operation. However, in reactor 2, where As^{V} was the only electron acceptor being reduced, the removal of As was consistently less than 5%. This evidence proves that the removal of As from water can be achieved by the precipitation of an ASM in a SO_4^{2-} -reducing bioreactor under slightly acidic conditions.

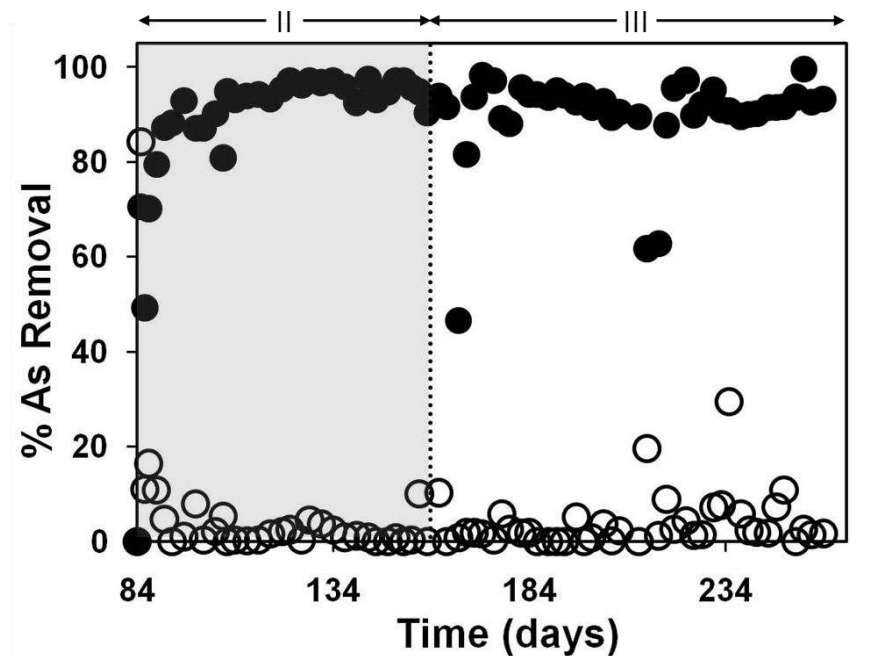


Fig. 6.4. Total As removal, expressed as the percentage of total As removed in the reactors over the time of operation in reactor 1 (●) and reactor 2 (○). The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation, right after the addition of As^{V} commenced.

6.4.7. Fate of the electron donor in the reactors

Ethanol was added to the reactors as an exogenous electron donor source. The fate of ethanol and the outcome of the reactions that took place depended on the reactor and the stage of the operation.

Reactor 1 was initially being fed with 0.83 ± 0.12 mM SO_4^{2-} and 1.00 ± 0.29 mM ethanol for 84 d (stage I). Fig 6.5A shows that ethanol was completely degraded in the reactor since day 1 of operation. The intermediate, acetate accumulated in the reactor during the first month, after that time acetate was effectively transformed to CH_4 by the methanogens. CH_4 production was observed since the first week, 2.7 mmol/ L_{liq} CH_4 was measured after 8 d of operation (Fig. 6.5B). The initial CH_4 detected was 1.8-fold higher than the theoretical value (1.5 mmol/ L_{liq}) if both acetate and H_2 were solely used by the methanogens. The initial CH_4 formed in excess of the ethanol consumption can be rationalized by the presence of endogenous substrates within the anaerobic biofilm. The endogenous decay of the methanogenic biofilm, measured in test tubes, was $0.6 e^-$ meq/g VSS.d (details presented in Appendix C).

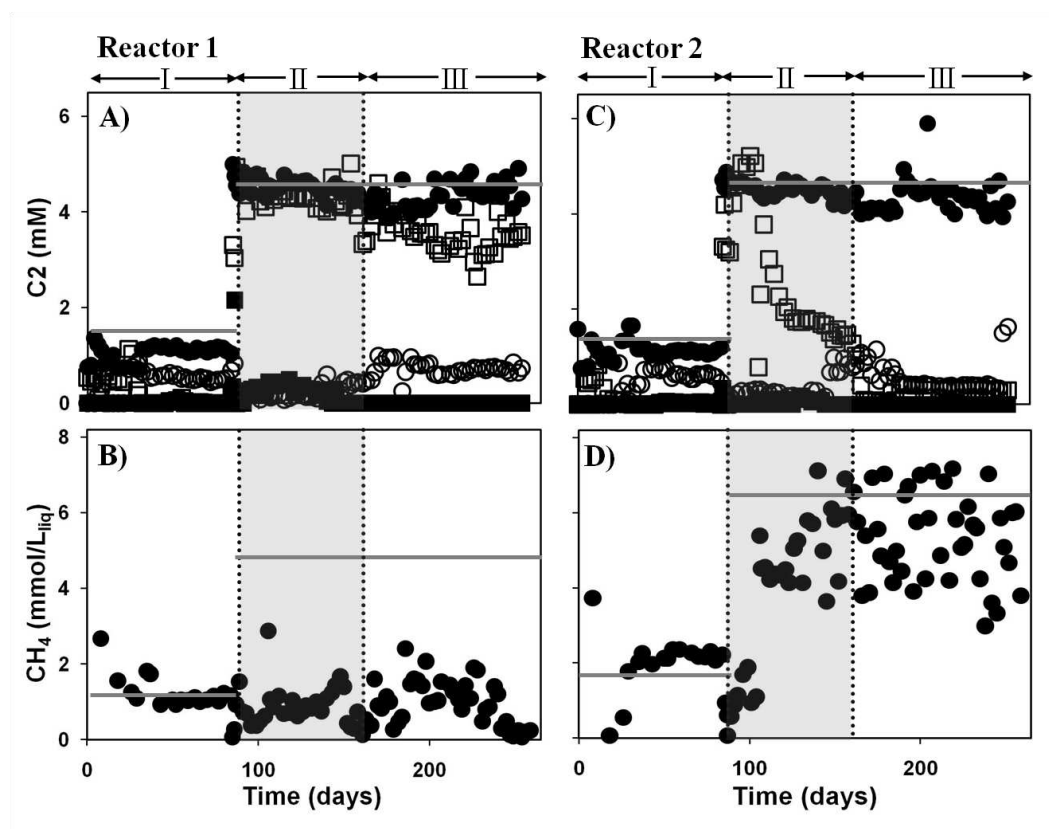


Fig. 6.5. Transformation of ethanol and production of acetate and CH_4 in the reactors. (A) ethanol and acetate concentration in reactor 1; (B) production of CH_4 in reactor 1; (C) ethanol and acetate concentration in reactor 2; (D) production of CH_4 in reactor 2. Ethanol in the influent ($\bullet$) and in the effluent ($\circ$); and acetate concentration in the influent ($\blacksquare$) and in the effluent ($\square$). CH_4 production in the reactors ($\bullet$). The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation, right after the addition of As^{V} . The horizontal grey lines represent the theoretical concentration of the species in the effluent if no transformation/immobilization would take place.

Following the initial CH₄ formation, the CH₄ production gradually decreased for the next 43 d until reaching a steady state concentration of 1.27±0.21 mmol/L_{liq} during stage I, which was 15.3% less than the expected 1.5 mmol/L_{liq} suggesting the use of some H₂ by the SO₄²⁻-reducers. 0.8 mM SO₄²⁻ were being reduced, which will required an input of 6.64 e⁻ meq/L. Ethanol was being added at a concentration of 1 mM, producing 1 mM of acetate and 2 mmol/L_{liq} of H₂. The use of acetate as electron donor for SO₄²⁻ reduction required long start-time, because the SO₄²⁻-reducers need to outcompete the acetoclastic methanogens community (Omil et al. 1998); however, H₂ is readily used by SO₄²⁻-reducers as electron donor, because SO₄²⁻-reducers rapidly outcompete the hydrogenotrophic methanogens (Weijma et al. 2002). The average concentration of CH₄ being produced was 1.3 mmol CH₄/L_{liq}. The 1 mM of acetate is used by the methanogens to produce 1 mM of CH₄; and, the 2 mmol/L_{liq} (4 e⁻ meq/L) of H₂ could reduce 0.5 mM of SO₄²⁻. There are still 0.3 mmol CH₄/L_{liq} produced and 0.3 mM SO₄²⁻ reduced which only can be explained by the activity of the endogenous substrate decay in the reactor. These results indicate the role of ethanol in the reactor, as well as the endogenous substrate, supporting methanogens and SO₄²⁻ reducing bacteria.

During stage II and III, 1.02±0.06 mM of As^V were fed into the reactor, and the concentrations of SO₄²⁻ and ethanol were increased to 1.49±0.15 mM and 4.42±0.26 mM, respectively. The addition of As inhibited the activity of the methanogenic archaea in the reactor. The concentration of acetate in the effluent increased to 3.31 mM in only 1 d, and

it reached a steady state which averaged 3.88 ± 0.53 mM over the rest of the experiment. The recovery of acetate in the effluent slightly decreased over time, from 4.29 ± 0.40 mM over stage II to 3.59 ± 0.40 mM over stage III. Since acetate was not being degraded, the production of CH_4 was limited to 0.92 ± 0.57 mmol/L_{liq} over the entire time of operation, which was 74.4% less than the expected if all the ethanol was entirely transformed to CH_4 , and after subtracting the e^- eq that could be used to reduce As^{V} ($2 e^-$ meq/L) and SO_4^{2-} ($12 e^-$ meq/L). Therefore, the formation of ASM seemed to have a sustained toxic effect on the acetoclastic methanogenic community.

The operation of the reactor 2 during the stage I was used to study the methanogenic activity of the anaerobic biofilm. During stage I, an average of 1.11 ± 0.28 mM ethanol was fed to reactor 2 (Fig. 6.5C). Initially (over the first 18 d), a small accumulation of acetate was detected, with a maximum concentration of 0.83 mM acetate on day 12. After this initial period, the concentration of acetate in the effluent decreased until it became negligible. CH_4 production started simultaneously with the acetate disappearance from the effluent (Fig. 6.5D), and it achieved a steady state production of 2.03 ± 0.12 mmol/L_{liq} over stage I. The theoretical value for the ethanol added in the reactor was 1.66 mmol/L_{liq}, suggesting a contribution of endogenous substrates in the anaerobic biofilm to the CH_4 production. Overall, the evidence indicates robust acetogenic and methanogenic activity of the microbial community to support ethanol conversion to CH_4 .

During stages II and III, 1.02 ± 0.06 mM As^{V} was being reduced in reactor 2, producing 0.91 ± 0.12 mM of As^{III} . At the start of stage II, the ethanol concentration was increased to 4.39 ± 0.30 mM. The formation of As^{III} from the reduction of As^{V} initially caused a strong inhibition in the CH_4 production. As a consequence of the inhibition, ethanol was converted to acetate rather than to methane. Immediately after the addition of As^{V} , the acetate concentration increased to 3.31 mM and the production of CH_4 dropped from 2.22 mmol/L_{liq} on day 84 to 0.07 mmol/L_{liq} on day 85. For the first 22 d of stage II, ethanol introduced into reactor 2 was recovered stoichiometrically as acetate. However, the methane production progressively recovered over time. After an initial period of adaptation, the acetate started to become degraded and CH_4 production increased until reaching a new steady-state production of 5.26 ± 1.10 mmol/L_{liq}, 82 d after the addition of As^{V} . This value is 25.5% less than the theoretical value if the entire ethanol amended in the reactor was transformed to CH_4 . A 4% loss in methane production can be accounted for by H_2 redirected towards As^{V} reduction, the remaining discrepancy may be due to cell yield. In conclusion, although the presence of As^{III} initially greatly lowered the methanogenic activity of the anaerobic biofilm, the data clearly suggest methanogens become tolerant to As^{III} after a period of adaptation. In order to completely understand the inhibitory effect of As in the methanogens, toxicity assays were performed and they are presented in the next section.

6.4.8. Toxicity of As^{III} and ASM to methanogenic activity

The addition of As to both of the reactors impacted the methanogenic activity of the anaerobic biofilm. A series of batch experiments were run to study the inhibitory effect of the products obtained in both reactors, the ASM precipitated in reactor 1 and the As^{III} obtained from the reduction of As^V in reactor 2.

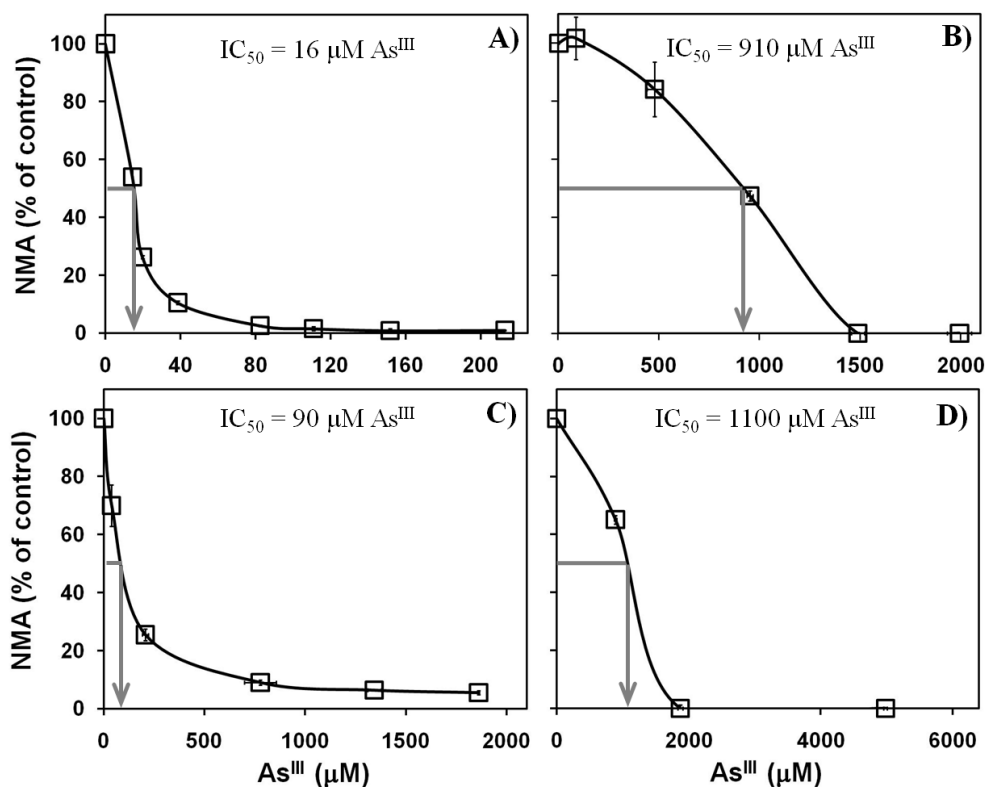


Fig. 6.6. Normalized methanogenic activity determined in the batch toxicity assays as a function of the concentration of the measured soluble As^{III} . The acetoclastic methanogenic activity of the (A) unexposed, original sludge; and, (B) adapted, reactor 2 sludge. The hydrogenotrophic methanogenic activity of the (C) unexposed, original sludge; and (D) adapted sludge, reactor 2. The IC_{50} values are shown in the corresponding graph.

The effect of As^{III} in the methanogenic community was evaluated with two different biofilms: the original biofilm which was not in contact with As; and, biofilm from reactor 2 after being exposed to As for more than 160 d. The toxicity of As^{III} to acetoclastic methanogenesis and hydrogenotrophic methanogenesis was studied. As^{III}

had a higher inhibitory effect in the acetoclastic compared to the hydrogenotrophic methanogenesis. Fig. 6.6 shows the NMA for different concentrations of As^{III} (the actual time courses of CH_4 production are presented in Fig. C.6 of the Appendix C). As it is shown in Fig. 6.6A, the IC_{50} for As^{III} was only 16 μM in the acetoclastic methanogenesis in the original biofilm; but after the pre-exposure, 910 μM of As^{III} was necessary to cause the same 50% inhibition (Fig. 6.6B), almost 57-fold more than the unadapted biofilm. In the case of the hydrogenotrophic methanogenesis, the IC_{50} for As^{III} increased from 90 μM (Fig. 6.6C) to 1100 μM (Fig. 6.6D), which is a 12-fold difference after adaptation. Consequently, very low concentration of As^{III} will initially inhibit the production of CH_4 in reactor 2, but after a period of adaptation, the methanogens will adapt and the IC_{50} of As will increase to approximately 1000 μM for both types of methanogenic activities (acetoclastic and hydrogenotrophic).

The toxicity of the precipitated ASM mineral was evaluated using commercial As_2S_3 and the biogenic mineral recovered from the reactor 1. The effect of the ASM on acetoclastic methanogenic activity of the original biofilm was analyzed. The presence of the biogenic mineral caused inhibition of CH_4 production in a concentration dependent fashion. The results of these experiments are presented in Fig. C.7 of the Appendix C. The formation of CH_4 over time for the different mineral concentrations is shown in Fig. C.7A for the commercial As_2S_3 , and in Fig. C.7B for the biogenic mineral. The actual concentration of As provided as a mineral was measured after acid digestion, therefore

the concentration of the mineral in the treatments can be expressed as mg As/g VSS. The IC_{50} concentration of the commercial As_2S_3 was 50 mg As_{solid}/g VSS (50 mg As_{solid}/L), whereas the IC_{50} concentration of the biogenic mineral was 12 mg As_{solid}/g VSS (12 mg As_{solid}/L) (Fig. C.7C). In order to discriminate between the toxicity of the mineral and the caused by any soluble As leached from the mineral, analysis of the soluble As were conducted and the results are shown in Fig. C.7D in the Appendix C. The fraction of As that became mobilized from the commercial As_2S_3 experiment was higher than the IC_{50} obtained for soluble As^{III} for the unadapted original sludge ($88.8 \pm 15.1 \mu M$ soluble As for the highest concentration of As_2S_3); however, the solubility of As in the biogenic mineral was lower than the IC_{50} for soluble As^{III} ($15.7 \mu M$ soluble As for the highest concentration of biogenic ASM). Thus, the presence of the biogenic mineral at low concentrations inhibits methanogenesis either by the presence of the mineral directly or by the release of localized toxic soluble concentrations of As in the vicinity of the biofilm.

6.5. Discussion

The results presented in this study demonstrate that an As-containing water stream can be remediated by promoting the concurrent biological reduction of As^{V} and SO_4^{2-} through the formation of insoluble ASM. This (bio)conversion was sustained over long-term bioreactor operation at slightly acidic pH and supplied with ethanol as an electron donating substrate. SO_4^{2-} and As^{V} were readily reduced by the anaerobic biofilm and subsequently precipitated as a mixture of AsS and As_2S_3 , resulting in more than 91% of soluble As removal. Formation of sulfide from sulfate reduction was essential to promote the formation of insoluble ASM since a control reactor in which SO_4^{2-} was excluded from the medium was unable to significantly remove soluble As. Instead the control reactor just reduced As^{V} to As^{III}

6.5.1. Arsenic immobilization due to the formation of ASM

The total concentration of As and S introduced in reactor 1 decreased by $91.2 \pm 8.3\%$ and $76.8 \pm 7.9\%$, respectively, only when As^{V} and SO_4^{2-} were fed simultaneously into the reactor 1. The removal rate of As during the operation averaged of $2.9 \pm 0.4 \text{ mg As}/(\text{L} \cdot \text{h})$ over stages II and III. This As removal rate is higher compared to a previous study examining the remediation of As in Fe-free systems in which a fixed-film sulfate reducing bioreactor treated an acidic (pH 2.7 to 5) effluent at a rate of 1.5-2.0

mg As/(L•h) using glycerol as electron donor, and 2.5 mg As/(L•h) using H₂ (Battaglia-Brunet et al. 2012). Other studies in the literature have focused in the interaction between the S, As and Fe cycles at a circumneutral pH. Altun et al. (2014) studied the precipitation of ASM in the presence of Fe in fixed-bed bioreactors with ethanol as the electron donor source. Their study contained control reactors lacking the addition of Fe, however the system was poorly buffered, and the pH of the medium increased from 4 to 7.4, limiting the removal of As to only 8%. Kirk et al. (2010) also observed insignificant As removal in a semi-continuous reactor, fed with As^V and SO₄²⁻, and acetate as electron donor, operated at pH 7.3. Collectively the results suggest that successful removal of As by the precipitation of ASM has only been feasible at slightly acidic to acidic pH and not feasible at slightly alkaline values.

The methanogenic sludge used as inoculum in the bioreactors was able to reduce SO₄²⁻ and As^V individually or concomitantly with ethanol as electron donor, even when it was not previously exposed to As. The reduction of SO₄²⁻ to DS and As^V to As^{III} caused the formation of ASM, which precipitated in reactor 1. SO₄²⁻ and As^V reduction readily occurred in reactor 1 and 2, respectively, when added separately. After the addition of As^V as an alternative electron acceptor in reactor 1, the SO₄²⁻-reducing activity was initially limited. The standard redox potential for the pair As^V/As^{III} (60 mV) is higher than that for SO₄²⁻/H₂S (-220 mV) (Hoeft et al. 2004); therefore, microorganisms will gain more energy by utilizing As^V as the terminal electron acceptor than by utilizing

SO_4^{2-} . Newman et al. (1997b) hypothesized that the inhibition of SO_4^{2-} -reducing activity after amending As^{V} to the treatment was due to a bioenergetic competition. If both As^{V} and SO_4^{2-} are present, microorganisms would use As^{V} under non-limiting conditions. If the substrate becomes limited, either by decreasing As concentrations (Newman et al. 1997a, Rodriguez-Freire et al. 2014) or by increasing bacterial numbers, microorganisms will start using SO_4^{2-} as electron acceptor. The reduction of As^{V} before SO_4^{2-} means that when DS starts to be produced, there will be As^{III} available to sequester the DS and precipitate it as ASM.

As^{V} - and SO_4^{2-} -reducing bacteria can commonly be found associated with each other in microbial communities, such as in alluvial aquifers (Saunders et al. 2005), hypersaline lakes (Kulp et al. 2006) and mining sites (Tomczyk-Zak et al. 2013). Occasionally, the ability to reduce As^{V} and SO_4^{2-} is present in a single microorganism, like the *Sulfurospirillum arsenophilum* MIT-13 (Ahmann et al. 1994) or the *Desulfomicrobium* sp. str. Ben-RB and *Desulfovibrio* sp. str. Ben-RA (Macy et al. 2000). Among all the As^{V} - and SO_4^{2-} -reducing microorganisms that have been reported, only a few of them are able to mineralize As. These microorganisms can precipitate ASM because of the preferential use of As^{V} over SO_4^{2-} (Huber et al. 2000, Ledbetter et al. 2007, Lee et al. 2007, Macy et al. 2000). *Desulfotomaculum auropigmentum* (Newman et al. 1997a), which precipitates As_2S_3 , starts reducing As^{V} and then SO_4^{2-} ; however, *Desulfobulbus propionicus* quickly reduces SO_4^{2-} before reducing As^{V} , leading to the

accumulation of DS in the medium and favoring the formation of thioarsenites, and as such was found to be unable to form an ASM. Consequently, the microbial reduction of As^{V} and SO_4^{2-} can enhance or limit the formation of ASM by controlling the rate of formation of As^{III} and DS.

There are two major constraints that will dictate the feasibility of the remediation of As-contaminated waters by precipitation of ASM: the pH and the concentration of DS. The solubility of ASM increases as the pH increases and at high DS concentrations due to the formation of soluble thioarsenite species (Wilkin et al. 2003). Fig. C.8 in Appendix C illustrates the stability of the mineral phases As_2S_3 and AsS as a function of the pH and the redox potential (E_{h}) (Fig. C.8A), and it shows how as the pH increases, the amount of DS required for the formation of thioarsenites decreases, at any given As concentration (Fig. C.8B). In this study, the pH of the medium was maintained between 6.25 - 6.5, and As^{V} and SO_4^{2-} were supplied in a molar relation 1:1.5 (As:S) to ensure the feasibility of the process. Battaglia-Brunet et al. (2012) worked at a lower pH range achieving lower As removal rates than the present study. Given the low pH, higher As removal rates would have been expected, however the acidic pH may have been suboptimal for the biological SO_4^{2-} and As^{V} reduction processes, or the higher SO_4^{2-} to As^{V} ratio may have caused a DS excess promoting the formation of soluble thioarsenite species, even at low pH. Newman et al. (1997a) observed that the precipitation of As_2S_3 occurred at pH lower than 7 for a DS concentration of 0.1 mM, and that by increasing the DS, the pH limiting

the formation of As_2S_3 will decrease. Furthermore, the study by Rodriguez-Freire et al. (2014) demonstrated that the As removal rate due to ASM formation of was positively correlated with decreasing pH values. Therefore, while the formation of ASM by the microbial reduction of As^{V} and SO_4^{2-} can be effectively used as a remediation technique, careful management of the pH and DS concentration must be achieved to successfully precipitate ASM.

6.5.2. *The mineralization of As led to the precipitation of a mixture of AsS and As_2S_3*

The reduction of As^{V} and SO_4^{2-} in reactor 1 produced a yellow precipitate which was visible 4 d after the addition of As. Microscopic analysis showed particles of As-S precipitate in with similar composition in the surroundings and on the surface of the bacteria. Newman et al. (1997a) previously reported the intra- and extra-cellular precipitation of As_2S_3 on *D. auripigmentum*; and Lee et al. (2007) observed filamentous As-S nanotubes growing inside *Shewanella* sp. HN-41. The biologically precipitated ASM are formed following the microbial reduction of As^{V} and SO_4^{2-} , which takes place inside of the cytoplasmic membrane (Slyemi and Bonnefoy 2012, Suess and Planer-Friedrich 2012). Therefore, the close physical association between the ASM and the bacteria is an indication of the microbial role in the formation of the mineral.

The biogenic mineral precipitated in reactor 1 was composed of AsS and As₂S₃. The ratio $S_{\text{loss}}/As_{\text{loss}}$ successfully predicted the composition of the precipitated over the long term incubation, which was confirmed by the XAS analysis. However, during stage II, the stoichiometric average ratio $S_{\text{loss}}/As_{\text{loss}}$ did not correspond with a mixture of 70% As₂S₃ and 30% AsS, this could be due to the stabilization of the reactor after the addition of As and the presence of residual DS from stage I and endogenous biomass decay that could have precipitated with the formed As^{III}. The overall ratio $S_{\text{loss}}/As_{\text{loss}}$ for stage II and III was 1.22 predicting a mixture of 44% As₂S₃ and 55% AsS, and the analysis of the solid phase confirmed a precipitated made of 40% As₂S₃ and the 60 % AsS. The increase in the proportion of AsS with the incubation time could be due to the maturation of the most crystalline form, AsS, confirming the results obtained by Lee et al. (2007) in which the strain *Shewanella* sp. Str. HN-41 started precipitating As₂S₃, and, as the incubation time increased, the As₂S₃ was transformed to AsS and duranusite (As₄S) and, finally, to As-S nanotubes.

The co-precipitation of As₂S₃ and AsS was previously observed in batch experiments in a pH range of 6.1 to 7.1 (Rodriguez-Freire et al. 2014). Two different hypotheses could explain the precipitation of both minerals. (1) Different microorganisms promoted the formation of each mineral. Demergasso et al. (2007) noticed the formation of AsS and As₂S₃ in laboratory experiments using enrichment cultures originating from sediment samples; solid phase analysis of the precipitate on the microbial surface

corresponded more closely to As_2S_3 , and the bulk precipitate corresponded closely to AsS. Among the microorganisms reported to precipitate ASM, two pure cultures, one strain from γ -Proteobacteria (Demergasso et al. 2007) and *D. auripigmentum* (Newman et al. 1997a), precipitate As_2S_3 ; the strain YeAs (Ledbetter et al. 2007) precipitated AsS; and, *Shewanella* sp. Str. HN-41 (Lee et al. 2007) initially precipitated As_2S_3 , AsS, As_4S and, finally, As-S nanotubes. (2) Chemical variations can lead to variations in the redox conditions inside reactor. The Pourbaix diagram (E_h -pH diagram) in Fig. C.8 in Appendix C predicts, that at the pH and E_h range (-256 ± 27 mV, measured) in the reactor, both minerals can be formed within the standard deviation of the measurements. For a fixed pH, AsS will be the predominant under more reducing conditions than As_2S_3 , and for a fixed E_h , AsS will be predominant at more acidic values of the pH. Since microbial activity will control the redox condition in the reactor, the co-precipitation of As_2S_3 and AsS can be most likely explained by a combination of process (1) and (2).

6.5.3. Soluble As initially inhibited the methanogenic activity

The methanogenic activity suffered an initial inhibition in reactor 2 due to the reduction of As^{V} and formation of 915 ± 82 μM of As^{III} , but the methanogens adapted to the toxicity after an acclimation period, as observed by the progressive increase in acetate consumption and CH_4 production in the reactor. The concentration of As^{III} in the reactor is 57-fold higher than the IC_{50} obtained for the acetoclastic methanogenesis (16 μM) and

10-fold higher than the IC_{50} for the hydrogenotrophic methanogenesis (90 μ M). Surprisingly, the methanogens became tolerant over time. The IC_{50} for the pre-exposed biofilm increased to 910 μ M for the acetoclastic methanogens and 1100 μ M for the hydrogenotrophs.

The IC_{50} obtained for acetoclastic methanogenesis of the unexposed biofilm is in close agreement with the 15.5 μ M obtained by Sierra-Alvarez et al. (2004). On the other hand, the IC_{50} obtained for hydrogenotrophic methanogenesis is 3.3-fold greater than the obtained by Sierra-Alvarez et al. The toxicity of As^{III} is based on its tendency to be associated with S, which will cause enzymatic inhibition if it occurs in the cell. *Ars* operon is the main mechanism responsible to the As^{III} tolerance of many microorganisms, which expels As^{III} from the cell through an ArsB periplasmic protein pump (Slyemi and Bonnefoy 2012). The ability of methanogens to gain tolerance to As^{III} has not been previously reported, but it could potentially be explained by the ability of the methanogens to express the *ars* operon in response to the high concentration of As^{III} .

6.5.4. The formation of the ASM completely inhibited the methanogenic activity

The co-precipitation of As_2S_3 and AsS during stage II and III in reactor 1 completely inhibited the acetoclastic methanogenic activity in the reactor as evidenced by the accumulation of acetate over stage II and III. This result contradicted a previous

observation that related the formation ASM with a decreased in toxicity due to the immobilization of the toxic As^{III} (Rodriguez-Freire et al. 2014). Several hypotheses can explain the toxicity observed on the methanogenic community following the formation of ASM. Firstly, the undissociated H_2S is known to cause inhibition to methanogens in sludge, with IC_{50} values of the order of 6 mM (O'Flaherty et al. 1998). However since this concentration is 20-fold higher than the DS concentration in the reactor (0.3 mM), a hypothesis based on H_2S toxicity must be rejected. Secondly, ASM could be cause inhibition of methanogens directly or indirectly. Indirectly could be due to the partial solubilization of the mineral. The levels of soluble As^{III} formed using the commercial As_2S_3 could account for methanogenic inhibition observed at the IC_{50} of 16 μM for this mineral, but the soluble fraction associated with the biogenic ASM is much lower than the IC_{50} for As^{III} . Thus either very localized concentrations of soluble As in the vicinity of the biofilm or the precipitate itself must be responsible for the toxicity. Directly, the presence of 12 mg $\text{As}_{\text{solid}}/\text{g VSS}$ of the biogenic mineral caused a 50% inhibition in the acetoclastic methanogens. The reactor immobilized 0.22 mg As/g VSS.h, therefore, the IC_{50} could be expected to be reached after only 55 h of operation, which explains the accumulation of acetate only a day after the addition of As.

No previous study has related the formation of As_2S_3 or AsS with methanogenic inhibition. The toxic effect of the biogenic minerals may be related to their intra- and extracellular formation, as was observed previously (Newman et al. 1997a, Lee et al.

2007, Focardi et al. 2010). Since the main microbial mechanism for As detoxification is through an efflux pump (Slyemi and Bonnefoy 2012), the transport can be impeded by the formation of a precipitate coating the cells. Intracellular precipitates can likewise form a As^{III} sink evading that As^{III} is pumped out of cells.

6.6. Conclusions

The formation of an ASM can be stimulated by promoting the concurrent biological reduction of As^{V} and SO_4^{2-} in a continuous bioreactor with a controlled pH ranging from 6.25 to 6.5. An anaerobic sludge was used as an inoculum and readily reduced As^{V} and SO_4^{2-} with ethanol as the electron donor. High As removal rates, of 2.9 mg $\text{As}/(\text{L}\cdot\text{h})$ were achieved and formed a precipitate composed of As_2S_3 and AsS , as characterized by the $\text{S}_{\text{loss}}/\text{As}_{\text{loss}}$ ratio and XANES analysis; whereas, negligible As immobilization was observed in the control reactor, lacking the addition of SO_4^{2-} . The control reactor only converted As^{V} to As^{III} which caused methanogenic inhibition but the methanogens adapted to the toxicity. However, acetoclastic methanogenesis was more permanently inhibited in the bioreactor producing ASM and this was due to the accumulation of ASM which were shown to be responsible for the inhibition.

Taken as a whole, these results confirm that the biomineralization of As as sulfide minerals can be used as remediation technique that can be sustained over long term

operation in continuous bioreactors. Further studies on the stability of the biogenic minerals under various conditions will be important to better understand the feasibility of the process.

CHAPTER VII:
ARSENIC MOBILIZATION FROM SULFIDE MINERALS IS HIGHLY
AFFECTED BY THE PH BUT NOT BY THE REDOX CONDITIONS OF THE
ENVIRONMENT

7.1. Abstract

Arsenic (As) toxicity and widespread occurrence in natural waters poses a serious risk to millions of people in the world. An important natural source of As in the environment is As-bearing sulfide minerals (ASM), which are known to contribute to high background As concentrations in groundwater; however, the immobilization of As can be harnessed by promoting the formation of ASM. Recent studies have successfully formed ASM by stimulating the biological reduction of arsenate (As^{V}) and sulfate (SO_4^{2-}) to arsenite (As^{III}) and sulfide (H_2S), which can precipitate forming orpiment (As_2S_3) and realgar (AsS). The objective of this study is to investigate the stability of a biogenic ASM composed of 60% AsS and 40% As_2S_3 at two different pH values (6.5 and 7.5) under three different redox conditions. The stability was evaluated in three different bioreactors: aerobic (R1); anoxic, with nitrate (NO_3^-) as alternative electron acceptor (R2); and anaerobic (R3). The dissolution of ASM was greatly affected by the pH value. The

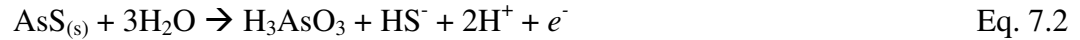
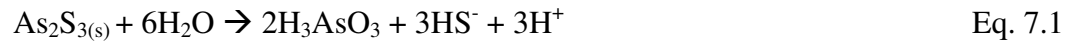
ASM was very stable at pH 6.5, with an As mobilization rate of 0.15-0.21 mg As/(L·h); however, the As mobilization rate was up to 7-fold higher at pH 7.5, likely due to the formation of thioarsenical species. The stability of ASM was also impacted by the electron acceptor. The As mobilization rate under anaerobic conditions was 1.17 mg As/(L·h), whereas under aerobic conditions it was only 0.66 mg As/(L·h), most likely due to the formation of secondary As-bearing minerals. The presence of NO_3^- did not have a significant effect on the dissolution of ASM. Therefore, the stability of ASM can be controlled by altering environmental conditions such as the pH or the presence of a suitable electron acceptor.

7.2. Introduction

Arsenic (As) is a toxic metalloid which is naturally present in the environment and it can be found at a wide range of concentrations in the groundwater around the world (Murcott 2012). The presence of As in drinking water supplies poses a serious health risk for the people where As levels are well above the acceptable limit of 10 $\mu\text{g/L}$ established by the World Health Organization (WHO) (WHO 1993) and the United States Environmental Protection Agency (US-EPA) (US-EPA 2001). Arsenic groundwater concentration ranges from a few ppb to thousands depending on anthropogenic activities (Garelick et al. 2008) and different geological factors (Smedley and Kinniburgh 2002)

High As concentrations are commonly associated with parent rock materials with a high background of sulfidic minerals. Realgar (AsS), orpiment (As_2S_3) and arsenopyrite (FeAsS) are naturally formed As-bearing sulfide minerals (ASM) (Demergasso et al. 2007, O'day et al. 2004) which are known to be a source of As contamination in the environment (Welch et al. 2000). Recent studies have focused in evaluating the potential of promoting the precipitation of ASM as an As bioremediation technique by stimulating the biological reduction of arsenate (As^{V}) and sulfate (SO_4^{2-}) to arsenite (As^{III}) and sulfide (H_2S) (Battaglia-Brunet et al. 2012, Keimowitz et al. 2007, Onstott et al. 2011, Rodriguez-Freire et al. 2014). The reliability of this novel remediation process depends on the stability of the biogenic ASM over time. There are several factors that can affect the mobilization of As from ASM, among them, the pH value, the redox conditions, and the microbial activity are the main ones to consider. In alkaline pH, ASM are not thermodynamically stable and their dissolution will lead to the formation of aqueous As-S species known as thioarsenical compounds (Suess and Planer-Friedrich 2012, Wilkin et al. 2003). In oxidizing environments, the ASM minerals will undergo weathering liberating soluble species of As (As^{V} and As^{III}) and S (SO_4^{2-} and H_2S) into water. Microbial activity has been shown to enhance the mobilization of As from ASM (e.g. *Acidithiobacillus ferrooxidans* (Jiang et al. 2008, Rhine et al. 2008, Tuovinen et al. 1994)), however there is some evidence that some microorganisms can decrease the dissolution rate of some ASM (e.g. *Thiobacillus caldus* (McGuire et al. 2001)).

The purpose of this study was to evaluate the stability of a biogenic ASM, composed by 60% AsS and 40% As₂S₃, at two different pH values (6.5 and 7.5) under different redox conditions. Three bioreactors were inoculated with anaerobic methanogenic sludge and amended with the biogenic ASM and operated under aerobic, anoxic (using nitrate (NO₃⁻) as electron acceptor), and anaerobic conditions. The dissolution of the ASM was evaluated by measuring the concentration of the different As and S species in the aqueous phase, since the reaction that describes the dissolution process of As₂S₃ and AsS can be defined as the following:



7.3. Materials and methods

7.3.1. Source of anaerobic inocula and biogenic ASM

The anaerobic inoculum and biogenic ASM used to pack the three bioreactors were obtained from a bioreactor used to study the biomineralization of As^V to ASM. The biomineralization bioreactor was run for 371 d with an influent containing 1 mM As^V, 1.5 mM sulfate SO₄²⁻, and 4.5 mM ethanol as electron donating substrate. The original inoculum in the reactor was an anaerobic granular biofilm obtained from an upflow

anaerobic sludge blanket treating wastewater from a beer brewery (Mahou, Guadalajara, Spain). The volatile suspended solids (VSS) composition of the sludge was 0.042 ± 0.002 g VSS/g wet wt and the endogenous substrate decay rate was $0.57 \text{ e}^- \text{ meq/g VSS.d}$. As was removed from the liquid medium at a rate of $2.9 \text{ mg As/(L}\cdot\text{h)}$, and a mixture of 60% AsS and 40% As_2S_3 was the product of the As immobilization. The arsenic concentration in the material was 1.9 mg As/mg VSS at the end of the operation in the reactor. The biomineralization reactor was opened in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) and the inoculum plus biogenic ASM was immediately transferred to the three bioreactors.

High purity sand, white quartz (40-100 mesh, Sigma-Aldrich Corp., St. Louis, MO) was used as a packing material in the bioreactors.

7.3.2. Mineral medium composition

The bioreactors were fed with mineral medium prepared using ultra-pure water (Milli-Q system; Millipore). The composition of the mineral medium was as follows (mg/L): $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (332); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (40); NH_4Cl (101); NaHCO_3 (660). 2 mL/L of a trace element solution that was added to the medium to provide a final concentration of ($\mu\text{g/L}$): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (2,000); $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$ (2,000); $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$ (500); $\text{AlCl}_3 \cdot 6 \text{H}_2\text{O}$ (90); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (30); ZnCl_2 (50); H_3BO_3 (50); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{H}_2\text{O}$ (50); $\text{Na}_2\text{SeO}_3 \cdot 5$

H₂O (100); NiCl₂·6H₂O (50); EDTA (1,000); resazurin (200); HCl 36% (1 µL). The media were buffer using the phosphate system using 580 mg/L K₂HPO₄ and 2267 mg/L KH₂PO₄ at pH 6.5, and 2320 mg/L K₂HPO₄ and 907 mg/L KH₂PO₄ at pH 7.5. If anaerobic conditions were required, the medium was flushed for 20 min with N₂:CO₂ (80:20). NO₃⁻ was supplied as the salt KNO₃ to the anoxic bioreactor, initially 25 mg/L KNO₃ and it was later increased to 89 mg/L KNO₃.

7.3.3. Bioreactors set-up

Three borosilicates 60 mL (25 mm internal diameter) columns (Kimble Chase Kontes, Rockwood, TN, USA) were used as laboratory-scale upflow bioreactors. The three bioreactors were operated in parallel as shown in Fig. 7.1. Reactor 1 (R1) was used to study the dissolution of ASM under aerobic conditions; reactor 2 (R2) was used to study the dissolution of ASM under anoxic conditions, using NO₃⁻ as an alternative electron acceptor; and, reactor 3 (R3) was used to study the dissolution of ASM under anaerobic conditions. The three reactors were in operation for 145 d and run at two different pH values over three stages: stage I run from day 0 to 54 at pH 6.5; stage II run from day 55 to 123 at pH 7.5; and, stage III run from day 124 to 145 at pH 6.5. The hydraulic retention time (HRT) in the reactors averaged 14.5±2.2 h. The reactors were maintained in an incubation room at a temperature of 30°C.

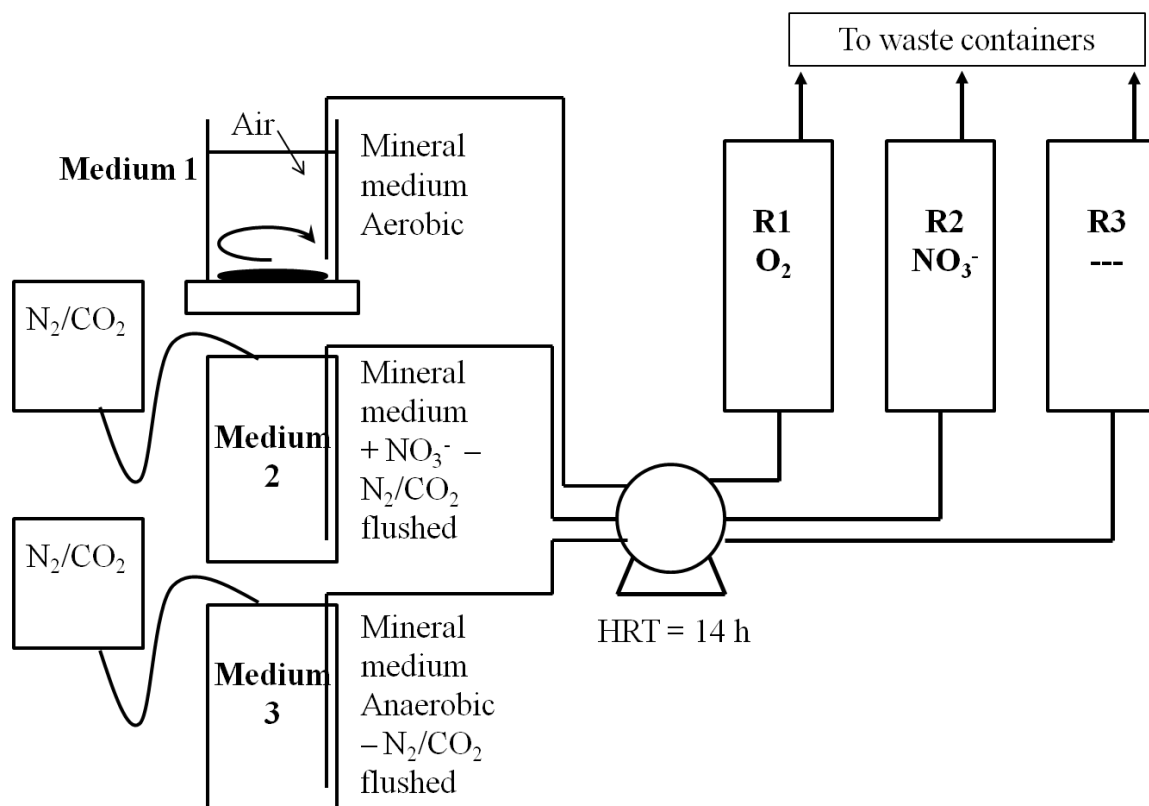


Fig. 7.1. Schematic of the set-up for the three bioreactors used in this study. Aerobic (R1); anoxic, with NO_3^- as alternative electron acceptor (R2); and anaerobic (R3).

R1 was packed with 14.6 g of the inocula and ASM material, 25 g of sand, leaving an empty volume in the reactor of 36 mL, which corresponded with 17.1 g VSS/L and 1.95 mg As/mg VSS. The mineral medium for R1 was maintain in an open beaker placed over a magnetic stirrer (Barnstead/Thermolyne Cimarec Magnetic Stirrer, Thermo Scientific, Pittsburgh, PA, USA) operating at 350 rpm to facilitate the aeration of the medium. R2 was packed with 14. 9 g of the inoculum and ASM material, 24.8 g of sand,

leaving an empty volume in the reactor of 40 mL, which corresponded with 15.6 g VSS/L and 1.95 mg As/mg VSS. 0.25 mM ($1.25 e^-$ meq/L) of NO_3^- was amended to R2 mineral medium over the first 25 d, which corresponded with the amount of electron equivalent supplied by 10 mg/L dissolved oxygen (DO) in R1. After the initial 25 d, the concentration of NO_3^- was increased to 0.88 mM ($4.38 e^-$ meq/L) which was maintained for the rest of the operation in the reactor. R3 was packed with 14.9 g of the inoculum and ASM material, 25.7 g of sand, leaving an empty volume in the reactor of 39 mL, which corresponded with 16.1 g VSS/L and 1.95 mg As/mg VSS. The mineral media for R2 and R3 were maintained in two different containers under anaerobic conditions using a gas bag (SKC-West Inc, Fullerton, CA) containing N_2/CO_2 (20:80).

Liquid samples from the influent and effluent were taken from the sampling ports. pH, dissolved oxygen (DO), and H_2S were measured immediately. Samples for As^{V} , total As (soluble), As speciation, SO_4^{2-} , and NO_3^- analysis were centrifuged (10 min, 14,000 g) and stored in polypropylene vials at -20°C until analysis. Solid samples were taken periodically from the reactors for mineral characterization. The solid samples were washed with anaerobic ultra-pure water to remove any soluble fraction of As, flushed with N_2 gas, and stored at -4°C .

7.3.4. Arsenic extraction and mass balance in the bioreactors

At the end of the stage III, the bioreactors were sealed and transferred to an anaerobic chamber. Subsequently, the reactor contents were sieved to separate the liquid and the solid phases. The liquid phase was collected in a serum bottle. The solid phase was homogenized and samples were taken for arsenic extraction and for solid characterization. The arsenic extraction method was adapted from a previously described protocol (Pereira de Andrade et al. 2012). Four different extraction medium were prepared, the first one consisted of 1 M MgCl_2 , at pH 8 and incubation of 2 h, in order to separate the exchangeable As; the second extraction consisted in 1 M NaHPO_3 , at pH 5 and incubation of 24 h, in order to separate strongly adsorbed As; the third extraction consisted of 1 M HCl , and incubation time of 2 h, to separate any As weakly precipitated; the last extraction was performed using aqua regia ($\text{HCl}:\text{HNO}_3$, 3:1), incubated for 24 h, to separate any As coprecipitated as H_2S minerals. The samples were prepared using 50 mg of solid phase and 5 mL of extraction media in 15 mL polypropylene (extractions 1 to 3) or borosilicate (extraction 4) tubes, and incubated at 30°C in a shaker set at 125 rpm during the time of the extraction. At the end of the operation time, the samples were centrifuged (4,500 rpm, 30 min) and analyzed for total As content. The extraction procedure was run in duplicate.

7.3.5. Pourbaix diagrams

Pourbaix (E_h -pH) diagrams were used to understand the stability of ASM under the experimental conditions previously described. As concentration was 0.5 mM and S concentration was 0.75 mM, while for the $\text{FeAsS}_{(s)}$. The thermodynamic data were obtained from MinTEQ and NBS databases and the diagrams were built using the W32-Stabcal modeling software (Huang and Young 1996).

7.3.6. Analytical techniques

Total As concentration was measured by using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system model Optima 2100 DV from Perkin–Elmer TM (Shelton, CT, USA) monitored at wavelength 193.7 nm. As^V and SO_4^{2-} were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 analytical column (4 x 250 mm) and AG16 guard column (4 mm x 40 mm). The injection eluent (KOH) was 30 mM for 10 min. Selected samples were sent for As speciation to detect As^{III} , As^V and the organics methylarsonic acid (MMA^V), and dimethylarsonic acid (DMA^V) was performed using a high pressure liquid chromatography system (Agilent 1100 HPLC, Agilent Technologies, Inc.) with a reverse-phase C18 column (Gemini 5u C18 110A, 150x4.60mm, Phenomenex, Torrance, CA) and guard cartridge. The mobile phase (pH

5.85) contained 4.7mM tetrabutylammonium hydroxide, 2mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 ml/min. The column temperature was maintained at 50°C and samples are kept at 4°C in a thermally controlled autosampler. Following the HPLC separation, the As species were detected using an ICP-mass spectrometer (ICP-MS) (Agilent 7500ce) with a Conikal nebulizer (Glass Expansion). The operating parameters were as follows: Rf power 1500 watts, plasma gas flow 15 L/min, carrier flow ~0.9 L/min, 0.15 L/min makeup, and arsenic was measured at 75 m/z. Low detection limits for the measured species are: 0.03 ppb As^{III}, 0.17 ppb As^V, 0.11 ppb DMA^V, 0.15 ppb MMA^V, 0.05 ppb total As.

The presence of thioarsenical compounds was qualitatively detected using a tandem ultra high performance liquid chromatograph with infusion quadrupole-time of flight (QToF) and mass spectrometry. The mass spectra was obtained from the injection of UHPLC samples run through the UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA) in the tripleTOF 5600 tandem mass spectrometer (AB Sciex, Framingham, MA) run 22 ul/min in negative electrospray ionization mode, with 0.05 ml/min of 50% aq MeOH co-infusion to help ionization.

DO was measured using an Orion 5 Star DO probe with a 081010 MD electrode (Thermo Scientific, Pittsburgh, PA, USA). H₂S in liquid samples was determined using the methylene blue method (Truper 1964) and measured using an UV-visible

spectrophotometer (Agilent 8453, Palo Alto, CA, USA). The total amount of dissolved sulfide (DS) was calculated by considering the H₂S speciation at the measured pH using the dissociation constant for H₂S (pK_{a1} = 6.99, pK_{a2} = 12.92 (Benjamin 2002)). Consequently,

$$DS = [H_2S]_{aq} + [HS^-] + [S^{2-}] \quad \text{Eq. 7.3}$$

Measurements of pH and VSS were conducted according to standard methods (APHA 1999).

7.4. Results

7.4.1. Dissolution of ASM under aerobic conditions (Reactor 1)

The dissolution of ASM under aerobic conditions was evaluated in reactor 1 (R1). The concentrations of As and S mobilized from the ASM and the DO consumption in R1 are shown in Fig. 7.2. The concentration of total As being mobilized at pH 6.5 during stage I (day 1-54) reached steady state after 3 d (Fig. 7.2A). The total As mobilized during that period accounted for 58.9±31.9% of the total As supplied to the column. The average concentration of total As and As^V in the effluent were 0.036±0.021 mM and 0.018±0.013 mM, respectively (Fig. 7.2A and 7.2B). The only other arsenic species detected during stage I was As^{III} (Table 7.1). The formation of DS (Fig. 7.2C) and SO₄²⁻

(Fig. 7.2D) followed a similar trend as the formation of As species. After 11 d of adaptation, the concentration of DS and SO_4^{2-} reached a steady state at 0.023 ± 0.018 mM and 0.009 ± 0.007 mM, respectively. Assuming that no other S species were formed, DS accounted for $68.8 \pm 21.8\%$ of the total S being produced. Overall, the dissolution of ASM was low at pH 6.5. Only 1.16% of the total As present in the solid phase became mobilized over the 54 d that lasted stage I.

The increase in the pH to 7.5 during stage II enhanced the dissolution of ASM. The concentration of total As being mobilized increased to 0.616 mM in less than 24 h after the pH increment. The mobilization of As slowly decreased during the first 22 d during stage II until reaching a steady concentration of 0.127 ± 0.030 mM (Fig. 7.2A). The average concentration of As^{V} in the effluent was 0.031 ± 0.024 mM, which only accounted for $24.9 \pm 17.8\%$ of the As supplied to the column (Fig. 7.2B). The total amount of As^{III} , DMA^{V} and MMA^{V} measured only account for $79.4 \pm 11.7\%$ of the total As concentration mobilized (Table 7.1), indicating the presence of other soluble As species, most likely thioarsenicals. The formation of DS also increased after the change in pH from 6.5 to 7.5. The concentration of DS detected in the effluent 24 h after the pH increase was 0.483 mM (Fig. 7.2C). The production of DS decreased slowly during the next 22 d when it reached a steady state concentration of 0.048 ± 0.022 mM. Whereas the DS concentration increased after pH rise, the concentration of SO_4^{2-} remained constant during the first 22 d and then it quickly increased by 3.2-fold to 0.029 ± 0.008 mM (Fig. 7.2D). Overall, 140.3

mg of As were mobilized during stage II, accounting for 11.7% of the total As initially present in the solid phase.

Table 7.1. Summary of the results from As speciation in R1. The samples where the total soluble As is not explained by the sum of the soluble As species measured ($\pm 10\%$) are shown in bold.

Time (d)	Total As (mM)	As ^{III} (mM)	As ^V (mM)	DMA ^V (mM)	MMA ^V (mM)	Σ As (mM)	% As recover
<i>Stage I</i>							
20.9	0.03	0.02	0.01	-----ND ^a -----		0.03	101.4
<i>Stage II</i>							
46.0	0.02	ND	0.02	-----ND-----		0.02	97.1
53.9	0.05	ND	0.04	-----ND-----		0.04	79.9
56.9	0.43	0.01	0.34	-----ND-----		0.35	83.0
61.9	0.45	0.20	0.14	-----ND-----		0.34	74.7
67.9	1.15	ND	0.95	-----ND-----		0.96	83.4
70.8	3.00	1.00	0.73	-----ND-----		1.73	57.6
80.9	0.03	ND	0.03	-----ND-----		0.03	89.8
88.8	0.04	ND	0.03	-----ND-----		0.03	87.8
95.9	0.12	0.06	0.03	ND	0.02	0.11	98.0
104.9	0.06	ND	0.04	-----ND-----		0.04	69.9
118.7	0.05	ND	0.03	-----ND-----		0.03	70.3
<i>Stage III</i>							
132.9	0.05	0.04	0.01	-----ND-----		0.05	92.1

^a ND.: below detectable limit, which are 0.03 ppb As^{III}, 0.17 ppb As^V, 0.11 ppb DMA^V, 0.15 ppb MMA^V, 0.05 ppb total As

The mobilization of As and S from the ASM decreased after decreasing the pH back to the initial value of 6.5 during the stage III. The average total As concentration detected in the effluent during this last stage was 0.031 ± 0.011 mM, which is 4.3-fold lower than in stage II (Fig. 7.2A). The average concentration of As^{V} was 0.016 ± 0.005 mM, which corresponds to $52.5 \pm 19.5\%$ of the total As solubilized (Fig. 7.2B). The sum of As^{III} and As^{V} concentrations accounted for the total As produced (Table 7.1). In contrast with As, the release of DS decreased immediately when the pH was lowered to 6.5 to an average concentration of 0.027 ± 0.012 mM (Fig. 7.2C). However, the decrease in the concentration of SO_4^{2-} was gradual, taking 13 d to reach the new steady state concentration of 0.018 ± 0.002 mM (Fig. 7.2D). The mobilization of As during stage III was only 0.24% of the total As initially present in the ASM.

The concentration of DO in the influent and effluent of R1 was monitored to ensure aerobic conditions throughout the operation in the reactor. Fig. 7.2E shows the actual concentration of DO fed in the reactor, which averaged 0.296 ± 0.042 mM ($1.2 e^-$ meq/L). The DO concentration in the effluent was 0.118 ± 0.036 mM ($0.5 e^-$ meq/L) which indicated that R1 was operating under oxic conditions during the entire experiment.

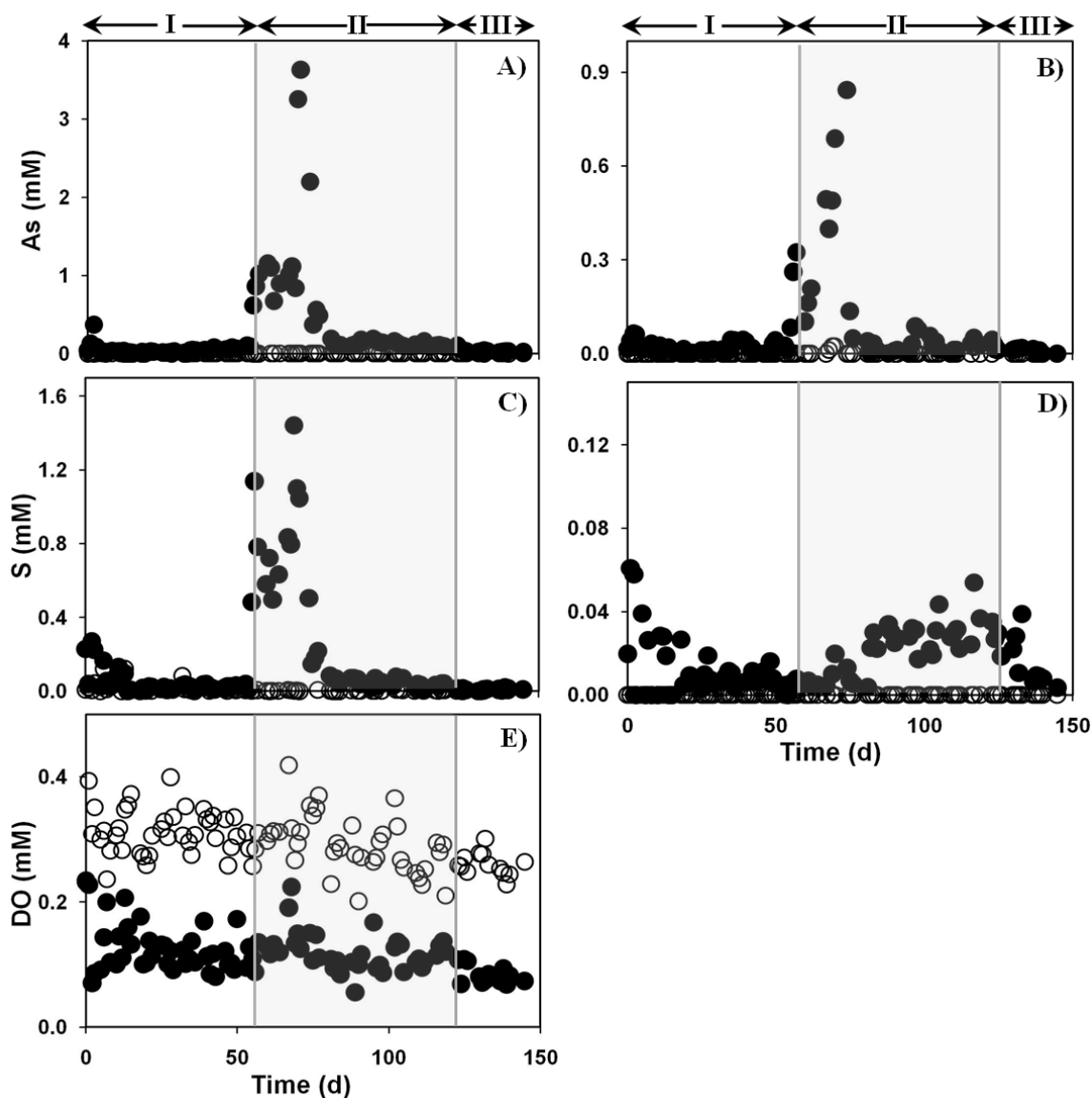


Fig. 7.2. As and S speciation and DO concentration in the influent (●) and in the effluent of reactor 1 (○). (A) total As concentration; (B) As^V concentration; (C) SO₄²⁻ concentration; (D) dissolved sulfide concentration; (E) dissolved oxygen concentration. The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation.

7.4.2. Dissolution of ASM under anoxic conditions using NO_3^- as alternative electron acceptor (Reactor 2)

The dissolution of ASM using NO_3^- as alternative electron acceptor was studied in reactor 2 (R2). Fig. 7.3 shows the concentrations of total As (Fig. 7.3A), As^{V} (Fig. 7.3B), DS (Fig. 7.3C), SO_4^{2-} (Fig. 7.3D) and NO_3^- (Fig. 7.3E) in the influent and effluent of R2. The mobilization of ASM in R2 followed a trend similar to that observed in R1. After an initial period of 6 d, the concentration of total As mobilized during stage I was 0.036 ± 0.022 mM (Fig. 7.3A), and the concentration of As^{V} was 0.011 ± 0.010 mM or $35.9 \pm 28.1\%$ of the total As mobilized (Fig. 7.3B). The difference between the total As and As^{V} concentrations can be attributed to As^{III} , since no other As species were detected (Table 7.2). The average concentrations of DS and SO_4^{2-} in R2 effluent were 0.016 ± 0.008 mM and 0.012 ± 0.010 mM, respectively. To sum up, the dissolution of ASM during the stage I in R2 was low, which only mobilized 1.03% of the total As immobilized as ASM.

When the pH was increased to 7.5, an increase in the release of As and S species into the aqueous phase was observed immediately. The total concentration of As mobilized quickly increased reaching a maximum concentration of 1.635 mM after 12 d. For the rest of the stage II, the concentration of total As progressively decreased to 0.293 mM on the last day of stage II (Fig. 7.3A). As^{V} only accounted for $25.5 \pm 20.1\%$ of the total As (Fig. 7.3B); and, similarly to what was observed in R1, the sum of all the

measured As species (As^{III} , As^{V} , DMA^{V} and MMA^{V}) only accounted for $72.0 \pm 22.6\%$ of the total As mobilized (Table 7.2), suggesting the formation of thioarsenical compounds. DS and SO_4^{2-} concentrations in R2 build up after the change in the pH, reaching up to 0.674 mM (after 13 d) and 0.129 mM (after 15 d), respectively. The amount of DS and SO_4^{2-} being released slowly decreased for the rest of the stage II, and the concentrations at the end of the stage were 0.077 mM of DS and 0.042 mM of SO_4^{2-} . During stage II, dissolution of ASM mobilized 205.5 mg of As or 16.8% of the total As present in the ASM.

During stage III, the pH was decreased to 6.5. Following the decrease in the pH, the mobilization of As and S species also decreased. The concentration of total As in the effluent decreased to levels similar to those observed in stage I in less than 36 h, and the average total As concentration was 0.037 ± 0.020 mM (Fig. 7.3A). The average concentration of As^{V} was 0.013 ± 0.003 mM, which accounted for $43.1 \pm 14.5\%$ of the total As mobilized (Fig. 7.3B). The sum of As^{III} and As^{V} accounted for the total As concentration (Table 7.2). The concentration of DS also decreased quickly to 0.018 mM in less than 24 h, and it averaged 0.010 ± 0.008 mM during this experimental phase (Fig. 7.3C). However, the decrease of SO_4^{2-} concentration in the effluent was slow and the average concentration was 0.027 ± 0.019 mM (Fig. 7.3D). Overall, the mobilization of As during stage III was low, and only 0.36% of the total As supplied to the reactor was released into the aqueous medium.

Table 7.2. Summary of the results from As speciation in R2. The samples where the total soluble As is not the explained by the sum of the soluble As species measured ($\pm 10\%$) are shown in bold.

Time (d)	Total As (mM)	As ^{III} (mM)	As ^V (mM)	DMA ^V (mM)	MMA ^V (mM)	Σ As (mM)	% As recover
<i>Stage I</i>							
20.9	0.03	0.02	0.01	-----ND ^a -----		0.03	95.7
<i>Stage II</i>							
46.0	0.02	ND	0.02	-----ND-----		0.02	94.0
53.9	0.03	ND	0.03	-----ND-----		0.03	91.7
56.9	0.05	ND	0.05	-----ND-----		0.05	103.8
61.9	1.50	0.66	0.44	-----ND-----		1.09	72.9
67.9	1.09	ND	0.71	0.05	ND	0.77	70.0
70.8	0.66	0.24	0.18	-----ND-----		0.42	64.2
80.9	0.70	ND	0.19	-----ND-----		0.19	27.4
88.8	0.31	ND	0.15	-----ND-----		0.15	47.5
95.9	0.33	0.11	0.11	ND	0.08	0.30	90.5
104.9	0.15	ND	0.12	0.01	ND	0.13	85.2
118.7	0.12	ND	0.08	-----ND-----		0.08	67.2
<i>Stage III</i>							
132.9	0.02	0.01	0.01	-----ND-----		0.02	95.2

^a ND.: below detectable limit, which are 0.03 ppb As^{III}, 0.17 ppb As^V, 0.11 ppb DMA^V, 0.15 ppb MMA^V, 0.05 ppb total As.

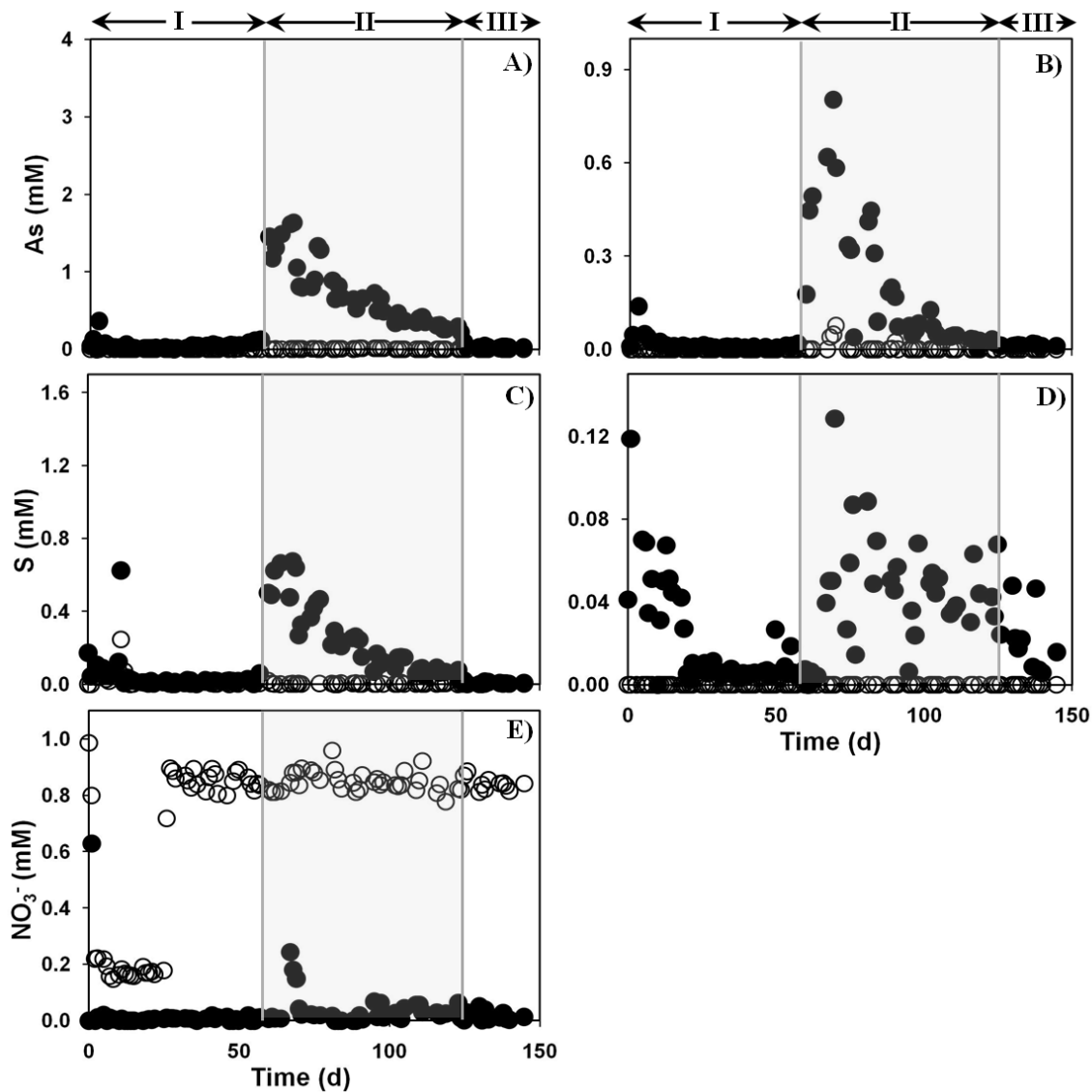


Fig. 7.3. As and S speciation and NO₃⁻ concentration in the influent (●) and in the effluent (○) of reactor 2. (A) total As concentration; (B) As^V concentration; (C) SO₄²⁻ concentration; (D) dissolved sulfide concentration; and (E) NO₃⁻ concentration. The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation.

The concentration of NO_3^- in the influent and effluent of R2 was measured in order to monitor the consumption of the electron acceptor in the reactor (Fig. 7.3E). The average concentration of NO_3^- in the influent was 0.178 ± 0.023 mM ($0.9 e^-$ meq/L) during the first 25 d, and 0.835 ± 0.107 mM ($4.2 e^-$ meq/L) during the rest of the experiment. The concentration of NO_3^- in the effluent was closed to practically zero over the entire time of operation, with the exception of a small accumulation of NO_3^- right after the change from stage I to stage II. In conclusion, the consumption of NO_3^- during the operation in R2 indicates that NO_3^- could be readily used as electron acceptor in the R2.

7.4.3. Dissolution of ASM under anaerobic conditions (Reactor 3)

The dissolution of ASM under anaerobic conditions was studied in reactor 3 (R3). The mobilization of As and S species from the ASM was studied and the results are shown in Fig. 7.4. The release of As and S species into the liquid medium was small at pH 6.5 during stage I. The total As concentration in the effluent averaged 0.035 ± 0.019 mM (Fig. 7.4A). The average As^{V} concentration was 0.009 ± 0.008 mM, which corresponds to $31.5 \pm 26.3\%$ of the total As concentration (Fig. 7.4B). The sum of the measured As^{III} and As^{V} was equivalent to the total As mobilized (Table 7.3). Like it occurred with the other two reactors, the steady state was reached later for the DS and SO_4^{2-} (after ca. 12 d) The average concentration of DS and sulfate was 0.014 ± 0.007 mM (Fig. 7.4C) and 0.005 ± 0.002 mM (Fig. 7.4D), respectively. The total amount of As

dissolved was 11.3 mg, which corresponds to 0.93% of the total As initially present in the ASM.

Table 7.3. Summary of the results from As speciation in R3. The samples where the total soluble As is not the explained by the sum of the soluble As species measured ($\pm 10\%$) are shown in bold.

Time (d)	Total As (mM)	As ^{III} (mM)	As ^V (mM)	DMA ^V (mM)	MMA ^V (mM)	Σ As (mM)	% As recover
<i>Stage I</i>							
20.9	0.03	0.02	0.01	-----ND ^a -----		0.03	97.0
<i>Stage II</i>							
46.0	0.02	ND	0.02	-----ND-----		0.02	91.0
53.9	0.02	ND	0.02	-----ND-----		0.02	100.9
56.9	0.04	ND	0.04	-----ND-----		0.04	102.5
61.9	1.39	0.63	0.38	-----ND-----		1.01	72.9
67.9	0.83	ND	0.54	-----ND-----		0.54	65.1
70.8	0.51	0.37	0.10	-----ND-----		0.47	91.1
80.9	0.35	ND	0.14	-----ND-----		0.14	39.2
88.8	0.19	ND	0.09	-----ND-----		0.09	49.1
95.9	0.26	0.11	0.07	ND	0.06	0.25	95.0
104.9	0.13	ND	0.09	-----ND-----		0.09	64.4
118.7	0.10	0.07	0.02	-----ND-----		0.09	94.8
<i>Stage III</i>							
132.9	0.02	0.01	0.01	-----ND-----		0.02	92.0

^a ND.: below detectable limit, which are 0.03 ppb As^{III}, 0.17 ppb As^V, 0.11 ppb DMA^V, 0.15 ppb MMA^V, 0.05 ppb total As

The mobilization of As and S increased during stage II at pH 7.5. The total As concentration dissolved increased up to 1.972 mM, 12 d into stage II. After this maximum, the concentration of total As decreased for the next 28 d and then it reached a steady state concentration of 0.267 ± 0.060 mM that lasted for the rest of the stage (Fig. 7.4A). The concentration of As^{V} in the effluent only accounted for $20.0 \pm 11.8\%$ of the total As (Fig. 7.4B). Only $77.5 \pm 22.6\%$ of the total As can be accounted for by the concentrations of As^{III} , As^{V} , DMA^{V} and MMA^{V} (Table 7.3), therefore suggesting the presence of thioarsenical species. The formation of DS also increased during the first 13 d of stage II up to 0.618 mM, and then it decreased progressively for 15 d until reaching a steady state concentration of 0.062 ± 0.029 mM (Fig. 7.4C). The increment in SO_4^{2-} concentration was more gradual, since the maximum concentration was not attained until 34 d after the change in the pH, then it slowly decreased for the next 13 d until a steady state concentration of 0.020 ± 0.003 mM (Fig. 7.4D). During stage II, 199.0 mg of As were mobilized, accounting for 16.3% of the total As supplied to R3.

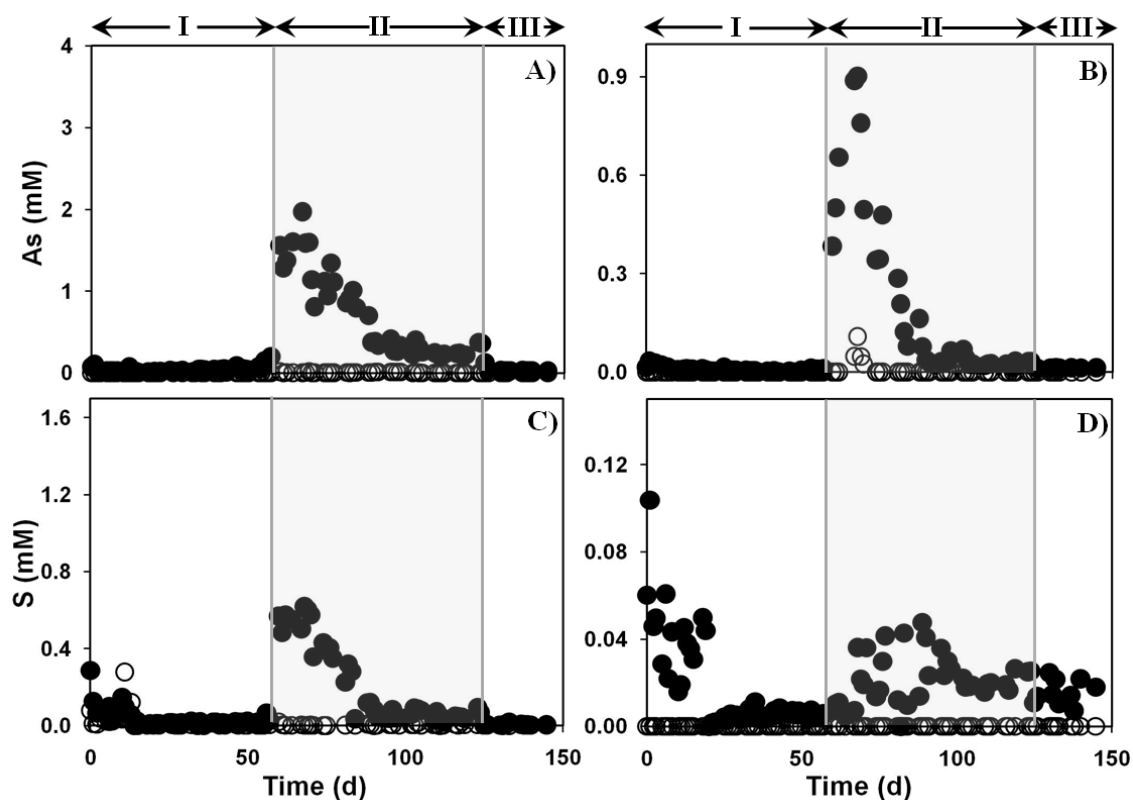


Fig. 7.4. As and S speciation in the influent (●) and in the effluent of reactor 3 (○). (A) total As concentration; (B) As^V concentration; (C) SO₄²⁻ concentration; and (D) dissolved sulfide concentration. The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation.

The mobilization of As and S from ASM during the stage III was comparable to that observed in stage I. The total As concentration decreased to 0.039 mM in less than 48 h and it remained relatively constant at an average of 0.030 ± 0.004 mM (Fig. 7.4A). The average concentration of As^V was 0.012 ± 0.001 mM (Fig. 7.4B) which accounts for $41.9 \pm 8.1\%$ of the total As in the effluent. As^{III} can be assumed to account for the

difference between total As and As^{V} , since the sum of the measured As^{III} and As^{V} was equal to the total As (Table 7.3). The steady state concentrations of DS and sulfate were 0.005 ± 0.005 mM (Fig. 7.4C) and SO_4^{2-} 0.016 ± 0.006 mM (Fig. 7.4D), respectively. The ASM dissolution during stage III was small, accounting for only 0.42% of the total As supplied initially to the reactor.

7.4.4. Effect of the pH in the As mobilization rate from ASM

The As mobilization rates were greatly affected by the pH of operation in the reactors. Fig. 7.5 shows the As mobilization rates compared with the actual pH values in the reactors. During stages I and III, the pH was set at 6.5 and the mobilization rates were comparable in the three reactors. The As mobilization rate was 0.21 ± 0.13 mg As/(L·h) in R1, 0.18 ± 0.11 mg As/(L·h) in R2 and 0.19 ± 0.10 mg As/(L·h) in R3 during stage I; and, 0.17 ± 0.06 mg As/(L·h) in R1, 0.15 ± 0.01 mg As/(L·h) in R2 and 0.15 ± 0.03 mg As/(L·h) in R3 during stage III. Therefore, the mobilization of As was slow at the slightly acidic pH.

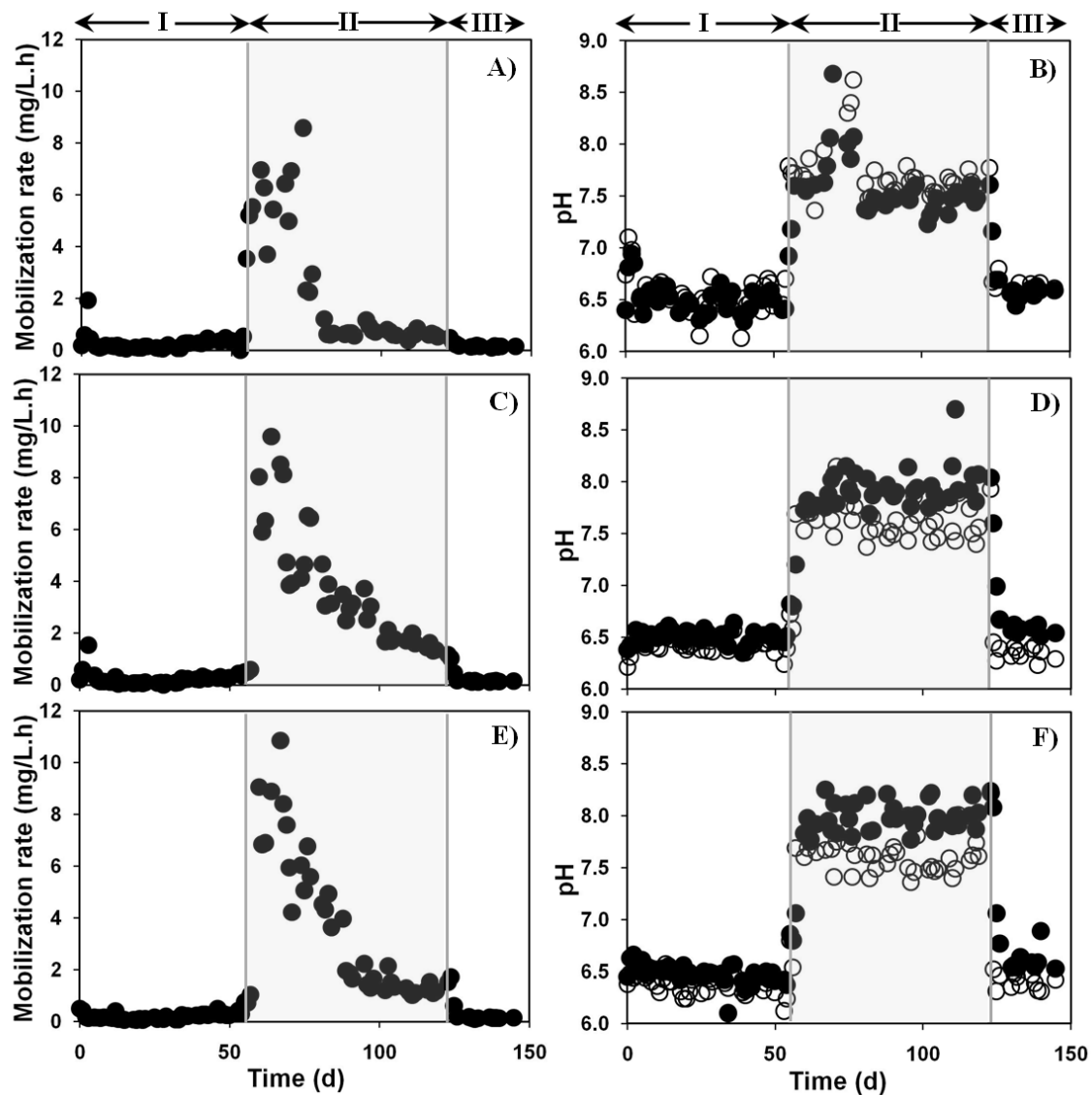


Fig. 7.5. Effect of the pH in the As mobilization rate for the three reactors. Reactor 1: (A) As mobilization rate and (B) pH in the influent (●) and in the effluent (○); Reactor 2: (C) As mobilization rate and (D) pH in the influent (●) and in the effluent (○); Reactor 3: (E) As mobilization rate and (F) pH in the influent (●) and in the effluent (○).

The increase in the pH during stage II (Fig. 7.5B, 7.5D and 7.5F) was associated with an increment in As mobilization rate in the three reactors (Fig. 7.5A, 7.5C and 7.5E). The As mobilization rate increased immediately after the change in pH until reaching maximum values of 8.61 mg As/(L·h) (after 19 d), 9.59 mg As/(L·h) (after 9 d) and 10.85 mg As/(L·h) (after 12 d) in R1, R2 and R3, respectively. After these maxima values, the rates of As mobilization progressively decreased. In R1, the steady state was attained 8 d after the maximum, and it was maintained at an average of 0.66 ± 0.16 mg As/(L·h); in R3, the steady state mobilization rate was an average of 1.36 ± 0.28 mg As/(L·h) reached 23 d after the maximum; in R2, the mobilization rate decreased very slowly until 1.17 mg As/(L·h) at the end of the stage II, but the steady state was not reached over the stage. These results demonstrate that a slightly alkaline pH can enhance the dissolution of ASM and, consequently, the mobilization of As from these ASM.

7.4.5. Qualitative evidence of thioarsenical compounds

The formation of thioarsenical compounds was observed to occur upon the dissolution of ASM at pH 7.5. Samples from four different days were analyzed for thioarsenical compounds, one sample from the stage I (day 21), and three samples were analyzed from Stage II (days 62, 71 and 96). Two thioarsenical species, $\text{As}(\text{OH})\text{S}_2^{2-}$ and AsS_3^{3-} , were detected in the samples from days 62 and 71, the evidence is presented in the Appendix D, Fig. D.1. The formation of thioarsenicals corresponded with samples

were only $79.0 \pm 10.5\%$ (day 62) and $65.5 \pm 8.6\%$ (day 71) of the total As concentration was recovery as the sum of the measured soluble As species; while, in the samples where the total As recovery was $100 \pm 10\%$ no thioarsenicals were detected. Therefore, the difference between the total soluble As measured and the sum of the As^{III} , As^{V} , DMA^{V} and MMA^{V} can be attributed to the formation of thioarsenical compounds. Future efforts will focus in the quantification of the different thioarsenicals in dissolution.

7.4.6. Effect of the redox conditions in the dissolution of ASM

The effect of the three studied redox conditions in the dissolution of ASM can be evaluated during the stage II of operation in the reactors, since it was the stage where the greatest As mobilization from the three reactors was observed. Under aerobic conditions the mobilization of As from the mineral was limited, accounting for only 140.3 mg. Under anoxic (nitrate as e-acceptor) and anaerobic conditions, the total amount of As dissolved in the same time period was somewhat higher, 205.5 and 199 mg As, respectively. These results suggest that aerobic conditions reduces As mobilization from ASM when compare with the mobilization occurring under anoxic and anaerobic conditions.

7.4.7. Arsenic mass balance in the reactors

The total amount of As mobilized in each reactor over the entire experiment was calculated and compared to the amount of As left in the sludge at the end of the operation obtained by the extraction procedure. The extraction method recovered between 80.4% (R1) and 86.6% (R2) of the total As estimated to be immobilized in the reactors at the end of operation. The difference between the measured and estimated As can be due to difference in the homogenization of the material in the reactors when collecting the samples combined with the loss of some colloidal As during the operation in the reactors. For the three reactors, 98.6% of the total As extracted using the extraction protocol was mobilized with aqua regia as extraction medium, therefore, the As extracted was coprecipitated as ASM. Therefore, these results demonstrate the relative stability of the ASM after being exposed to three different redox conditions at two different pH values.

7.5. Discussion

7.5.1. Effect of the pH in the mobilization of As and S from ASM

The aqueous solubility of ASM was greatly enhanced under neutral to alkaline pH. The total As mobilized over the 145 d of operation of the reactors accounted for 13.1-18.2% of the initial As in the biogenic ASM. The majority of ASM dissolution took

place after increasing the pH to 7.5, with 7.8-9.3% of the total As mobilization occurring during the first 22 d at the beginning of stage II, and then the As mobilization rate decreased until reaching 0.7-1.4 mg As/(L·h). However, a small pH change from 7.5 to 6.5 decreased the As mobilization rate 5- to 7-fold. These results are in agreement with previous studies reporting a higher solubility of As_2S_3 and AsS under neutral to alkaline pH. For example, Lengke and Tempel (2002, 2003) have shown that the rate of As_2S_3 and AsS dissolution increased as the pH increased in experiments performed under aerobic conditions at a pH ranging from 6.8 to 8.8. The dissolution of As_2S_3 under anoxic conditions has also been reported to depend on the pH of incubation, with increasing dissolution rates with increasing pHs in a nitrogen (N_2) environment (Floroiu et al. 2004).

The higher solubility of ASM under alkaline pH is likely due to the formation of As-S complexes, or thioarsenical species, which will contribute to the mobilization of As and S (Suess and Planer-Friedrich 2012, Wilkin et al. 2003). During the stage II of operation in the reactors, only 72-79% of the measured soluble total As was recovered as As^{III} and As^{V} , which suggests the presence of other aqueous As species in the solution. The presence of thioarsenical compounds was qualitatively confirmed in samples where the recovery of total As was less than $100\pm 10\%$.

The Pourbaix diagram presented in Fig. 7.6 illustrates the thermodynamic stability of As_2S_3 and AsS as a function of the reduction potential (E_h) and the pH for a total soluble As concentration of 0.5 mM and total S concentration of 0.75 mM. At pH 6.5 and with an E_h below -0.15 V, As_2S_3 and AsS are going to be the predominant species, indicating the stability of the solid phase; however, the increase of the pH to 7.5 makes the aqueous thioarsenical, AsS(OH)HS^- , the predominant phase under those conditions. Similar behavior was predicted by the Pourbaix diagrams created by Saunders et al. (2008) and Couture and Van Cappellen (2011), however the pH at which thioarsenical species start to be formed depends on the DS concentration in the treatment. Rodriguez-Freire et al. (2014) predicted that as the concentration of DS increases the formation of thioarsenicals occurs at lower pH values. Therefore, the dissolution of ASM will be enhanced at pH higher than 7, where free OH^- can interact with the surface of the mineral promoting the formation of thioarsenical species.

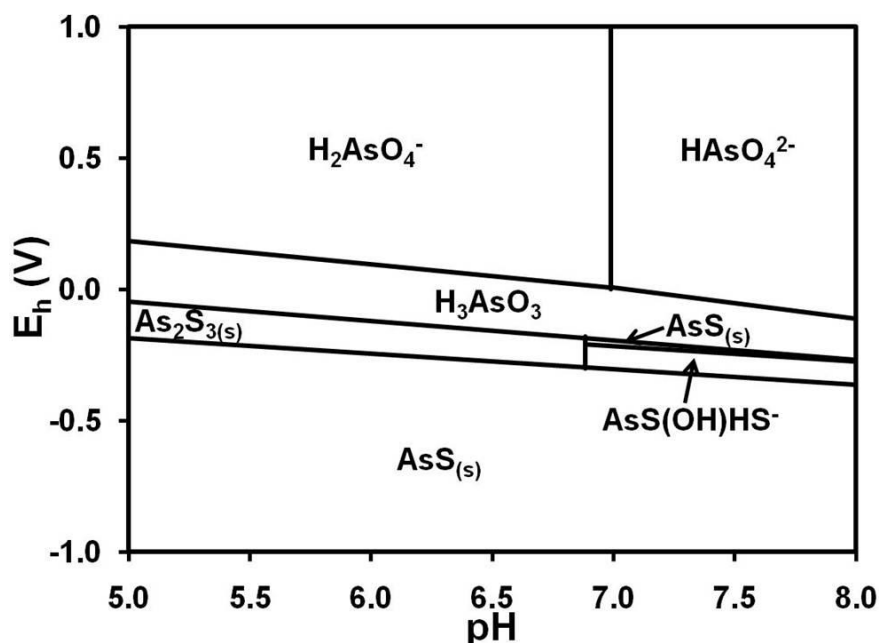
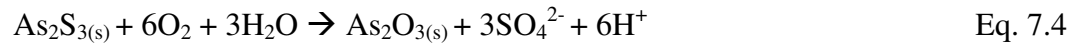


Fig. 7.6. Predicted stable mineral and aqueous phases at equilibrium, including thioarsenite species. Pourbaix diagram for As minerals at 25°C and 1 atm showing the stability fields for solid phases at the conditions the orpiment and realgar, in a solution with 0.5 mM of As and 0.75 mM of S.

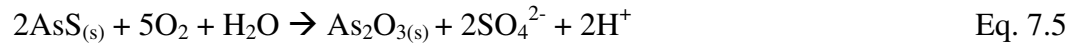
7.5.2. Effect of the electron acceptor in the mobilization of As and S

The effect of the electron acceptor on the mobilization of As and S was more subtle than the effect of the pH. Comparison of the performance of the aerobic and anaerobic reactors indicates that the presence of DO decreased the mobilization of As. The total As dissolved under aerobic conditions was 37.2% less than that mobilized under anaerobic conditions. Lengke and Tempel studied the oxidative dissolution of As_2S_3

(Lengke and Tempel 2002) and AsS (Lengke and Tempel 2003) at different DO concentrations and their observations show a positively correlation between the ASM dissolution rate and DO concentration, which seems to contradict the results presented in this study. However, it is known that the oxidation of As₂S₃ and AsS can lead to the formation of secondary minerals like claudetite (monoclinic As₂O₃) and arsenolite (cubic As₂O₃) (Drahota and Filippi 2009), which would explain the lower As mobilization rate observed under aerobic conditions. The aerobic oxidation of As₂S₃ to As₂O₃ can be described by the following reaction (Foley and Ayuso 2008):



Similarly, the reaction of AsS oxidation to As₂O₃ can be described as follows:



Lacking information about the mineral phase transformation in the reactors, the formation of secondary As-bearing minerals during the operation in R1 can be proved by studying the total amount of As and S released to the aqueous phase during stage II. While the total As concentration was measured, the total S concentration can be estimated by assuming that it is the sum of DS and SO₄²⁻ produced, which can only be used as a comparison between the reactors and not for quantification, since a significant

amount of the soluble S will be incorporated in the thioarsenical species. While the total As mobilized is lower in R1, the total S mobilized in R1 is 43-74% more during the stage II than in R2 and R3. Overall, the higher release of S in R1 than in the other reactors confirms the greater transformation of As_2S_3 and AsS under aerobic conditions due to oxidative dissolution; however, the formation of secondary As-bearing minerals may have prevented the mobilization of As from the solid phase.

The presence of NO_3^- as an alternative electron acceptor did not have a significant impact on the As mobilization rate. The total As mobilized under anoxic conditions was only 3.1% greater than under anaerobic conditions. The small increase in the mobilization of As can be explained by the oxidative dissolution of AsS, which is favored in the presence of an electron acceptor (Eq. 7.2). Several studies have proven that the dissolution of other sulfide minerals, such as pyrite (FeS), can be enhanced by the addition of NO_3^- (Haaijer et al. 2007, Torrento et al. 2010, Torrento et al. 2011). However, NO_3^- did not enhance the mobilization of As from FeAsS under acidic conditions (McKibben et al. 2008).

The mobilization of As and S under anaerobic conditions demonstrates that a non-oxidative dissolution process took place in the reactors. Floroiu et al. (2004) studied the dissolution of As_2S_3 under a N_2 atmosphere. The mechanism of dissolution of As_2S_3 under circumneutral and alkaline pH can be explained by the reaction of free hydroxide

(OH⁻) and deprotonated H₂S (HS⁻) on the surface of the mineral, which would polarize the As-S bond, resulting in the release of As-S-OH complexes, or thioarsenical species, into the aqueous phase.

The redox conditions in the reactors not only affected the mobilization of As from ASM but also the products of such dissolution. The presence of DO and NO₃⁻ promoted the oxidation of reduced As and S species to As^V and SO₄²⁻. The formation of thioarsenical species in the reactors and the different behavior among the reactors during stage II complicates the analysis of the species in this stage, however during stage I and II the three reactors have a similar dissolution pattern which facilitate the comparison. The percentage of As^V in the effluent was higher in R1 (58.9% during stage I and 52.5% during stage III) than in R2 (35.9% during stage I and 43.1% during stage III) and R3 (31.5% during stage I and 41.9% during stage III). However, the percentage of SO₄²⁻ was higher in R2 (41.1% during stage I and 74.8% during stage III) than in R1 (31.2% stage I and 60.4% stage III) and R3 (35.5% stage I and 68.7% stage III). Therefore, the presence of DO stimulated the oxidation of As to As^V while NO₃⁻ stimulated the oxidation of S to SO₄²⁻ in the reactors.

7.6. Conclusions

The stability of biogenic ASM was evaluated at pH of 6.5 and 7.5 in three different bioreactors operating under different redox conditions: aerobic (R1); anoxic, with NO_3^- as alternative electron acceptor (R2); and, anaerobic (R3). The dissolution of ASM showed a great dependency on the pH, as the mobilization of As from the ASM was up to 7-fold lower at the slightly acidic pH (6.5) than at slightly alkaline pH (7.5). The formation of thioarsenical species can explain the greater solubility of ASM at pH 7.5. The stability of ASM was also impacted by the electron acceptor. Aerobic conditions caused a decrease in the mobilization of As in R1 in 37% compare to anaerobic conditions, most likely due to the formation of secondary As-bearing minerals. The presence of NO_3^- did not cause a significant effect on the dissolution of ASM, suggesting a non-oxidative mechanism for the dissolution of As_2S_3 under the experimental conditions. Current work is focused in the identification of the minerals at the different stages of the reactors and the design of a effective method to quantify the different thioarsenical species formed from the dissolution of ASM at pH 7.5.

CHAPTER VIII:

CONCLUSIONS

Arsenic (As) is a highly toxic metalloid, widely known by its carcinogenic effects, which is found at relatively high concentrations in groundwater around the world. The fate of As in groundwater depends on the activity of microorganisms which transform As either by using arsenite (As^{III}) as an electron donor (As^{III} -oxidizing bacteria), or by using arsenate (As^{V}) as an electron acceptor (dissimilatory As^{V} -reducing bacteria). The mobility of As in the environment is highly dependent on its speciation. While As^{V} can be strongly adsorbed on iron (Fe)-rich sediments under aerobic conditions, As^{III} can form As-bearing sulfide minerals (ASM) under anaerobic conditions. Therefore, it is imperative to gain a better knowledge on the microbial processes governing the (im)mobilization of As in the environment, in order to prevent, control, and remediate As contamination. Hereby, the main aim of this research was to study the role of microorganisms on the biogeochemical cycle of As in the environment.

Firstly, three As^{III} -oxidizing bacteria, *Azoarcus* sp. pb-1 strain EC1, *Azoarcus* sp. pb-1 strain EC3, and *Diaphorobacter* sp. pb-1 strain MC, isolated from pristine, non As-contaminated environments were metabolically characterized. These bacterial strains are

able to oxidize As^{III} under aerobic and anoxic conditions, using nitrate (NO_3^-) as an alternative electron acceptor (e-acceptor), thus demonstrating a great adaptability to changing redox conditions in the environment. Furthermore, the isolates are able to survive with environmentally relevant electron donors (e-donors) such as hydrogen (H_2) and acetate, which can be expected in anaerobic conditions where organic matter is decomposing. The ability of As^{III} -oxidizing strains to survive in environments in which As is present in negligible concentrations is most likely related to their flexibility in using different e-donors. These results taken as a whole suggest that As utilizing bacteria are probably more widespread in the environment than previously thought due to the high level of adaptability to redox conditions and flexibility in the use of substrates.

Secondly, the stability of two ASM, orpiment (As_2S_3) and arsenopyrite (FeAsS), was investigated in order to assess the possibility of using the formation of ASM as an As remediation technique. The minerals were incubated in abiotic and biological microcosms, at circumneutral pH (7.2) and under aerobic- and anoxic (nitrate as e-acceptor) conditions. The complexity of the As system in the environment was evident in the fact that higher ASM dissolution does not imply higher As mobilization. While the dissolution of ASM was higher under biotic than abiotic conditions (higher SO_4^{2-} concentrations), the greatest As mobilization was observed under abiotic anoxic conditions, with 87% of the total As solubilized from As_2S_3 and 33% from FeAsS . The mobilization of As was limited in the biological assays due to the formation of secondary

As-bearing minerals under aerobic conditions (claudetite, As_2O_3 , or scorodite, $\text{FeAsO}_4 \cdot 2\text{H}_2\text{O}$) and due to As adsorption onto the anaerobic biofilm under anoxic conditions, a process that might also occur in the environment where different substrates can act as sinks for As immobilization. The biological As mobilization from As_2S_3 was not affected by the redox conditions, whereas As mobilization from FeAsS was greater when NO_3^- was used as the e-acceptor, however, the role of NO_3^- as e-acceptor seemed to be more related to its ability to oxidize As^{III} and sulfide to As^{V} and SO_4^{2-} , respectively, than to the direct oxidation of FeAsS . Overall, the results obtained indicate that As mobilization from ASM can be impacted by the redox conditions and by microbially-mediated mechanisms; therefore, the stability of ASM should be evaluated under actual environmental conditions to predict and prevent As contamination.

Thirdly, this research studied the precipitation of ASM throughout the biological reduction of As^{V} and sulfate (SO_4^{2-}). A series of microcosm experiments were run at pH values ranging from 6.1 to 7.2 in order to determine the effect of the pH on the rate, extent, and type of ASM precipitation. A methanogenic mixed culture was used as inoculum in the biological treatments. The formation of a mineral composed by orpiment (As_2S_3) and realgar (AsS) was observed in the biological treatments amended with As^{V} , SO_4^{2-} and ethanol as e-donor. The extent and rate of As removal was highly influenced by the pH. Slightly acidic pH promoted the formation of the mineral. The As removal rate was 17-fold higher at pH 6.1 than at pH 7.2; also, the percentage of As removed was 94%

at pH 6.5 and only 78% at pH 7.2. The pH did also impact the mineralogical composition of the ASM, with an increase in orpiment compared to realgar at neutral pH. In conclusion, these results indicate that the biological formation of ASM can be promoted at circumneutral pH; however, small pH variations would affect the nature of the mineral and the extent and rate of the As immobilization.

Fourthly, the feasibility of a long-term biological process based on the formation of an ASM for the remediation of As-contaminated groundwater was assessed. This study demonstrated that the formation of ASM can be stimulated by promoting the concurrent biological reduction of As^{V} and SO_4^{2-} in a continuous anaerobic bioreactor with a controlled pH ranging from 6.25 to 6.50 for over 175 d. As^{V} and SO_4^{2-} were readily reduced by an anaerobic mixed culture using ethanol as the e-donor. A control reactor lacking SO_4^{2-} was run in parallel. High As removal rates (2.9 mg As/(L·h)) were achieved and sustained over long periods of operation (175 d) in the sulfate-amended reactor and formation of a precipitate composed of a mixture of As_2S_3 and AsS was observed. In contrast, in the control reactor As^{V} was reduced to As^{III} but a negligible As immobilization was recorded. Taken as a whole, these results confirm that biomineralization of As by formation of As-S minerals in sulfate-amended bioreactors is a promising approach for the remediation of As-contaminated groundwater. .

Fifthly, the potential toxicity of dissolved As species and ASM formed in anaerobic bioreactors towards the methanogenic community was evaluated. A series of batch experiments were run to evaluate As^{III} toxicity in the acetoclastic and hydrogenotrophic methanogenic community of an anaerobic biofilm without previous As exposure and after 160 d of pre-exposure to 1 mM As^{III}. The toxicity of commercial As₂S₃ and biogenic ASM in the methanogenic community was also evaluated in a separate set of batch experiments. The presence of soluble As^{III} in the unadapted biofilm caused a great inhibition in the methanogenic activity, with 50% inhibition concentrations (IC₅₀) of 16 µM for the acetoclastic methanogens and 910 µM for the hydrogenotrophic methanogens. However, the methanogens demonstrated to have the ability to adapt to the As^{III} toxicity, and the IC₅₀ increased to 90 µM and 1100 µM for the acetoclastic and hydrogenotrophic microorganisms, respectively, after the pre-exposure of the sludge to As^{III}. While the methanogenic community was able to overcome the toxicity of soluble As^{III}, the formation of ASM permanently inhibited the acetoclastic methanogenesis. It was determined that only 12 mg As/g VSS of the biogenic ASM or and 50 mg As/g VSS of commercial As₂S₃ were enough to cause 50% methanogenic inhibition. In conclusion, while the methanogens were able to adapt to the As^{III} toxicity, the increasing formation of ASM led to complete inhibition of the methanogens in the bioreactors.

Lastly, the long-term stability of a biogenic ASM was assessed under different environmental conditions. The dissolution of biogenic ASM composed by 40% As₂S₃ and

60% AsS was studied at pH of 6.5 and 7.5, in three bioreactors operating under either aerobic, anoxic (nitrate as e-acceptor), and anaerobic conditions. Similar to the the formation of ASM, the ASM dissolution showed a great dependency on the pH. ASM dissolution increased with increasing pH (i.e., 7-fold higher As dissolution at pH 7.5 compared to pH 6.5), as the formation of soluble As-sulfide complexes becomes more favorable. The stability of ASM was also impacted by the e-acceptor present. While the ASM dissolution rate is expected to be higher under aerobic conditions, the mobilization of As was actually 37% lower than under anaerobic conditions, most likely due to the formation of secondary As-bearing minerals. However, the presence of NO_3^- did not have a significant effect on the dissolution of ASM, with similar As mobilization rate than under anaerobic conditions, suggesting a non-oxidative mechanism controlling the dissolution of As_2S_3 under the experimental conditions utilized. Overall, the biogenic ASM was found to be very stable under slightly acidic pH, but unstable at pH values greater than 7. Furthermore, contrary to the expectations, the mobilization of As from ASM was limited under aerobic conditions due to the formation of secondary As-bearing minerals, such as As_2O_3 . Therefore, altering the pH and the redox conditions of the environment are important tools to control the release of As from ASM.

In conclusion, this research has provided additional evidence to support the important role of microbial transformations in environmental cycling of As. It has demonstrated that As-utilizing bacteria can be more widespread than initially thought,

thanks to their flexibility to use different substrates and adapt to changing redox conditions. It has also proven that the stimulation of biological As^{V} and SO_4^{2-} reduction will promote the precipitation of As-S minerals (ASM), a process that can be effectively used to remediate As-contaminated waters. While ASM dissolution is greatly affected by the pH, the presence of microorganisms could prevent the mobilization of As from the ASM due to As adsorption in anaerobic biofilm under anoxic-anaerobic conditions or by stimulating the formation of secondary As-bearing minerals under aerobic conditions. And finally, even though As^{III} can be highly toxic to microorganisms such as methanogens, some microorganisms were found to overcome the initial As toxicity and become tolerant to increasing As concentrations.

APPENDIX A: SUPPLEMENTARY INFORMATION CHAPTER III
FLEXIBLE BACTERIAL STRAINS THAT OXIDIZE ARSENITE IN
ANOXIC OR AEROBIC CONDITIONS AND UTILIZE HYDROGEN OR
ACETATE AS ALTERNATIVE ELECTRON DONORS

A1. Materials and methods

A1.1. PCR primers for targeting the nitrous oxides reductase and RuBisCO genes.

Two sets of PCR primers were used to target the *nosZ* gene, including primer set *nosZ661F* and *nosZ1527R*, and primer set *nosZ661F* and *nosZ1773R* (Scala and Kerkhof 1998). Optimal PCR conditions for the primer sets were 56°C annealing temperature, 0.4 µM of each primer. The cycling parameters were as follows: reactions were initially heated to 94°C for 5 min, followed by 35 cycles of 95°C for 0.5 min, 56°C for 1.5 min, 72°C for 2 min, with a final 10-min 72°C extension period. Two specifically designed PCR primer sets were used to identify two RuBisCO type genes, both *cbbL* and *cbbM* genes (Elsaied and Naganuma 2001). The optimal PCR conditions for primer set for RuBisCO type I *cbbL* gene were 49°C annealing temperature, 0.3 µM of each primer. Optimal PCR conditions for the primer set of RuBisCO type II *cbbM* gene were 62°C annealing temperature, 0.3 µM of each primer. The cycling parameters were set up as

follows: reactions were initially heated to 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, annealing temperature for 1 min, 72°C for 3 min, with a final 15-min 72°C extension period.

A1.2. PCR primers for targeting As^{III} oxidase and As^V reductase genes.

Four primer sets were used to target the As^{III} oxidase (*aroA*) gene, which included *aroA1*-85F and *aroA1*-593R (set #1) (Inskeep et al. 2007), *aroA2*-85F and *aroA2*-602R (set #2) (Inskeep et al. 2007), *aroA3*-69F and *aroA3*-1374R (set #3) (Rhine et al. 2007) and *aroA4*-85F and *aroA4*-622R (set #4) (Hoeft et al. 2007). The PCR conditions for primer set *aroA1*-85F and *aroA1*-593R were: 95°C for 4 min followed by nine cycles of 95°C for 45 s, 50°C (decreased by 0.5°C after each cycle) for 45 s, 72°C for 50 s, followed by 25 cycles of 95°C for 45 s, 46°C for 45 s, and 72°C for 50 s, and a final extension of 72°C for 5 min. The concentration of each primer was 1 uM. The PCR conditions for two primer sets *aroA2*-85F and *aroA2*-602R, and *aroA4*-85F and *aroA4*-622R were: 90°C for 3 min followed by 40 cycles of 92°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min, and a final extension of 72°C for 5 min. The final concentration of each primer was 0.21 and 1 uM, respectively. The PCR conditions for primer set *aroA3*-69F and *aroA3*-1374R were: 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min, and a final extension of 72°C for 10 min. The concentration of each primer was 1 uM. Two primer sets were used to amplify the As^V

reductase genes (*arrA*), which included CHarrAfwd and CHarrArev (set #1) (Malasarn et al. 2004) and HAarrA-D1F and HAarrA-G2R (set #2) (Kulp et al. 2006). The PCR conditions for primer set of CHarrAfwd and CHarrArev include 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 50°C for 40 s, and 72°C for 1 min, and a final extension of 72°C for 10 min. The concentration of each primer was 0.5 µM. For primer set of HAarrA-D1F and HAarrA-G2R, the PCR conditions were 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 53.5°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 5 min. The final concentration of each primer was 0.33 µM.

A1.3. Cloning of purified PCR products.

To verify the integrity of the amplification, a negative control (no template DNA) was utilized and wherever possible a positive control (suspected to contain target DNA) was also utilized. PCR results were checked on a 1.5% agarose gel. The identities of amplicons were confirmed by verifying the molecular sizes of the amplicons on the gel with electrophoresis. The purified PCR products from successfully amplified functional genes were cloned into plasmid vector pCR 2.1-TOPO using the TOPO TA cloning system (Invitrogen, Carlsbad, CA) to identify the sequences. The detailed procedure was described by Sun et al. (Sun et al. 2009). Selected clone amplified with specific primer set obtained in pure culture was deposited in the GenBank database.

A2. Figures

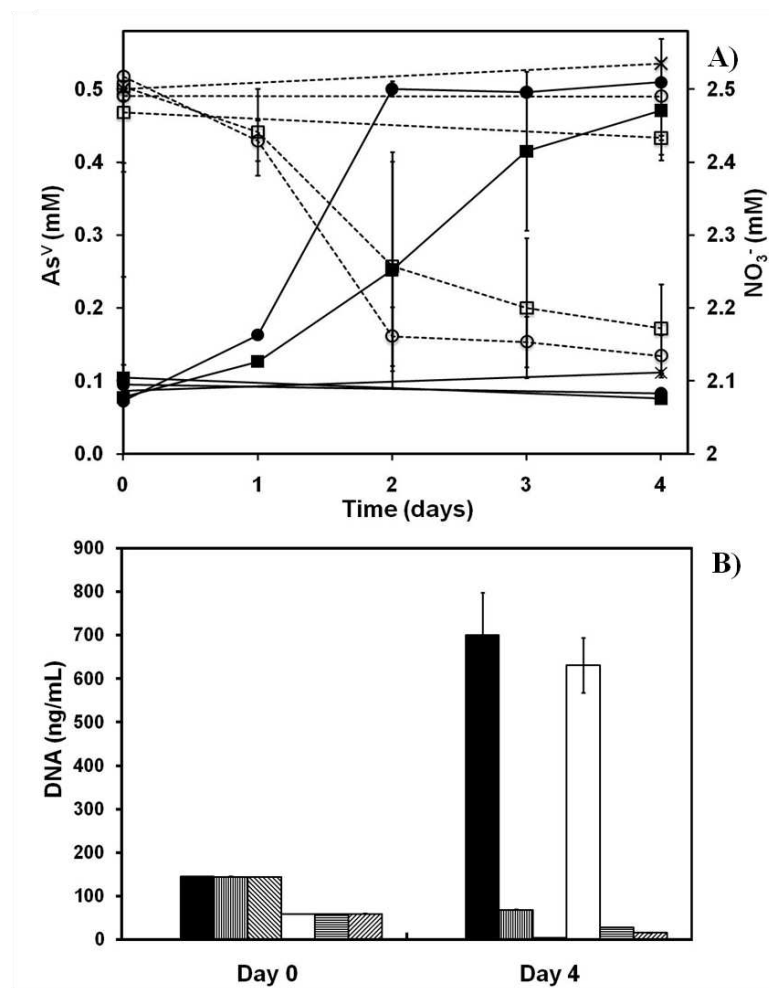


Fig. A.1. Growth of pure cultures EC1 and EC3 by oxidation of As^{III} (0.5 mM) linked to NO₃⁻ (2.5 mM) reduction. Legends: panel A, As^V formation (primary axis, continuous lines) and NO₃⁻ reduction (secondary axis, dashed lines), complete treatments containing As^{III} and NO₃⁻ and inoculated with EC1 (■) and EC3 (●); biological controls: containing As^{III} but lacking NO₃⁻, continuous line, EC1 (□) and EC3 (○); containing NO₃⁻ but lacking As^{III}: dashed line, EC1 (□) and EC3 (○); non inoculated control (x); panel B, DNA concentration: complete treatments containing As^{III} and NO₃⁻ and inoculated with EC1 (full) and EC3 (empty); biological controls: containing As^{III} but lacking NO₃⁻, EC1 (vertical stripes) and EC3 (horizontal stripes); containing NO₃⁻ but lacking As^{III}, EC1 (stripes to the left) and EC3 (stripes to the right).

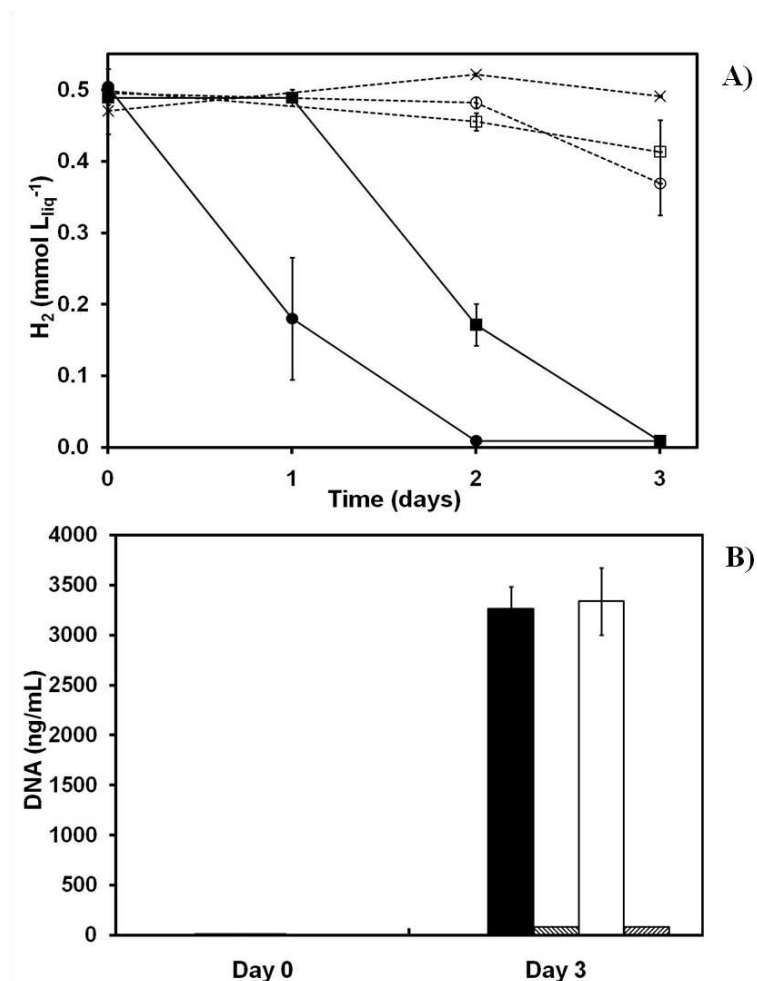


Fig. A.2. Growth of pure cultures EC1 and EC3 by oxidation of H₂ (0.5 mmol/L_{liq}) linked to O₂ (0.25 mmol/L_{liq}) reduction. Legends: panel A, H₂ consumption (primary axis, continuous lines) and NO₃⁻ reduction (secondary axis, dashed lines), complete treatments containing As^{III} and NO₃⁻ and inoculated with EC1 (■) and EC3 (●); biological treatments supplied with O₂ and H₂, EC1 (■) and EC3 (●); controls (dashed lines) supplied with H₂ but no O₂, EC1 (□) and EC3 (○); non inoculated control (x); panel B, DNA concentration: complete treatments containing H₂ and O₂ and inoculated with EC1 (full) and EC3 (empty); biological controls: containing H₂ but lacking O₂, EC1 (stripes to the left) and EC3 (stripes to the right).



Fig. A.3. The PCR gel for the functional gene PCR of RuBisCO *cbbL* gene primer set. MW = molecular weight ladder indicated in base pair length. Lane 1 = positive control, an autotrophic anaerobic ammonium oxidizing (anammox) enrichment culture (gift of R. Mendez-Pampin, University of Santiago de Compostela, Spain), Lanes 2 , 3 and 4 are amplicons from PCR reaction with DNA from strains EC1, EC3, and MC, respectively. Lane 5 = negative control, demineralized water.

APPENDIX B: SUPPLEMENTARY DATA CHAPTER V
BIOMINERALIZATION OF ARSENATE TO ARSENIC SULFIDES IS
GREATLY ENHANCED AT MILDLY ACIDIC CONDITIONS

B1. Materials and Methods

B1.1. Solid phase characterization

Scanning Electron Microscopy (SEM) was performed in a Hitachi S-4800N Type II with a cold field emission electron gun and an accelerant voltage of 20 kV. The SEM was combined with a ThermoNORAN microanalyzer for energy dispersive spectroscopy (EDS). The samples were vacuum dry and crushed to a powder material. Then, the samples were adhered to a metallic base and coated with platinum. Arsenic K-edge X-ray absorption spectra (XAS) were acquired at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 11-2 under dedicated conditions (3 GeV, 300 mA) using an unfocused beam detuned 40% to remove high energy harmonics calibrated to As⁰ foil edge jump inflection defined as 11867 eV. Solid samples were characterized by XAS with X-ray absorption near-edge structure (XANES) and extended x-ray absorption fine-structure (EXAFS). Samples were loaded as wet pastes into 0.5 mm thick aluminum sample holders and sealed with kapton tape in an anaerobic glovebag (Coy, MI). To

avoid X-ray beam induced redox changes, all samples were collected in a LN₂ cryostat sample holder (77 K), with the sample position moved at least 1 mm (always greater than the X-ray beam size) between scans to expose a fresh cross-sectional area of sample to the X-ray beam. Collected spectra (3-5 scans) were averaged in SixPack and background subtracted using a linear fit through the pre-edge region, spectra were normalized to unity about the average height of the post-edge absorbance oscillation, and a cubic spline was fit through the EXAFS region above the absorption edge using EXAFSPAK software packages. Normalized EXAFS spectra were fit by linear combination least squares fits to model spectra of orpiment and realgar using the Datfit package in EXAFSPAK.

B2. Figures

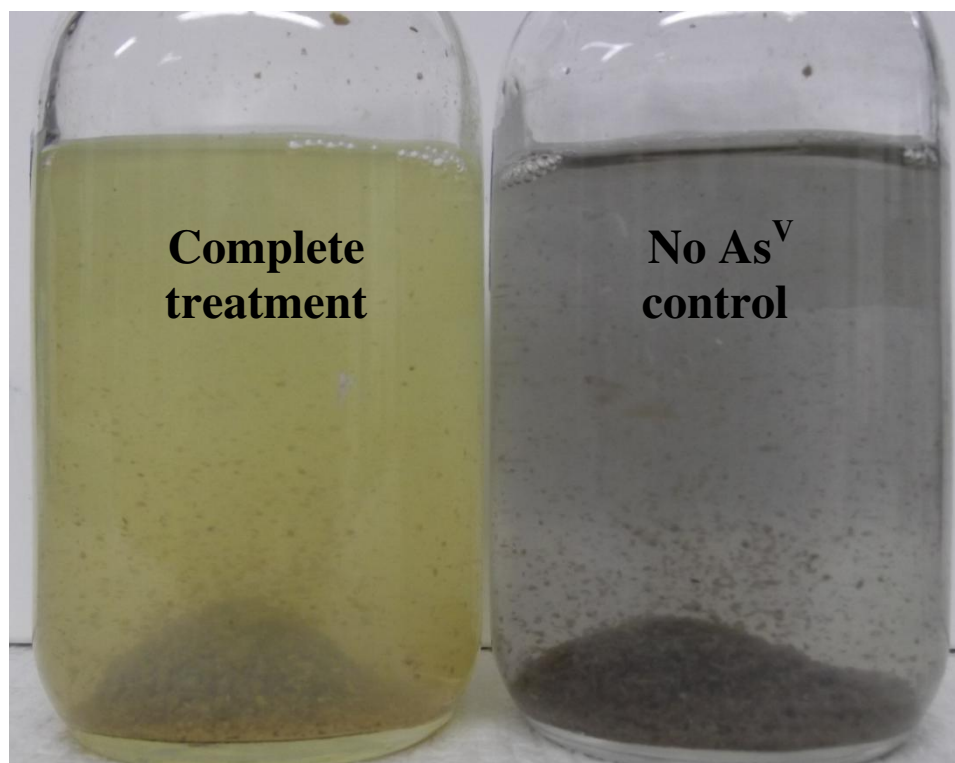


Fig. B1. Visual evidence of As-S mineral precipitation for the experiment at pH 6.1 after 12 days of incubation. Comparison between the complete inoculated treatment containing 0.5 mM As^V, 0.75 mM SO₄²⁻ and 14 mM ethanol and the control treatment lacking the addition of As^V.

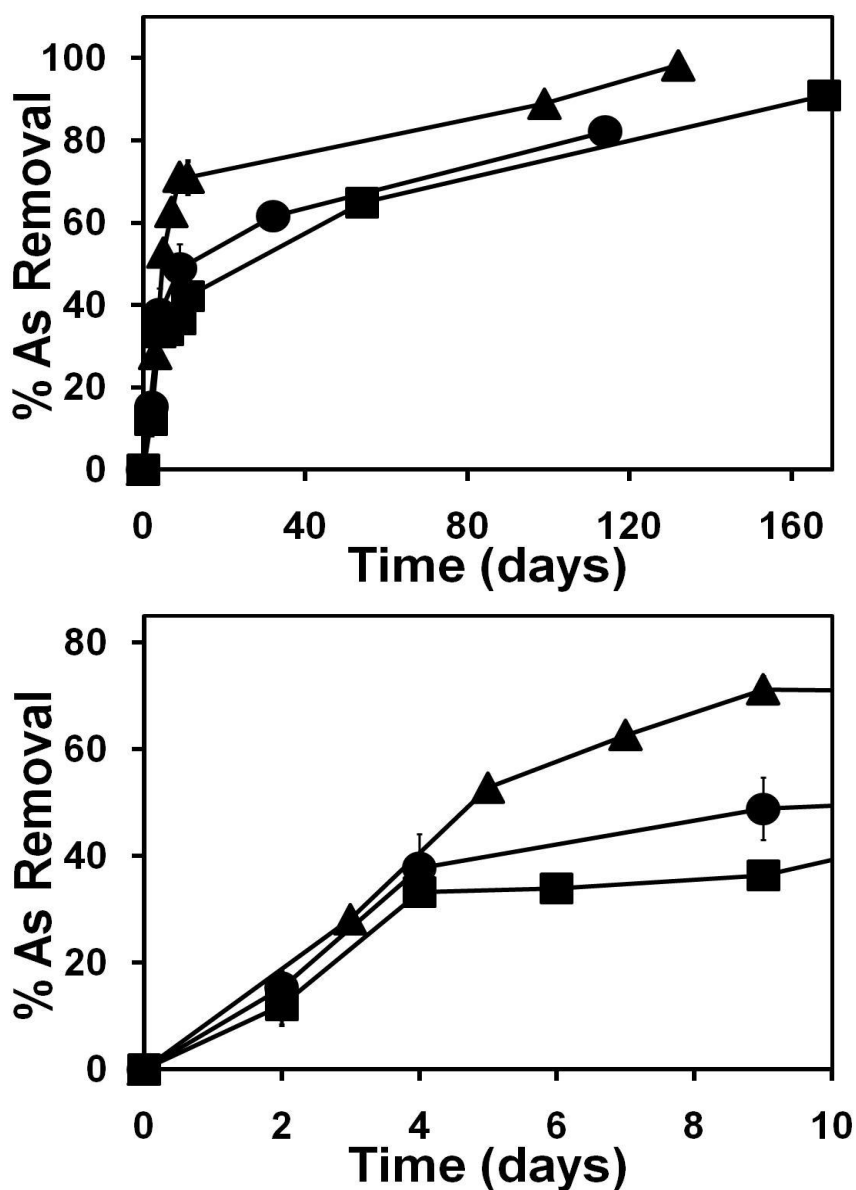


Fig. B.2. Total As removal as a function of the different pH for the old sludge. Panel (A) shows the percentage of total As removal for the long incubation time of the experiments. Panel (B) shows the As removal for the first 10 days of incubation to get a better look at the arsenic removal rate for the different pH. Legends: pH 7.2 (■), 6.8 (●) and 6.2 (▲).

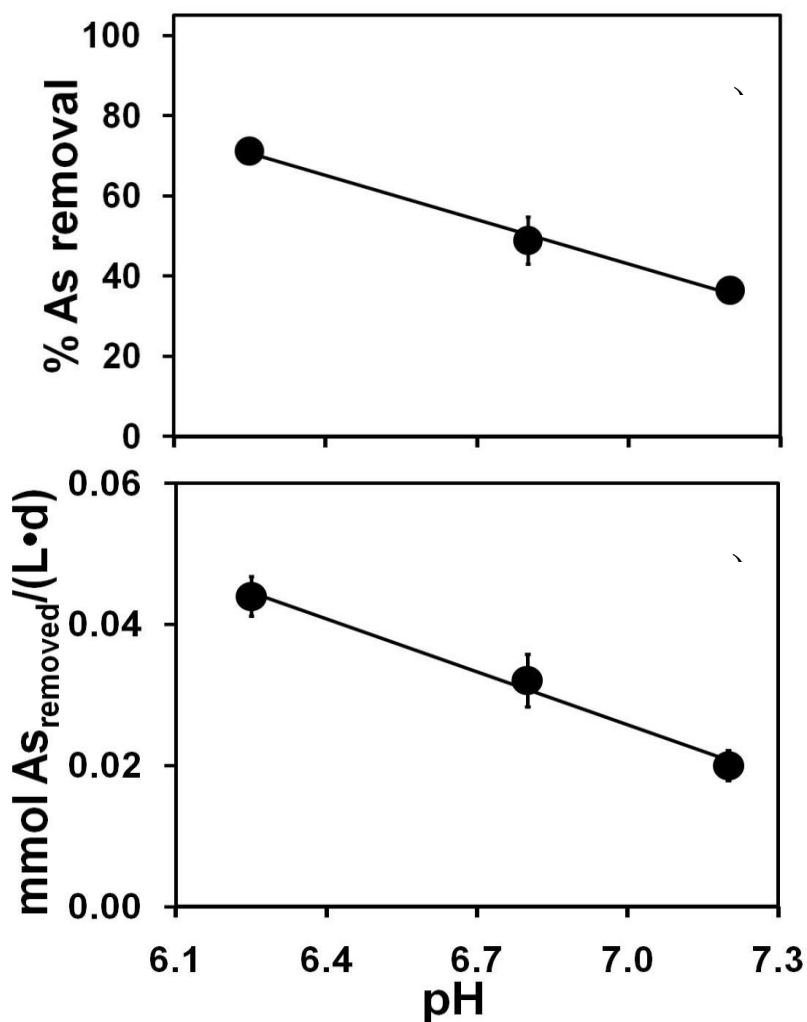


Fig. B.3. Relationship between the total As removal and the pH. Panel (A) illustrates the trend and linear regression line for As removal as a function of the pH at day 9 of incubation; the linear regression equations were obtained and the relationship between the As removal and pH can be represented by the linear equation $\%As_{\text{removal}} = A - B \cdot \text{pH}$. The constants and the R-squared are: $A = 301 \text{ \%As/d}$, $B = 36.9\%As/d$, $R^2 = 0.9949$. Panel (B) shows the total As removal rate as a function of the pH. The rate of As removal is related to the pH by the linear regression equation with constants $A = 0.2009 \text{ mmolAs/(L} \cdot \text{d)}$, $B = 0.025 \text{ mmolAs/(L} \cdot \text{d)}$, and $R^2 = 0.9913$.

APPENDIX C: SUPPLEMENTARY INFORMATION CHAPTER VI:
ARSENIC BIOREMEDIATION BY FORMATION OF ARSENIC
SULFIDE MINERALS IN A CONTINUOUS-FLOW ANAEROBIC
BIOREACTOR

C1. Materials and methods

C1.1. Solid phase characterization

Scanning Electron Microscopy (SEM) was performed in a Hitachi S-4800N Type II with a cold field emission electron gun and an accelerant voltage of 20 kV. The SEM was combined with a ThermoNORAN microanalyzer for energy dispersive spectroscopy (EDS). The samples were vacuum dry and crushed to a powder material. Then, the samples were adhered to a metallic base and coated with platinum. Arsenic K-edge X-ray absorption spectra (XAS) were acquired at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 11-2 under dedicated conditions (3 GeV, 300 mA) using an unfocused beam detuned 40% to remove high energy harmonics calibrated to As⁰ foil edge jump inflection defined as 11867 eV. Solid samples were characterized by XAS with X-ray absorption near-edge structure (XANES) and extended x-ray absorption

fine-structure (EXAFS). Samples were loaded as wet pastes into 0.5 mm thick aluminum sample holders and sealed with kapton tape in an anaerobic glovebag (Coy, MI). To avoid X-ray beam induced redox changes, all samples were collected in a LN₂ cryostat sample holder (77 K), with the sample position moved at least 1 mm (always greater than the X-ray beam size) between scans to expose a fresh cross-sectional area of sample to the X-ray beam. Collected spectra (3-5 scans) were averaged in SixPack and background subtracted using a linear fit through the pre-edge region, spectra were normalized to unity about the average height of the post-edge absorbance oscillation, and a cubic spline was fit through the EXAFS region above the absorption edge using EXAFSPAK software packages. Normalized EXAFS spectra were fit by linear combination least squares fits to model spectra of orpiment and realgar using the Datfit package in EXAFSPAK.

C2. Results

C2.1. Endogenous activity of the inocula

The endogenous substrate decay of the anaerobic biofilm was obtained by measuring the CH₄ produced by 50 mg wet wt of sludge incubated with 10 mL of mineral medium over a 7 d period. The experiment was performed in 25 mL culture tubes, closed with butyl rubber septum and aluminum seals, and flushed with N₂/CO₂ to ensure anaerobic conditions. 0.21±0.04 mM of CH₄ were produced after 7 d, which

corresponded to $8\ e^- \text{ meq/g VSS}$. The endogenous decay rate was as high as $2.57\ e^- \text{ meq/g VSS.d}$ during the first two days of incubation and then it stabilized at $0.57\ e^- \text{ meq/g VSS.d}$ for the rest of the experiment.

Table C.1. Actual values of the samples send for As speciation.

Reactor 1						Effluent				
Date	Influent									
	DMA ^V (mM)	MMA ^V (mM)	As ^{III} (mM)	As ^V (mM)	Total As (mM)	DMA ^V (mM)	MMA ^V (mM)	As ^{III} (mM)	As ^V (mM)	Total As (mM)
1/10/2013		below LOD ^a		1.16	1.10		below LOD		0.12	0.14
2/1/2014		below LOD		1.02	1.00		below LOD		0.11	0.12
3/7/2014		below LOD		1.03	1.01		below LOD		0.12	0.12
4/5/2014		below LOD		0.93	0.92		below LOD		0.09	0.08
5/1/2013		below LOD		1.08	1.05		below LOD		0.16	0.16
6/5/2013		below LOD		0.95	0.92		below LOD		0.16	0.16
6/26/2014		below LOD		1.00	1.01		below LOD		0.12	0.12
Reactor 2						Effluent				
Date	Influent									
	DMA ^V (mM)	MMA ^V (mM)	As ^{III} (mM)	As ^V (mM)	Total As (mM)	DMA ^V (mM)	MMA ^V (mM)	As ^{III} (mM)	As ^V (mM)	Total As (mM)
1/10/2013		below LOD		1.13	1.10		below LOD	0.72	0.10	0.76
2/1/2014		below LOD		1.02	0.99		below LOD	1.00	0.04	0.99
3/7/2014		below LOD		1.05	1.03		below LOD	0.98	0.09	1.02
4/5/2014		below LOD		1.00	0.95		below LOD	1.00	0.08	1.01
5/1/2013		below LOD		1.15	1.15		below LOD	0.84	0.19	1.02
6/5/2013		below LOD		1.01	0.96		below LOD	0.81	0.15	1.00
6/26/2014		below LOD		0.96	0.92		below LOD	0.87	0.12	0.97

^(a) LOD, low detection limit, 0.03 ppb As^{III}, 0.17 ppb As^V, 0.11 ppb DMA^V, 0.15 ppb MMA^V, 0.05 ppb total As

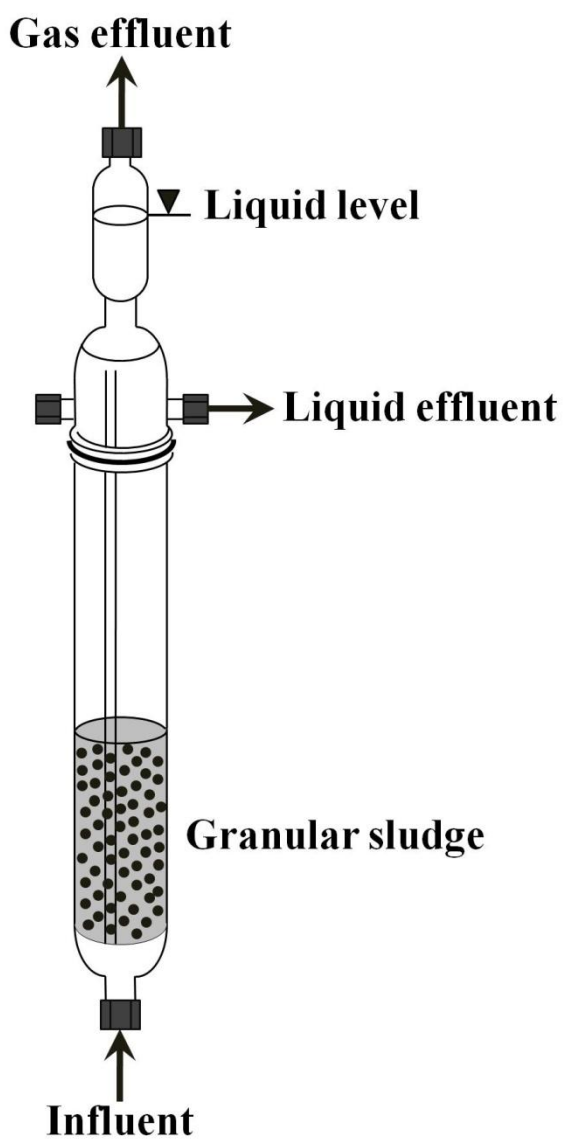


Fig. C.1. Schematic of the bioreactors used in this study.

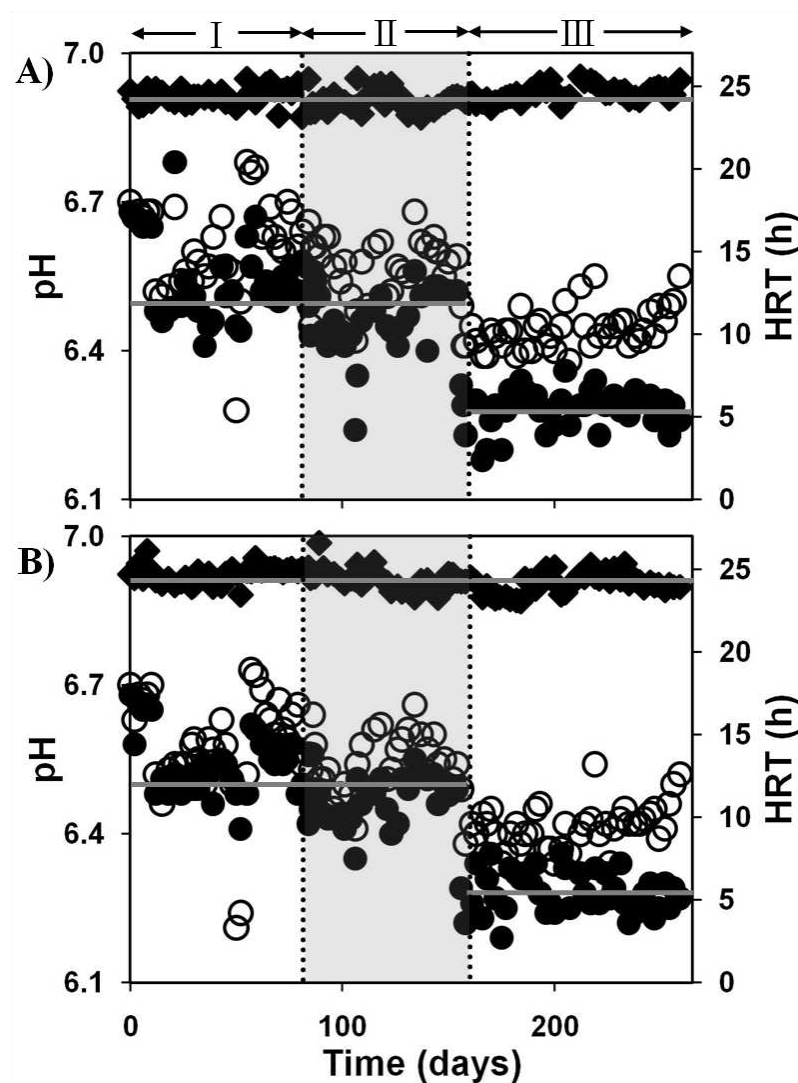


Fig. C.2. Actual pH and HRT of the reactor 1 (A) and reactor 2 (B). (♦) HRT; (●) pH of the influent; (○) pH on the effluent. The vertical dashed lines indicate the separation between the three stages in the reactor. The shaded area designates the stage II of operation, right after the addition of As^{V} . The horizontal grey lines represent the theoretical target values of pH and HRT.



Fig. C.3. Evidence of mineral precipitation. The picture was taken 70 d after the addition of As^{V} . A yellow precipitate is clearly present in reactor 1 (left), in the liquid medium and on top of the sludge. No precipitate is observed in reactor 2 (right), where the medium appears clear and the sludge clean.

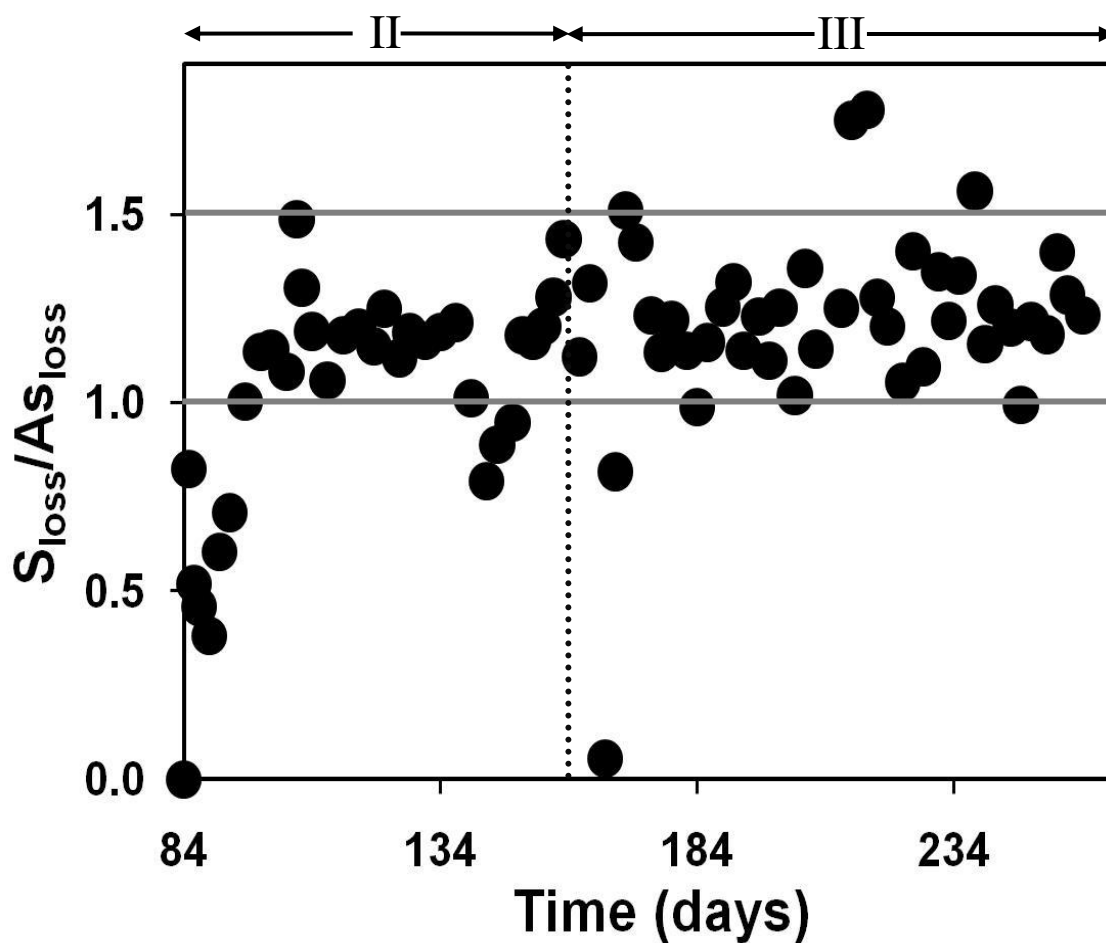


Fig. C.4. Calculated $S_{\text{loss}}/As_{\text{loss}}$ ratio over the operation in reactor 1. The vertical dashed line indicates the separation between stages II and III in the reactor. The shaded area designates the stage II of operation, right after the addition of As^V . The horizontal grey lines represent the theoretical stoichiometric values for the precipitation of AsS (1) and for the precipitation of As_2S_3 (1.5).

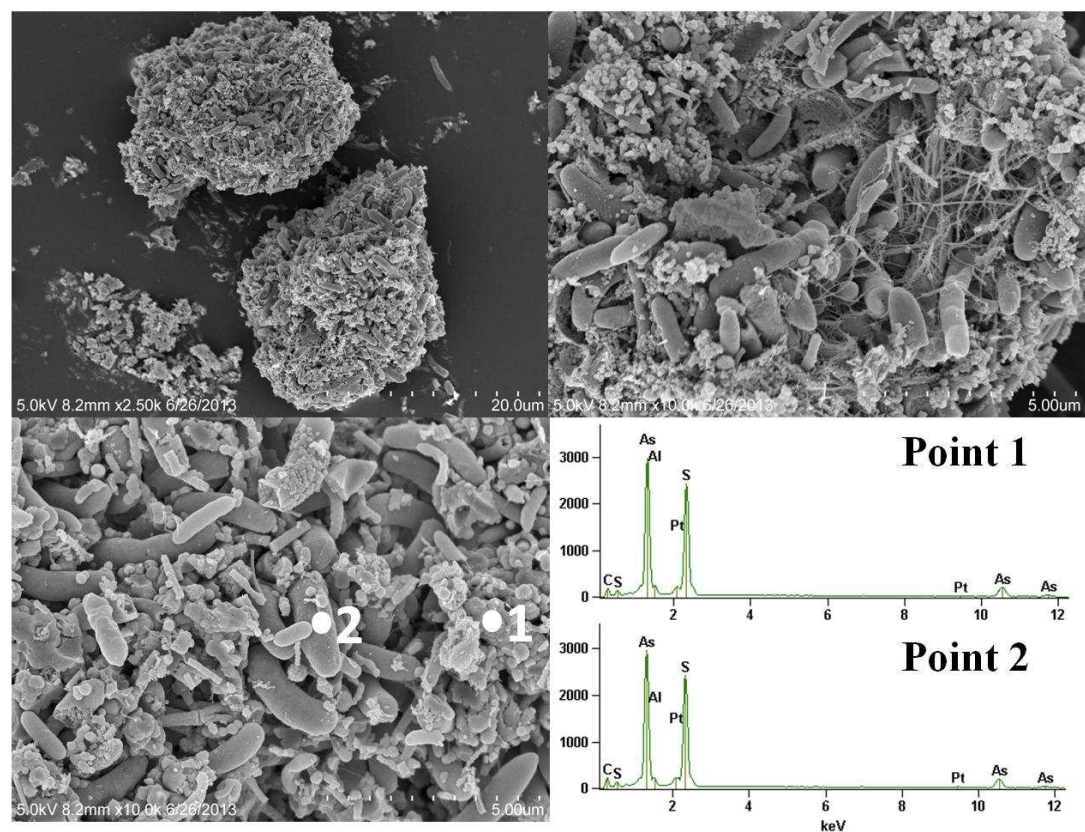


Fig. C.5. SEM – EDS analysis of the precipitate from reactor 1. Sample was taken 170 d after the addition of As^{V} . The image on the left shows the granules packed with bacteria and precipitate. The image on the top right is a zoom in on one of the granules, where it can be notice the different shapes of bacteria and the precipitate. Finally, the bottom left shows the image of the bacterium and precipitate that were used for the EDS analysis.

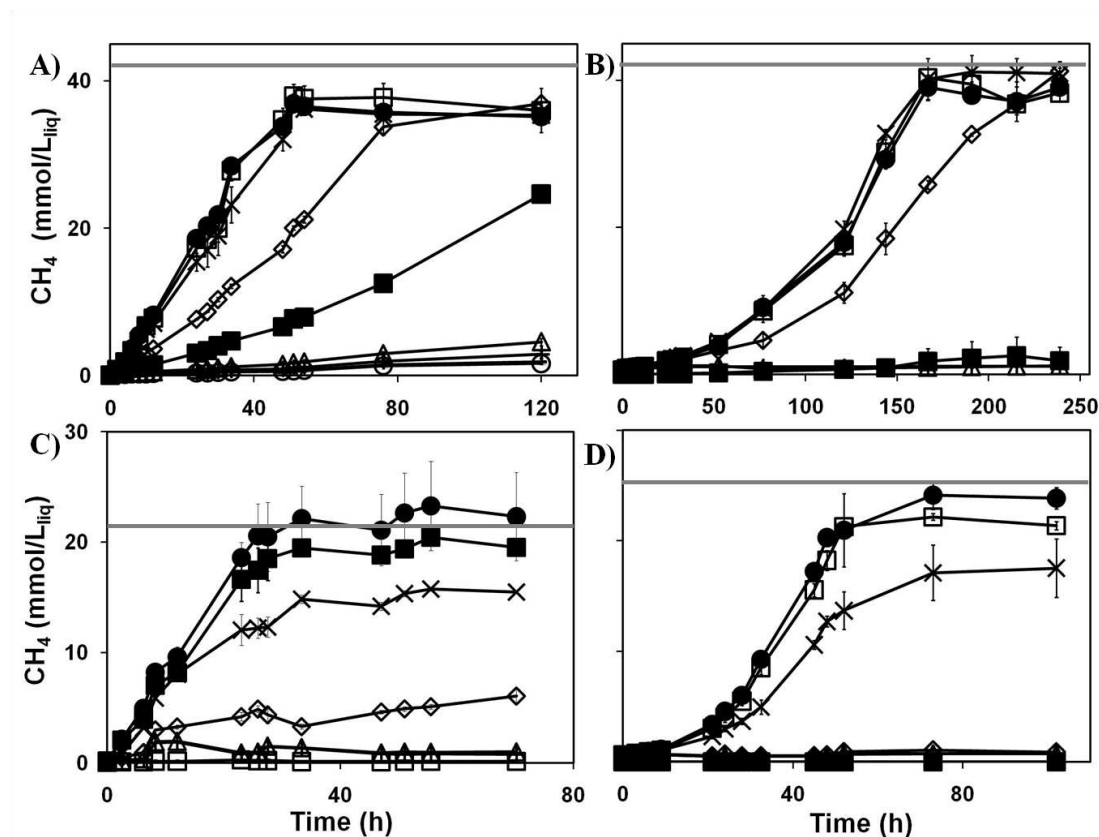


Fig. C.6. CH₄ production in the experiments assessing the toxicity of As^{III} on: (A) acetoclastic methanogens from the original biofilm: (□) no As^{III} control 1, (●) no As^{III} control 2, (x) 10 μM As^{III}, (◇) 20 μM As^{III}, (■) 40 μM As^{III}, (Δ) 80 μM As^{III}, (+) 100 μM As^{III}, (-) 140 μM As^{III}, (○) 200 μM As^{III}; (B) acetoclastic methanogens from the pre-exposed biofilm from reactor 2: (□) no As^{III} control 1, (●) no As^{III} control 2, (x) 100 μM As^{III}, (◇) 500 μM As^{III}, (■) 1000 μM As^{III}, (Δ) 1500 μM As^{III}, (+) 2000 μM As^{III}; (C) hydrogenotrophic methanogens from the original biofilm: (■) no As^{III} control 1, (●) no As^{III} control 2, (x) 100 μM As^{III}, (◇) 500 μM As^{III}, (□) 1000 μM As^{III}, (Δ) 1500 μM As^{III}, (+) 2000 μM As^{III}; (D) hydrogenotrophic methanogens from pre-exposed biofilm from reactor 2: (□) no As^{III} control 1, (●) no As^{III} control 2, (x) 1000 μM As^{III}, (◇) 2000 μM As^{III}, (■) 5000 μM As^{III}, (Δ) 7500 μM As^{III}, (+) 10000 μM As^{III}. The horizontal grey lines represent the theoretical concentration of CH₄ that can be produced.

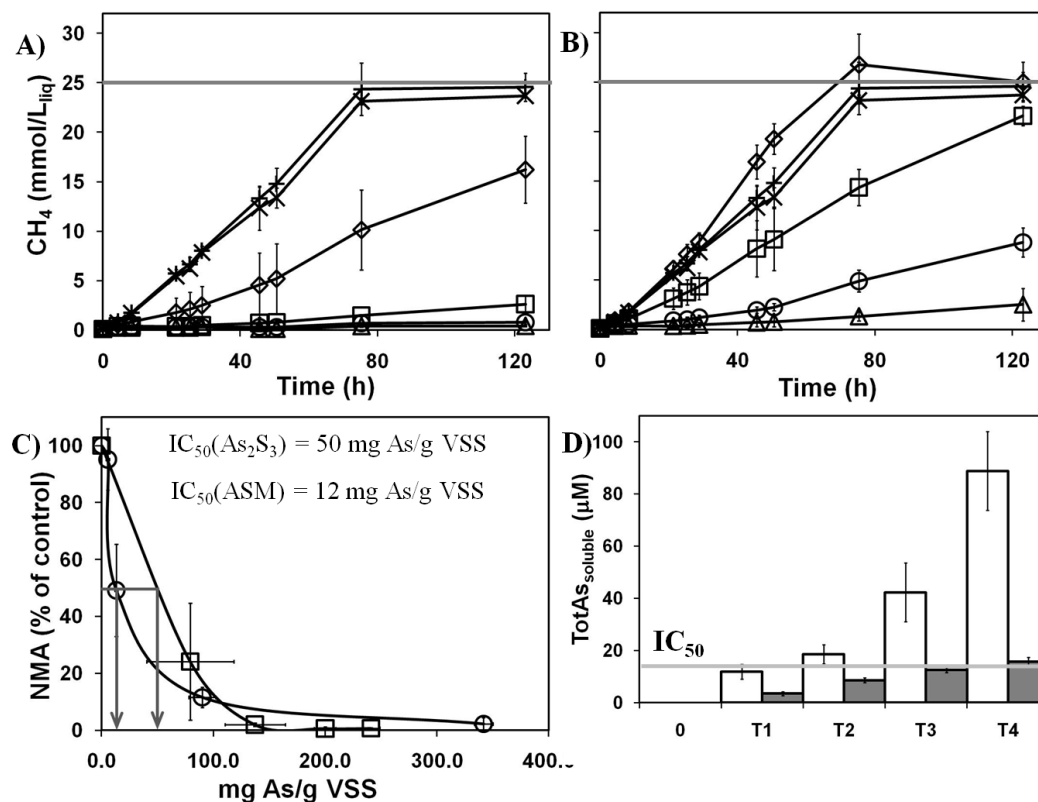


Fig. C.7. Toxicity effect of ASM minerals on the acetoclastic methanogenic activity. **(A)** CH₄ production in the experiments assessing the toxicity of commercial As₂S₃: (x) no mineral control 1, (+) no mineral control 2, (◇) 79 mg As/g VSS, (□) 137 mg As/g VSS, (○) 200 mg As/g VSS, (Δ) 241 mg As/g VSS; **(B)** CH₄ production in the experiments assessing the toxicity of biogenic ASM: (x) no mineral control 1, (+) no mineral control 2, (◇) 5.6 mg As/g VSS, (□) 13.1 mg As/g VSS, (○) 90 mg As/g VSS, (Δ) 341 mg As/g VSS. The horizontal grey lines in panel A and B represent the theoretical concentration of CH₄ that can be produced. **(C)** Normalized methanogenic activity as a function of the concentration of the measured mg As/g VSS: (□) commercial As₂S₃, (○) biogenic ASM, the IC₅₀ are shown in the graph; **(D)** Measured total soluble As concentration in the experiments for the commercial As₂S₃ (empty bar) and for the biogenic ASM (grey bar). The treatment name as 0 is for the control lacking mineral addition. The names T1-T4 represent the different treatments with increasing mineral concentration. The horizontal grey line represents the calculated IC 50 for AsIII for acetoclastic methanogenic and the original sludge (16 μM).

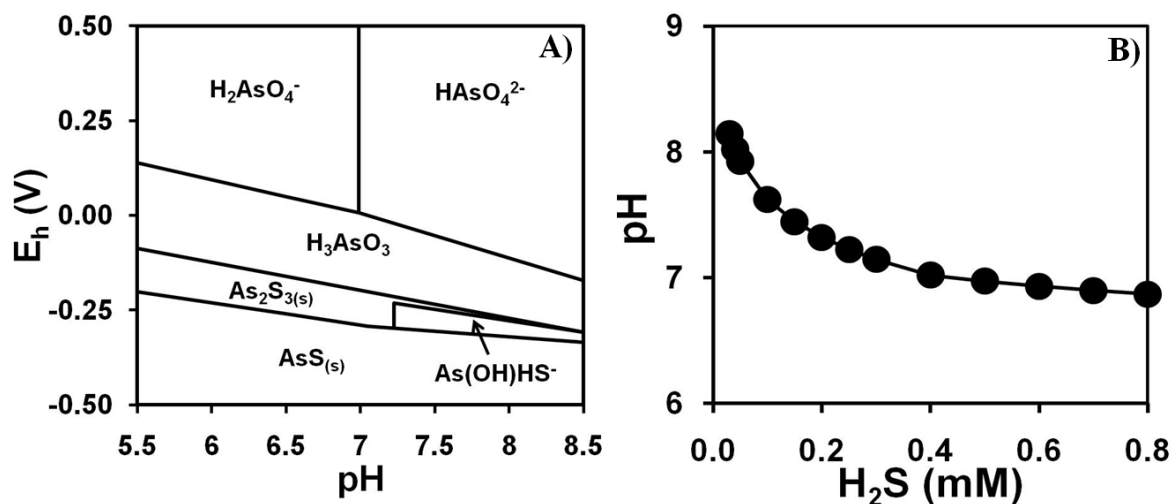


Fig. C.8. Predicted stable mineral and aqueous phases at equilibrium, including thioarsenite species. (A) Pourbaix diagram for As minerals at 25°C and 1 atm showing the stability fields for solid phases at the conditions the orpiment and realgar, in a solution with 0.5 mM of As and 0.25 mM of S. (B) Minimum pH enabling formation of thioarsenite species as a function of the H_2S equilibrium concentration, ranging from 0 to 0.75 mM (Rodriguez-Freire et al. 2014).

APPENDIX D: SUPPLEMENTARY MATERIAL CHAPTER VII:
ARSENIC MOBILIZATION FROM SULFIDE MINERALS IS HIGHLY
AFFECTED BY THE PH BUT NOT BY THE REDOX CONDITIONS OF THE
ENVIRONMENT

C1. Figures

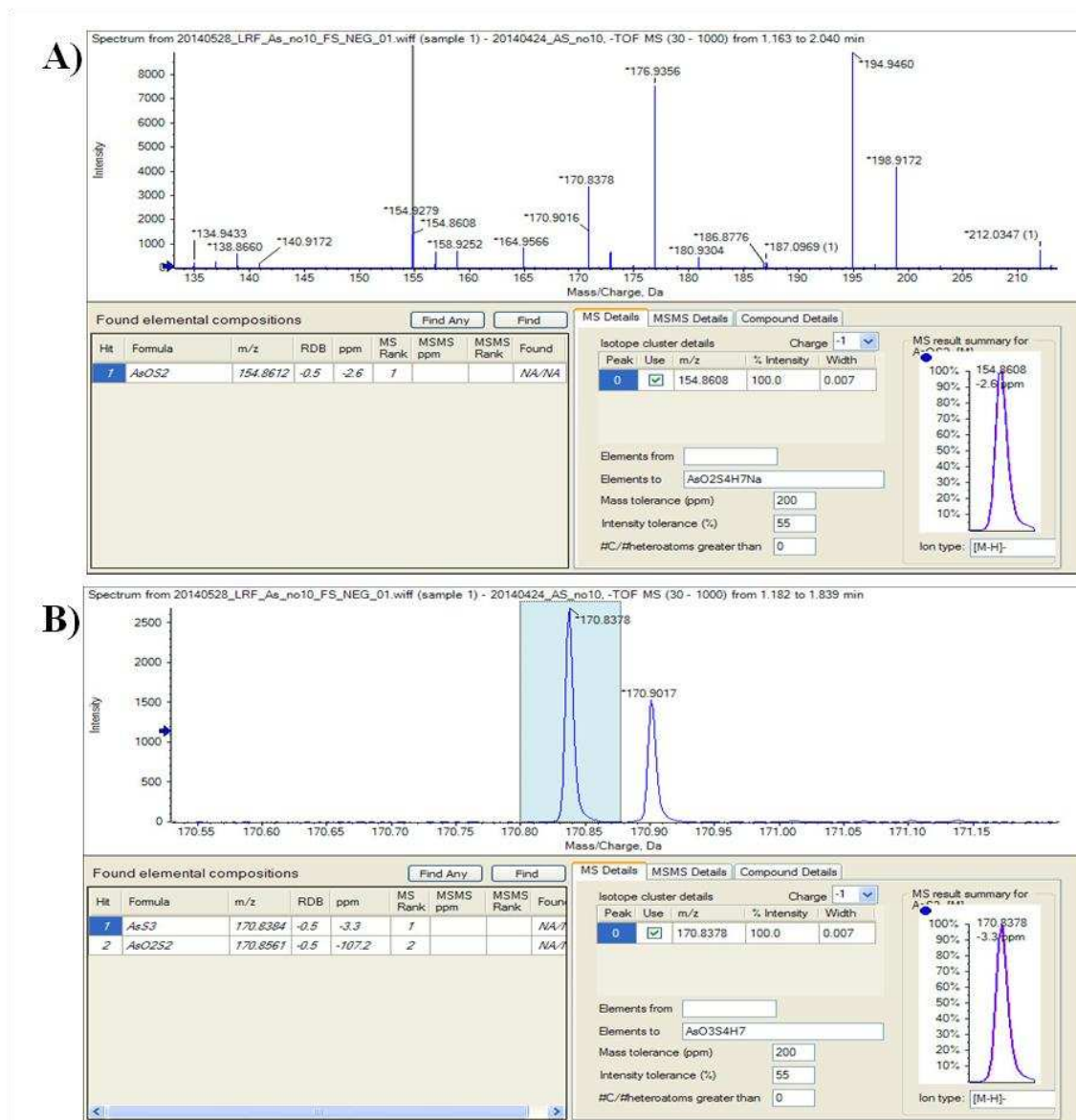


Fig. C.1. Qualitative evidence of the formation of thioarsenical compounds during the dissolution of ASM at pH 7.5. Mass spectrum of a sample from reactor 1 taken 7 d after the pH was increased to 7.5 showing the presence of As(OH)S₂²⁻ (A) and AsS₃³⁻ (B).

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