EXPLORING THE METAL-MICROBE INTERFACE USING ADVANCED MASS SPECTROMETRY TECHNIQUES

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science and Engineering

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Abstract

Exploring the metal-microbe interphase using advanced mass spectrometry techniques

Rebeca Lopez Adams
Doctor of Philosophy
The University of Manchester, 2019

Arsenic (As) is a poisonous metalloid that is persistent in many soils and aquifers worldwide. This element has a high affinity for iron (Fe) and sulfur (S) minerals, and in anoxic environments As is thought to be mobilised through the oxidation and reduction of these minerals, where bacterial metabolisms are directly involved. In these conditions some bacteria derive energy from arsenate [As(V)] respiration, releasing arsenite [As(III)], the more toxic and mobile As oxyanion; other bacteria catalyse the reverse reaction, where As(III) is used as an electron donor and oxidised to the least mobile As(V). Electron microscopy and metagenomics-proteomics are the tools conventionally used to study these systems, however, they are limited to spatially correlate metabolically active microorganisms. Thus, the bacterial mechanisms of As and Fe oxidation/reduction remain poorly understood. In this project, model As and Fe reducing/oxidising bacteria were analysed using nanoscale secondary ion mass spectrometry (NanoSIMS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) for the first time. These techniques were selected due to their high spatial resolution, high sensitivity and accurate isotope ratio analysis, where stable isotope probing was used to image metabolically active bacteria using NanoSIMS.

The model As(V) and Fe(III)-reducing bacteria, Geobacter sulfurreducens and Shewanella sp. strain ANA-3, were assessed in order to evaluate As mobilisation from sorbed As(V) on an Fe(III)-oxyhydroxide mineral. Scanning electron microscopy (SEM) and NanoSIMS allowed the inference of the predominant extracellular electron transport (EET) mechanisms, where G. sulfurreducens requires direct cell-mineral contact, in contrast to S. ANA-3 that uses flavins as electron shuttles. Additionally, a multistep As mobilisation mechanism was proposed for S. ANA-3. ToF-SIMS was further used for the identification of key molecules involved in the EET process in S. ANA-3, although single cells could not be imaged and biomarkers, for instance from flavins, were scarcely detected. Furthermore, two recently isolated denitrifying As(III) and Fe(II)-oxidising bacteria, Acidovorax sp. strain ST3 and Paracoccus sp. strain QY30, were studied as well as their biominal products. In these systems high concentrations of As(III) were removed via the formation of Fe(III) precipitates, and quantitatively analysed using transmission electron microscopy and energy dispersive X-ray spectroscopy (S/TEM-EDS) complementary to NanoSIMS, SEM and X-ray diffraction analysis (XRD). Periplasmic encrustation and cell surface coating with Fe minerals were also identified. Moreover, single cells of both As-Fe reducing/oxidising systems were depth-profiled in NanoSIMS, where the subcellular As and Fe spatial distributions were imaged and modelled in 3D.

This multi-technique approach successfully analysed active As and Fe respiring bacteria at the nanoscale, and NanoSIMS could rapidly become an established imaging tool used in Geomicrobiology and diverse redox systems.
The author declares that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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About the Author

The author obtained a BSc degree in Biochemical Engineering from the Technological Institute of Tijuana (Tijuana, Mexico) in 2011. After graduating, she worked in the Biomedical Industry for 6 months before starting her Master of Science studies in Biotechnology and Bioengineering in 2012, at the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV, Mexico City). After graduating from her MSc in 2014 she took a scientific break before starting this PhD in 2015.
This thesis is presented in the journal format. It is comprised of one published article (Chapter 4) presented in the published journal format, and two manuscripts (Chapters 5 and 7) which are presented in a format suitable for publication, as outlined below, additional to a manuscript of unpublishable results presented as a report (Chapter 6). Note that the figures and tables in the manuscripts presented in Chapters 5 and 7 are presented next to the appropriate text to ease readability, including the supporting information, which is presented after each Chapter.

**Chapter 4- NanoSIMS Imaging of Extracellular Electron Transport Processes During Microbial Iron(III) Reduction (research article 1)**


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**Chapter 5- A Single-cell Approach to Elucidate the Mobilisation Mechanism of Adsorbed Arsenate in *Shewanella* sp. Strain ANA-3 (research article 2)**

Lopez-Adams, R., Moore, K.L, Lloyd, J.R. and Lyon, I.C.

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Chapter 7- Elucidating Iron Biomineralisation Patterns in Denitrifying As(III)-Oxidising Bacteria: Implications for Arsenic Immobilisation (research article 3)

Lopez-Adams, R., Moore, K.L., Lyon, I.C., Fairclough, S.M., Zhao, F. and Lloyd, J.R.

R. Lopez-Adams: Principal author, experiment design, sample preparation, geochemical analysis, SEM, XRD and NanoSIMS data collection and analysis
K.L. Moore: NanoSIMS support and manuscript review
I.C. Lyon: NanoSIMS support and manuscript review
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F. Zhao: Concept development and manuscript review
J.R. Lloyd: Concept development and manuscript review
1.1 Research context

The general aim of the research described in this thesis was to understand the mechanisms of arsenic and iron redox transformations mediated by bacteria in the environment. The research described here was an interdisciplinary study that combined geomicrobiology, geochemistry and imaging approaches, in particular two secondary ion mass spectrometry techniques. These techniques, in contrast to conventional imaging methods, such as SEM, allow the location of microbes that are actively reducing or oxidising As-Fe minerals. Even though there is a developing consensus on the types of microorganisms and their mechanisms involved in As mobilisation, important questions persist. Experiments using bacteria that reduce or oxidise As and Fe were designed in order to identify the underpinning components of these electron transfer processes. The knowledge gained on what controls these microbial arsenic and iron metabolisms at a cellular scale could be used to predict their impact in bioremediation.

1.2 Thesis outline

This thesis comprises eight chapters of which four (Chapters 4 to 7) contain the main findings of the experiments designed and undertaken, preceded by a literature review (Chapter 2) and a methodology section (Chapter 3), where the sample preparation and analytical techniques are explained in detail. This thesis concludes with Chapter 8 in which a summary and possible future work paths are presented.

Chapter 2: Literature review. This chapter contains the literature review and gives an overview of what is known and what remains to be determined in the research field. Arsenic and iron transformations mediated by bacteria in the environment are at the centre of this chapter as well as the abiotic factors that influence these processes. This chapter also gives an overview of secondary ion mass spectrometry to illustrate why it is suitable to answer the posed research questions.

Chapter 3: Methodology. This chapter explains in detail the preparation of the experiments and all the analysis performed, including experimental set-up, sample preservation as well as the analytical techniques used in this research.
Chapter 4: Research article I. This chapter contains the published article titled “NanoSIMS imaging of extracellular electron transport processes during microbial iron(III) reduction”. A novel experimental set-up was designed and nanoscale secondary ion mass spectrometry (NanoSIMS) was applied to study the reductive dissolution of Fe(III) and arsenate [As(V)] at high spatial resolution, comparing two model bacteria, Geobacter sulfurreducens and Shewanella sp. strain ANA-3. The aim was to understand more about their extracellular electron transport mechanisms. Active cells were detected by their $^{13}$C accumulation in NanoSIMS and studied regarding their location with the Fe(III) mineral they were respiring. Active G. sulfurreducens cells showed direct contact with the Fe(III) mineral and an enrichment of presumably As(V) around the cells was assessed, given that G. sulfurreducens does not respire As(V). Active S. ANA-3 cells showed no direct contact with the Fe(III) mineral they were respiring, inferring an endogenous electron shuttle was being used in this system. This article presented evidence of the predominant Fe(III) reduction mechanism in both bacterial strains tested, indicating that NanoSIMS could be applied to investigate other environmentally relevant redox systems, for instance, manganese or sulfur transformations by bacteria.

Chapter 5: Research article II. This chapter contains the manuscript for the research article titled: “A single-cell approach to elucidate the mobilisation mechanism of adsorbed arsenate in Shewanella sp. strain ANA-3”. This article follows on from the previous chapter and uses NanoSIMS to study the electron transfer mechanism in the Fe(III) and As(V) respiration by S. ANA-3, contrasting it with a mutant strain that does not respire As(V). The aim of this article was to map the subcellular distribution of Fe and As alongside complementary aqueous phase analysis, in order to infer the bacterial mobilisation mechanism of this trace element. In this study the central questions were: what happens to As(V) during reductive Fe(III) dissolution? Is As(V) reduced and remains in solution as arsenite [As(III)] or does it resorb in these subsurface conditions? How and where does the As(V) respiratory reductase gain access to sorbed arsenate? And how far can a bacterial cell be from the Fe(III) mineral to transfer electrons? Both strains of S. ANA-3 mobilised considerable amounts of As, although only the As(V)-respiring strain mobilised As(III). NanoSIMS depth-profiles of single-cells revealed the subcellular association of As and the preferential affinity of As(III) for the cell surface in the As(V)-respiring S. ANA-3 strain. A multistep mechanism of As mobilisation by S. ANA-3 in these conditions is proposed.
Chapter 6: Molecular SIMS analysis of the Fe(III) respiration by *Shewanella* sp. strain ANA-3. This chapter contains the preliminary time-of-flight secondary ion mass spectrometry (ToF-SIMS) work performed on samples of *S. ANA-3* respiring an Fe(III)-oxyhydroxide mineral. The hypothesis was that ToF-SIMS would allow the detection of biomarker signals from flavins, cytochromes/proteins and flavocytochromes and, under certain settings, it would be possible to allocate the identified biomarkers to single-cells. Analysis of ToF-SIMS spectra can be challenging, and for this reason tests with reference materials were performed prior to the analysis of the samples in order to determine the significant peaks that could be yielded by this technique. Two ToF-SIMS instruments were used (one equipped with C$_{60}^+$ and one with a Au$_n^+$ ion beam), generating different spatial resolutions and secondary molecular patterns, and these differences are discussed.

Chapter 7: Research article III. This chapter presents the article manuscript titled: “Elucidating iron biomineralisation patterns in denitrifying As(III)-oxidising bacteria: implications for arsenic immobilisation”. This manuscript presents the work undertaken on the recently isolated *Acidovorax* sp. strain ST3 cultured under denitrifying conditions in order to assess simultaneous As(III) and Fe(II) oxidation, as a mechanism proposed for the immobilisation of As(III), by transforming it to As(V). In this study the following research questions were investigated: Is Fe(II) oxidation an enzymatic or indirectly biotic mechanism? Where is Fe(III) mineralised and what is the effect of this Fe mineralisation on the cells? What kind of minerals are produced and how effective are these biominerals for As removal? NanoSIMS was used to study the active cells (again determined by mapping $^{13}$C) and the relationship with biomineralisation of cells, as well as to study the As and Fe distribution on cells and biominerals. Scanning transmission electron microscopy (STEM) was used to characterise the biominerals either on the surface of cells or extracellularly. Complementary powder X-ray diffraction (XRD) was performed to characterise the bulk biominerals. The results showed that most cells were encrusted with Fe minerals at the end of the incubation and the cells that were metabolically active only showed low levels of encrustation, implying that high levels of encrustation are deleterious for ST3 cells. XRD identified lepidocrocite (Fe$^{3+}$OOH) as the main biomineral produced, whereas STEM helped to identify the periplasm and cell surface as the sites of mineralisation. Additionally, two biomineral morphologies were
identified, amorphous nanoparticles and crystalline “flakes”, where the former accumulated 20-30 % of As (normalised to Fe) and the latter only accumulated 2-10 % of As/Fe. This indicates that the amorphous nanoparticles were better hosts for As sequestration.

Chapter 8: Conclusions and future work. In this work the key findings of all the previous chapters are summarised as well as the environmental implications and possibilities of future research paths that could be pursued to deepen our understanding of As and Fe transformations by bacteria in natural locations.

Appendix A. This appendix contains results on the preliminary TEM and NanoSIMS work done with the chemolithoautotrophic As(III)-oxidising and denitrifying bacterium Paracoccus sp. strain QY30.
2.1 Arsenic in the environment and health risk

Arsenic (As) is a “popular” poison in history whose toxicity has been well-known for centuries. Its name derives from the greek word “arsenikon” meaning “potent”, although its toxicity greatly depends on its solubility and oxidation state (Bowell et al., 2014). Arsenic is a ubiquitous element that naturally occurs in rocks, sediments and groundwater, although generally at low abundance. Arsenic is present in many geological environments including sedimentary basins and is typically associated with geothermal waters and hydrothermal mineral deposits. This toxic element has recently gained recognition as one of the most important water pollutants due to its prevalence in aquifers in many countries including Chile, Argentina, Mexico, USA, China, Mongolia and many regions in South East Asia, in particular East India, Vietnam, Cambodia and Bangladesh. In fact, the World Health Organisation (WHO) considered the groundwater As pollution in Bangladesh as “the largest mass poisoning of a population in history” (Argos et al., 2010). The WHO guidelines recommend a maximum of 10 µg mL⁻¹ or ppb in drinking water, although it is estimated that currently more than 150 million people worldwide are exposed to levels of As above 50 ppb in drinking water, severely endangering their health by long-term exposure to this toxic element (Abdul et al., 2015; Singh et al., 2015). Chronic exposure to arsenic has been linked to different disorders in the skin, digestive, respiratory, cardiovascular, endocrine, renal, neurological and reproductive systems, and at more advanced stages can lead to organ failure and different types of cancer (Vishnoi et al., 2014). This environmental and health crisis mainly originated by natural processes, where the As is believed to be originally trapped in buried sediments before being dissolved into the water that was destined for human consumption (Fig. 2.1) (Polya and Charlet, 2006; Lloyd et al., 2011). However, humans have also contributed to the mobilisation of As in the environment, particularly through the use of pesticides and herbicides in agriculture, by the combustion of fossil fuels and through mining and mineral processing (Bowell et al., 2014).

Arsenic exists in four oxidation states: arsenate (As⁵⁺), arsenite (As³⁺), elemental arsenic (As⁰) and the gas arsine (As⁻³), although it is predominantly found in its inorganic forms
arsenite (H₃AsO₃⁻) and arsenate (ocean: HAsO₄²⁻ / fresh water: H₂AsO₄⁻), where their solubility is affected by pH (Singh et al., 2015). Arsenate is more thermodynamically stable in aerobic environments, whereas in anoxic environments arsenite is expected to be the predominant oxyanion (Cullen and Reimer, 1989).

Arsenate is structurally analogous to phosphate and can therefore enter living cells via transporters meant for the uptake of this nutrient, once within the cell it can replace phosphate in biochemical reactions, for example in adenosine 5’-triphosphate (ATP) production. Arsenite is generally more soluble and enters the cells through aquaglyceroporins (water and glycerol channels), reacting with thiol groups in proteins, inhibiting their activity and interfering with a wider variety of metabolic processes, and for this reason arsenite is considered the most toxic form of As, representing an even bigger health hazard than arsenate (Shen et al., 2013).

2.2 Bacterial reduction of As and Fe

2.2.1 Arsenic and iron minerals

Arsenic is a component of more than 500 known minerals (Bowell et al., 2014). Iron (Fe) oxides and sulphide minerals accumulate As, either as part of their structure or adsorbed to their surfaces, most notably in the ores arsenopyrite (FeAsS), orpiment (As₂S₃) and...
realgar (As$_4$S$_4$) (Bowell et al., 2014). Iron is the second most abundant metal in the earth’s crust just after aluminium and is a necessary element for practically all life forms, even though it is usually required in low concentrations. Physicochemical conditions such as pH, redox potential and oxygen availability determine the chemical behaviour of Fe in the environment, where Fe$^{2+}$ and Fe$^{3+}$ are the most common oxidation states (Hedrich et al., 2011). Ferric iron (Fe$^{3+}$) predominantly occurs in oxygen rich conditions and ferrous iron (Fe$^{2+}$) mainly occurs in anoxic environments; Fe$^{2+}$ is labile to spontaneous oxidation by oxygen (Hedrich et al., 2011).

Arsenate and arsenite have different sorption rates to Fe mineral surfaces. Arsenate sorbs more readily to Fe(III) oxides, making it the generally least mobile oxyanion. As(V) tends to interact more strongly with Fe(III) than As(III) with Fe(III), but this depends on the pH and reducing conditions. Redox potentials are greatly influenced by bacteria degrading organic matter and coupling this oxidation to the reduction of available oxidants (e.g. Fe(III), Mn(IV), O$_2$, NO$_3^-$, etc.) (Cullen and Reimer, 1989).

2.2.2 Microorganisms that respire Fe and As

Microorganisms have evolved mechanisms to transfer electrons from sources that are impossible to access to other life forms. These processes allow the microorganisms to trap, store and release energy by intracellular and extracellular mechanisms (Weber et al., 2006), but in the subsurface and sediments, where oxygen is absent or depleted, bacteria and archaea harness other molecules to conserve energy. Fe minerals are one example of a hard-to-access energy source that bacteria and archaea have used for billions of years to scavenge energy (Lloyd, 2003). Microbiological and geochemical evidence suggest that Fe(III) reduction was the first form of respiration in the early earth (Vargas et al., 1998; Lloyd, 2003), as opposed to sulfur respiration, formerly thought to be the first form of respiration (Lovley et al., 2004). Dissimilatory reduction of ferric iron is mediated by bacteria and archaea that couple Fe(III) reduction to the oxidation of organic matter in the absence of oxygen, conserving energy in this electron transfer process (Lovley and Phillips, 1988). During this process Fe is not transported into the cells nor incorporated into proteins (Lovley, 2002).

Microorganisms have evolved multiple strategies to cope with As toxicity. These strategies include: (i) minimizing the amount of As entering the cell by increasing the specificity of phosphate uptake, (ii) methylation with production of monomethylarsinic
acid and dimethylarsinic acid, (iii) volatilisation through the production of arsines, and (iv) reduction of arsenate and pumping out the reduced arsenite, via the ArsDABC reductase/transport system. As(V) reduction is extended in bacteria and yeast (Cullen and Reimer, 1989; Oremland and Stolz, 2003), although the arsenate-resistant microbes do not gain energy from the reduction of As(V) to As(III), but use it as a means of detoxification to deal with high levels of arsenic in the environment (Oremland and Stolz, 2005). A restricted group of prokaryotes can reduce arsenate and couple this reduction to the oxidation of organic matter to generate energy, this group of microbes is known as the dissimilatory arsenate-reducing prokaryotes (DARPs) (Oremland and Stolz, 2003). The As(V)-respiring microorganisms can typically use a wide range of electron acceptors other than arsenate, and this could explain why no obligate DARPs have been found (Oremland and Stolz, 2003). Some bacteria have both respiratory and detoxifying arsenate systems (Oremland and Stolz, 2003).

2.2.3 Pioneering work in dissimilatory arsenate-reducing prokaryotes (DARPs)

Pioneering work studying DARPs was done in sediments from Mono Lake, a saline lake in California, USA (Dowdle et al., 1996). Arsenate respiration was first observed in anoxic incubations of these sediments where the rate of reduction of As(V) to As(III) was enhanced by the addition of electron donors. A competitive effect between nitrate (NO$_3^-$) and arsenate as potential electron acceptors was also observed in early works, suggesting a separate enzymatic system for the reduction of As(V) and NO$_3^-$ (Oremland et al., 2000). NO$_3^-$ is a thermodynamically more favourable terminal electron acceptor, although Mono Lake has low NO$_3^-$ abundance, and supports the use of As(V) as the most abundant soluble terminal electron acceptor for bacterial respiration in this unique environment (Oremland et al., 2000). Further work performed under similar extreme salinity conditions revealed the unequivocal role of bacteria in the transformations of As and the implications of dissimilatory metal-reducing bacteria, not only for As but also for other abundant metals like Fe and Mn and for carbon cycling in sediments. *Sulfitobacter barnesii* was isolated from Mono Lake sediments, and its metabolism was further studied in incubations with As(V) in solution or As(V) sorbed to ferrihydrite (an amorphous Fe(III) mineral), producing more evidence to support the hypothesis that As(III) is present in aquifers as a consequence of the redox conditions and as the product of bacterial respiration (Zobrist et al., 2000). Further sediment and water column
incubation studies with different electron acceptors and donors revealed the metabolic diversity of the microbial communities in this As-rich environment. As(III)-oxidisers in anoxic conditions had remained unnoticed in previous studies, but As(III) oxidation to As(V) was first observed in these extreme salinity conditions, introducing another piece in the puzzle of As cycling (Hoeft et al., 2002; Oremland et al., 2002). In this closed saline lake system it was estimated that the contribution to CO₂ production by microbial As(V) respiration is up to 14 %, an effect that had been underestimated for DARPs and which has important implications for organic matter oxidation and carbon cycling (Oremland et al., 2004). Subsequent studies on Mono Lake bacteria suggest that different species are performing different tasks given the elements and environmental conditions, where some utilise organic carbon (chemotrophs) while others use inorganic carbon (chemoautotrophs) coupled to the reduction or oxidation of As, overall contributing to an As cycle where bacteria play an essential role (Oremland and Stolz, 2003). Therefore, the biogeochemical cycle of As is complex and involves its two main oxidation states and their biotic and abiotic transformations in the environment.

The mechanism of dissimilatory As(V) respiration was eventually characterised and the enzyme ArrA was identified as the reductase responsible for this reduction. The arsenate reductase from Chrysiogenes arsenatis was the first arsenate reductase to be fully characterised (Stolz and Oremland, 1999). Phylogenetically distant microorganisms are capable of As(V) respiration, although the fully characterised arsenate reductases to date have shown high identity, in particular the reductases from Bacillus selenitireducens, Shewanella sp. strain ANA-3 and Desulfitobacterium hafniense (Oremland et al., 2004).

2.2.4 The respiratory diversity of the Shewanella genus

The Shewanella genus is comprised of approximately 40 known species of Gram-negative facultative anaerobes characterised by their capacity to respire a wide variety of terminal electron acceptors in addition to oxygen, including nitrate, fumarate, Fe(III) minerals, manganese-Mn(IV), As(V) and radionuclides such as uranium-U(VI) or technetium-Tc(VII) (Hau and Gralnick, 2007; Fredrickson et al., 2008). This respiratory diversity confers advantages to these organisms commonly found in aquatic and sedimentary environments (Hau and Gralnick, 2007).
2.2.4.1 *Shewanella* sp. strain ANA-3: a model As(V)-respiring organism

*Shewanella* sp. strain ANA-3 has two systems to cope with As(V): the As(V) detoxification system and the dissimilatory As(V) respiratory system. The As(V) detoxification system, encoded in the *ars*DABC operon, as mentioned previously, confers As(V) resistance and is advantageous for the cells in the presence of arsenic; it can be active in both aerobic and anaerobic conditions (Saltikov et al., 2003). The *ars* operon contains a cytoplasmic arsenate reductase (*ArsC*), an arsenite efflux pump (*ArsB*), an ATPase (*ArsA*) that interacts with *ArsB* to extrude As(III), and an *ars* gene expression regulator (*ArsD*) (Saltikov and Newman, 2003; Saltikov et al., 2005). The *S*. ANA-3 As(V) respiration system, a two-gene cluster termed *arrAB* located in the periplasm, contains the reductase *ArrA* that anaerobically binds As(V) and reduces it to As(III), coupling this reduction to the oxidation of an electron donor in an energy conserving process. *ArrB* is an electron transporter protein that conducts electrons derived from *c*-type cytochromes (*c*-type cytochromes); see Figure 2.2 for a schematic representation of these enzymes in Gram-negative bacteria. Both gene subunits are essential for As(V) respiration (Saltikov and Newman, 2003; Saltikov et al., 2005). *S*. ANA-3, like all *Shewanella* species, preferably oxidises lactate to acetate via a lactate dehydrogenase and the electrons produced in these reactions are transferred to the *ArrAB* enzyme cluster (van Lis et al., 2013). In this bacterium, the *arr* operon is induced in the presence of nanomolar concentrations of As(V), whereas the induction of the *ars* operon requires approximately 1000 times more As(V) to induce this gene transcription. The accumulation of As(III) induces the expression of the *ars* detoxification system, which appears to be a protection mechanism at high concentrations of As(III); however, the *ars* operon is not essential for As(V) respiration (Saltikov et al., 2003; Saltikov et al., 2005; Malasarn et al., 2008). Furthermore, the X-ray crystal structure of the *arr* reductase complex of *S*. ANA-3 was recently obtained. In this two-subunits protein, phosphate was predicted to affect As(V) reduction via competitive enzymatic inhibition, but it was determined that phosphate likely exerts a greater effect by promoting As(V) desorption, given that the arsenate reductase *ArrA* could bind phosphate but has a higher affinity for arsenate (Glasser et al., 2018).
2.2.5 Extracellular electron transfer in _Shewanella_ species

Most of the preferred electron acceptors used by prokaryotes (oxygen, sulfate and carbon dioxide) are soluble, while environmental Fe(III) exists predominantly as poorly soluble oxyhydroxides or oxide minerals at circumneutral pH. Thus, in anoxic environments, Fe(III)-respiring bacteria must cope with the poor solubility of Fe(III) minerals. (Nevin and Lovley, 2002). The first example of dissimilatory microbial Fe(III) reduction coupled to organic matter oxidation dates back a few decades, when the strictly anaerobic Gram-negative bacterium _Geobacter metallireducens_ (formerly known as strain GS-15) was isolated, where the authors proposed the role of reductases to explain Fe(III) reduction (Lovley and Phillips, 1988). It is now clear that membrane-associated multiheme c-type cytochromes play a fundamental role in electron transfer from the cytoplasmic membrane to the outer membrane and cell surface of dissimilatory metal-reducing bacteria, in the presence of insoluble terminal electron acceptors (Shi et al., 2007). However, not all Fe(III)-reducing microorganisms require direct contact with the Fe(III) mineral they are respiring (Nevin and Lovley, 2002).
*Shewanella* species can reduce poorly soluble Fe(III) minerals at a distance; this means that direct cell-mineral contact is not required (Nevin and Lovley, 2002). These cells have developed mechanisms to transfer electrons from multiheme c-type cytochromes in the outer cell membrane to the surface of a suitable terminal electron acceptor. There are four proposed mechanisms for the reduction of poorly soluble Fe(III) minerals by *Shewanella* species: indirect electron transfer i) via an endogenous or exogenous electron shuttle (e.g. flavins or humic acids), or ii) through chelators which dissolve the Fe(III), followed direct electron transfer, or by iii) direct mineral contact through the multiheme c-type cytochromes on the outer membrane or iv) through electrically conductive nanowires, assumed to be outer membrane (OM) protein extensions. However, more than one mechanism could be at play at the same time (Coursolle et al., 2010).

*Geobacter* species appear to be one of the most abundant species in Fe(III)-reducing environments (Lovley et al., 2004) although the mechanism used by *Shewanella* species, where no direct contact with Fe(III) minerals is required, may be advantageous, for instance, when accessing minerals trapped in tight pore spaces in sediments or during growth in biofilms, where there are higher microbial densities and heterogeneous transfer of nutrients (Lovley et al., 1998; Nevin and Lovley, 2002).

### 2.2.5.1 Electron shuttle for the reduction of insoluble Fe(III) by S. ANA-3

Over a decade ago evidence of an endogenous molecule produced by *Shewanella* cells was first presented, and the presence of a chelator or an electron shuttle as the potential pathway for Fe(III) reduction at a distance was proposed (Lies et al., 2005). In a work by von Canstein et al. two flavins were identified for the first time as the electron shuttles used by different *Shewanella* species. Flavin mononucleotide (FMN) and riboflavin were both secreted, whereas flavin adenine dinucleotide (FAD) was produced intracellularly but not released to the medium (von Canstein et al., 2008). Flavins secreted by planktonic cultures ranged between 0.1 to 0.6 µM/g of cell protein in this work, much lower than what has been reported for other microorganisms, but they showed to be effective for Fe(III) reduction at nanomolar concentrations, moreover, it has been observed that above 1 µM the rate of Fe(III) dissolution is not enhanced. Flavins were produced in both aerobic and anaerobic cultures of *Shewanella* cells, which may appear as an energetic waste, but the flavins produced during aerobic conditions are possibly
used during anaerobic respiration, given that *Shewanella* species are typically found in the oxic/anoxic interphase in the environment (von Canstein et al., 2008). The reason why FMN and riboflavin are secreted extracellularly, in contrast to FAD, could be related to a membrane transporter specific for FMN and riboflavin that excludes FAD (von Canstein et al., 2008), although the mechanism by which flavins cross the membrane (secretion) is still unknown. In a work conducted by Covington et al. to investigate the mechanism of flavin secretion in *S. oneidensis*, it was found that FAD is first exported from the cytoplasm to the periplasm where UshA (a nucleotidase) hydrolyses FAD, forming FMN and adenosine monophosphate (AMP), FMN then diffuses outside of the cell, through OM porins, where a fraction is also hydrolysed to riboflavin in the medium (Covington et al., 2010). When *Shewanella* sp. MR-1 and MR-4 are growing in biofilms on electrodes (used as terminal electron acceptors), riboflavin is the predominant flavin secreted and accumulated in a range of 0.25-0.50 µM (Marsili et al., 2008). As noted above, the biosynthesis of these biomolecules can seem energetically costly, but in this work it was estimated to be <0.1 % of the cell’s ATP budget (Marsili et al., 2008). Overall, the metabolic cost of producing electron shuttles is considered to be minimal for microbial communities growing in redox limited conditions (Glasser et al., 2017). Flavins can also act as chelators (the isoalloxazine ring can chelate iron), but evidence strongly suggests that electron shuttling is the main mediator in Fe(III) oxide reduction. This is supported by the fact that *Shewanella* cells can transfer electrons to surfaces that cannot be chelated, such as electrodes, and known chelators have lower Fe(III) reduction rates than flavins (Covington et al., 2010). The reasons why the secretion of flavins had been overlooked as mediators of Fe(III) reduction in previous studies could be their light sensitivity and the fact that flavins easily bind to filters (used in filter-sterilisation), moreover, flavins are effective at very low concentrations (nM) and their characteristic yellow colour is only visible when flavins are oxidised and present at concentrations above 1µM (Marsili et al., 2008).

**2.2.5.2 Free flavins or cytochrome-bound flavins?**

The proposed electron shuttling mechanism was challenged when Okamoto et al. tested the effect of free flavins with reduced flavins bound to OM c-type cytochromes (MtrC) in biofilms of *S. oneidensis* MR-1. It was observed that bacterial respiration electrons are transferred to electrodes primarily via oxidised FMN (semiquinone) bound to MtrC and
the reaction rate was $10^3$-$10^5$-fold faster than that of free flavins. These results suggest that the flavocytochrome complex (flavin-MtrC interaction) regulates extracellular electron transfer (EET) coupled to intracellular metabolic activity (Okamoto et al., 2013). Further work with new body of biochemical, genetic, spectroscopic and structural evidence supported these observations, suggesting that flavins can bind to cytochromes, forming flavocytochrome complexes (MtrC-flavin) under anaerobic conditions that are involved in EET (Edwards et al., 2015). This complex was observed to rapidly lose the flavin cofactor upon exposure to oxygen, implying that this complex is oxygen-sensitive. In the presence of oxygen a complex like this could be harmful for the cells because it would increase the production of reactive oxygen species, increasing cellular oxidative damage (Edwards et al., 2015). Xu et al. also investigated the role of free versus cytochrome-bound flavins in extracellular electron transfer of *S. oneidensis* MR-1 using electrodes as final electron acceptors and concluded that free flavins did not contribute to EET from MR-1 to electrodes, implying that the current observed for MR-1 was mediated by cytochrome-bound flavins that function as redox cofactors (Xu et al., 2016). It was only until recently that flavins were finally acknowledged as the main mediators of poorly soluble Fe(III) mineral reduction in *Shewanella* species (except for *S. denitrificans* that lacks the Mtr respiratory pathway genes) (Brutinel and Gralnick, 2012), and it has been estimated that electron shuttling accounts for up to 75 % of the respiratory capacity in the model bacterium *S. oneidensis* under laboratory conditions. The remaining EET may be accomplished by direct contact of OM c-type cytochromes with the mineral surface (Kotloski and Gralnick, 2013).

![Figure 2.3. Riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) structures. Obtained from (Abbas and Sibirny, 2011).](image-url)
2.2.5.3 Electron conductive nanowires in *Shewanella*

One of the proposed models for long-range electron transfer from *Shewanella* cells was thought to be through electron conductive nanowires, analogous to *Geobacter* nanowires (Malvankar et al., 2014; Adhikari et al., 2016; Reguera, 2018). The hypothesis proposed a mechanism of electron hopping through nanowires, that more than pilin-based were thought to be extensions of the OM and periplasm, containing OM cytochromes (Pirbadian et al., 2014). This theory has been recently questioned given that the cytochromes in these nanowires are too distanced to support electron transfer or electron hop from cytochrome to cytochrome, and rather, diffusion with electron shuttles and direct electron hopping might explain the transfer of electrons in these nanowires (Subramanian et al., 2018).

2.2.5.4 Humic substances as mediators of Fe(III) and As(V) reduction

Humic substances (HS) are complex high molecular weight substances present in aquifers and soils. These substances are recalcitrant and were previously thought to not be involved in microbial metabolisms. However, the capability of HS to actively transfer electrons in the environment in interactions with a diversity of bacteria and simple organic molecules has been documented (Lovley et al., 1996), where the quinones present in HS are thought to be the sites for electron transfer (Lovley et al., 1998; Scott et al., 1998). In the proposed mechanism, bacteria reduce the HS coupling this reduction to the oxidation of simple organics such as acetate, which is fully oxidised to CO$_2$ (Fig. 2.4). The reduced HS can further reduce insoluble minerals such as Fe(III)-(oxyhydr)oxides, implying that HS are shuttling electrons in an energy conserving mechanism for the cells under anoxic conditions.

Furthermore, the quinone fraction in HS is redox active and can effect As speciation. The oxidised form of quinones has no direct effect, but semiquinone radicals and hydroquinones can affect As redox species, particularly As(III) (Jiang et al., 2009). This mechanism had been overlooked in studies of As mobilisation in the environment until

![Figure 2.4. Schematic representing the role of humic substances (HS) in bacteria-mediated Fe(III) reduction (Scott et al., 1998).](image)
recently; HS could be used to oxidise and inmobilise As(III) by changing its speciation to As(V). Although this process is pH dependant and the concentrations of semiquinone radicals and hydroquinones as well as As(V) and As(III) will effect this reaction. Additionally, HS have different composition in different sites and some have negligible quinone fractions (Jiang et al., 2009).

2.2.6 Intracellular electron transfer in Shewanella

The current mechanism of intracellular electron flow in Shewanella species involves the Mtr cytochrome pathway (see Figure 2.5). Electrons are transported from the menaquinone pool or the cytochrome CymA, anchored in the inner cell membrane, which are then transferred to MtrA in the periplasm. Then, electrons are translocated to MtrB, anchored in the OM, and from there, electrons are transferred to the cytochromes MtrC and OmcA located in the outer surface of the cell, where they reduce extracellular substrates, including flavins (Shi et al., 2007). Reduced flavins can transfer electrons to an insoluble electron acceptor and be re-reduced by the Mtr pathway. Flavins could easily diffuse from the cells, therefore the proposed mechanism involves the electron transport from the cytoplasm to the outer surface of the cell through the Mtr pathway, and the transport of electrons from flavin molecule to molecule (via electron hopping) to the surface of an insoluble electron acceptor (Covington et al., 2010). The specific role of all the Mtr pathway associated proteins (OmcA, MtrC, MtrA,
MtrB and CymA) in flavin reduction and electron shuttling to insoluble Fe(III) in S. oneidensis strain MR-1 were tested in a key study (Coursolle and Gralnick, 2010). In this study, flavins were discarded as chelators under these conditions and it was also proved that flavins are not consumed or destroyed, instead they may be recycled. Regarding the role of Mtr proteins in the reduction of flavins, it was determined that MtrA and MtrB are essential (Coursolle et al., 2010).

2.3 Bacterial oxidation of As and Fe

2.3.1 As(III)-oxidising bacteria

Abiotic arsenite oxidation is a slow process, but this process can be catalysed by diverse bacterial species with notable members of phylogenetically distant genera such as *Pseudomonas*, *Bacillus*, *Achromobacter* and *Alcaligenes*. However, most of these heterotrophic bacteria have been found to oxidise arsenite for detoxification purposes, using oxygen as a terminal electron acceptor, and not as a means to derive energy (Weeger et al., 1999).

As(III) is the predominant As species in acid mine waters, where heterotrophic As(III)-oxidisers are significant for the detoxification of this environment from this oxyanion (Miot et al., 2009a). Santini et al. isolated the first chemolithoautotrophic As(III)-oxidiser, from a gold mine in the Northern Territory of Australia, *Rhizobium* sp. strain NT-26, which showed rapid growth using As(III) as electron donor and CO₂ as carbon source (Santini et al., 2000). Strain NT-26 was the first characterised bacterium to conserve energy from the oxidation of arsenite and not as a detoxification mechanism.

Anaerobic As(III) oxidation with nitrate as a terminal electron acceptor was first observed in incubations of bottom waters of Mono Lake, California (Hoeft et al., 2002), which resulted in the isolation and characterisation of the facultative chemoautotroph *Alkalilimnicola ehrlichii* strain MLHE-1 (Oremland et al., 2002). This organism was found to oxidise arsenite to conserve energy.

2.3.1.2 Enzymatic arsenite oxidation

Arsenite is oxidised enzymatically through an oxidase in bacteria and transcription of this enzyme is promoted by the presence of As(III) in most of the oxidases studied (Lett et al., 2012). The first arsenite oxidase to be purified and characterised was from the heterotrophic soil bacterium *Alcaligenes faecalis* (Anderson et al., 1992). The arsenite
oxidase of *A. faecalis* is located in the outer surface of the inner membrane. The As(III) oxidase of strain NT-26, located in the periplasm, has also been characterised (Santini and vanden Hoven, 2004). This oxidase contains two subunits termed AroA (large subunit) and AroB (small subunit) which were later renamed AioA and AioB, respectively, in order to unify the nomenclature of arsenite-oxidising enzymes in bacteria (Lett et al., 2012). Dozens of organisms capable of catalysing As(III) oxidation, but metabolically and phylogenetically diverse, have been characterised along with the enzymes responsible for this oxidation (Stolz et al., 2010). Most of the arsenite oxidases studied are encoded by the *aio* operon, part of the same reductase family as the *arr* arsenate reductase: the dimethyl sulfoxide (DMSO) reductase family of molybdenum-containing enzymes (Kumari and Jagadevan, 2016). A notable exception is the As(III) oxidase from the alkaliphilic *A. ehrlichii* strain MLHE-1, with two subunits denominated ArxA and ArxB, which are phylogenetically closer to the *arr* reductase enzymes than to the *aio* oxidase enzymes (Zargar et al., 2010; Zargar et al., 2012). Nevertheless, given their widespread distribution in different As-polluted environments and its conservation in both heterotrophic and autotrophic bacteria, AioA genes could be used as markers for culture-independent identification of As(III)-oxidisers in the environment (Kumari and Jagadevan, 2016), analogous to ArrA genes as markers for As(V)-respirers (Gnanaprakasam et al., 2017).

### 2.3.2 As(III) and Fe(II) oxidation for the immobilisation of As

Arsenite transformation to the less mobile and less toxic As(V) by arsenite-oxidisers is a mechanism that has been proposed to immobilise and remove As from soils and groundwaters. This process could be more efficient if the oxidised As(V) has a suitable surface to sorb to, for example Fe(III) minerals. Indeed, Fe(II)-oxidising bacteria have been tested to perform this task, for instance, *Acidithiobacillus ferrooxidans*, precipitates Fe(III) and, contrary to the initial assumption, immobilises As as As(III), confirming that *A. ferrooxidans* is not an As(III)-oxidiser (Miot et al., 2009b). In contrast, bacteria such as *Thiomonas* sp. catalyse As(III) oxidation and precipitation with Fe(III) in acidic mine environments (Casiot et al., 2003). Neutrophilic Fe(II)-oxidising bacteria can also precipitate Fe(III) and mixed-valence As, however, As can modify the crystallinity of the Fe(III) minerals, as observed for *Acidovorax* sp. strain BoFeN1 (Hohmann et al., 2010).
Some denitrifying bacteria also possess arsenite oxidases. In the absence of oxygen, denitrifying bacteria use NO$_3^-$ as terminal electron acceptor, coupled to the oxidation of suitable electron donors (such as arsenite), during respiratory denitrification (Tiedje, 1988). These bacteria have often been isolated from As-contaminated soils after the stimulation of As(III) oxidation with addition of NO$_3^-$. Recent discoveries include the identification of the periplasmic AioAB arsenite oxidase cluster in the chemoautotroph Paracoccus sp. strain SY (Zhang et al., 2015) and in the heterotroph Acidovorax sp. strain ST3 (Zhang et al., 2017).

### 2.3.2.1 Denitrification and nitrate-dependent iron(II) oxidation (NDFO)

Denitrification is widespread in bacteria, where NO$_3^-$ is reduced to N$_2$ through a reductase enzymatic pathway comprised by the NAR, NIR, NOR and NOS enzymes (Fig. 2.6). Oxygen is a terminal electron acceptor energetically more favourable than nitrate, but upon depleting oxygen levels, the production of the nitrate reductases enzymatic pathway is triggered (Lycus et al., 2018). The current model assumes that all denitrifying bacteria synthesise NOS, but the expression of NAR, NIR and NOR is stochastic (Lycus et al., 2018). Denitrifying bacteria are thought to inherently catalyse Fe(II) oxidation, in a process known as nitrate-dependent iron(II) oxidation (NDFO). NO$_2^-$ and NO produced and accumulated during NO$_3^-$ reduction can abiotically oxidise Fe(II) (Kappler et al., 2005), and thus, considered responsible for some of the Fe(II) oxidation observed with denitrifying bacteria. Therefore, NDFO is a result of both biotic and indirectly biotic phenomena (Carlson et al., 2013).

**Figure 2.6.** The enzymatic reduction pathway from nitrate to nitrogen in denitrifying bacteria. Nitrogen species are in circles and the reductases are shown in arrows. Abbreviations: NAR, nitrate (NO$_3^-$) reductase; NIR, nitrite (NO$_2^-$) reductase; NOR, nitric oxide (NO) reductase; NOS, nitrous oxide (N$_2$O) reductase (Lycus et al., 2018).

### 2.3.2.2 Fe(II) oxidation pathways

The Fe(II) oxidation mechanism by denitrifying organisms remains poorly understood. Fe(II) is largely assumed to be oxidised by the NO$_3^-$ derived species NO$_2^-$ and NO, although enzymatic Fe(II) oxidation to conserve energy has also been proposed (Carlson et al., 2012). Moreover, Fe(II) oxidation as a detoxification mechanism, rather than an
energy yielding process, is another possible pathway (Ilbert and Bonnefoy, 2013). A stoichiometric balance of the mixotrophic conditions in which denitrifiers are usually cultured, (containing acetate, Fe(II), Fe(III), NO$_3^-$ and NO$_2^-$) is puzzling (Tominski et al., 2018), and the contribution of NO$_2^-$ or NO in abiotic Fe(II) oxidation has been challenged (Kappler et al., 2005; Klueglein and Kappler, 2013). Laboratory investigations of abiotic Fe(II) oxidation with NO$_2^-$ at neutral pH have shown that equivalent Fe(II) oxidation is detected only at concentrations of NO$_2^-$ (>2 mM) much higher than what is usually detected in cultures with denitrifying bacteria (<1 mM) (Carlson et al., 2013; Ilbert and Bonnefoy, 2013). Thus, additional mechanisms might be at play, for instance, enzymatic Fe(II) oxidation (Klueglein and Kappler, 2013). In the hypothetical enzymatic mechanism, c-type cytochromes associated to the cell membrane are proposed as Fe(II) oxidation catalysts (Liu et al., 2017), analogous to aerobic Fe(II) oxidation by At. ferrooxidans in acid mine drainage conditions (Ilbert and Bonnefoy, 2013). However, to this day no dedicated ferroxidase has been identified in denitrifying bacteria (Carlson et al., 2013; Price et al., 2018). The presence of an enzymatic system oxidising Fe(II) would likely include an Fe(III) exporting system, which would need to overcome the barrier imposed by the low solubility of Fe(III). A proteomic study showed that Acidovorax ebreus expressed efflux pumps in the membrane, used by the cells to export toxic metals, potentially as a response to nitrosative/oxidative stress caused by Fe(II) and nitrogen species in anoxic conditions (Carlson et al., 2013), whereby this study discards the energetic benefit of Fe(II) oxidation and supports the detoxification model (Ilbert and Bonnefoy, 2013).

2.3.2.3 Biomineralisation in cells

As a result of Fe(II)-oxidation, bacteria form mixed-valence Fe biominerals, which can be formed inside the cell or extracellularly, in a process widely studied in many microorganisms. Bacteria can precipitate Fe in different sites, including the periplasm, the cell surface and extracellularly (Benzerara et al., 2008; Miot et al., 2009a; Suzuki et al., 2011; Baumgartner et al., 2013; Miot et al., 2016a; Miot et al., 2016b).

2.3.2.4 Periplasmic encrustation and its effects on the cells

Encrustation of the periplasm with Fe minerals is common in NDFO bacteria (Kappler et al., 2005; Schmid et al., 2014). The periplasm is an essential cellular compartment in Gram-negative bacteria because many metabolic reactions take place there, and it is a
site of transit of nutrients and waste. Therefore, encrustation of the periplasm is predicted to be harmful to the cells. This encrustation has been associated with an increased production of periplasmic proteins that might act as mineral nucleation sites (Miot et al., 2009a). Periplasmic encrustation might also be a consequence of the activity of periplasmic enzymes producing (probably indirectly) insoluble products that precipitate at that location, such as Fe(III) minerals (Schädler et al., 2009). Environmental stress could also support the aggregation of misfolded proteins in the periplasm, which could also accumulate at the cell poles as an indicator of aging in cells (Miot et al., 2009a). Additional to periplasmic encrustation, the poles of the cells are the sites where higher encrustation has been observed. Glasauer et al. (2007) identified intracellular mixed-valence Fe granules preferentially located at the cell poles of *S. putrefaciens* CN32 and postulated that this region attracted Fe oxides and was an Fe uptake facilitator. However, this is the only work where these intracellular granules have been observed in dissimilatory metal-reducing bacteria. Protein aggregates accumulate at the older cell poles during cell division, and thus, pole encrustation due to cell aging seems to be a more reasonable explanation for this phenomenon (Lindner et al., 2008).

Biomineralisation in the periplasm could cause a series of effects that eventually lead to loss of functions in the cell: (i) it may lead to an increased production of porins (membrane proteins) promoting pore formation as a step forward to cell death (Bednarska et al., 2013), (ii) protein aggregates accumulate in the old poles of the cells as a means to prevent cell toxicity and conserve cell integrity and (iii) protein aggregation added to mineral accumulation in the periplasm may induce a nitrosative/redox stress response including reduction of membrane permeability (Carlson et al., 2013; Miot et al., 2015).

### 2.3.2.5 Extracellular biomineralisation

Cell mineralisation is widespread, however some Fe(II)-oxidisers are capable of keeping the cells free of encrustation. The production of extracellular polymeric substances (EPS) works as a nucleation site for Fe(III) mineral precipitation in strains of the microaerophilic Fe(II)-oxidisers *Gallionella* sp. and *Leptothrix* sp. (Fig. 2.7), which form extracellular organics in the shape of stalks or sheaths (Chan et al., 2009; Chan et al., 2011).
2.3.3 Model nitrate-dependant iron(II)-oxidising organisms

*Acidovorax* is a genus of facultative bacteria that typically inhabit soil and aquatic environments. Most *Acidovorax* strains are denitrifiers and some are known plant pathogens (Huang et al., 2012). *Acidovorax* sp. strain BoFeN1 could be considered the model organism for the study of neutrophilic NDFO (Kappler et al., 2005). This bacterium and its biomineralisation patterns have been studied through imaging and spectroscopic techniques since it was first isolated (Kappler et al., 2005), however, other neutrophilic Fe(II)-oxidising bacteria have been discovered and studied since then (Hegler et al., 2008; Miot et al., 2009b). The biomineralisation patterns in BoFeN1 were initially studied with scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray powder diffraction (XRD) and confocal laser scanning microscopy (CLSM) (Kappler et al., 2005). BoFeN1 presented periplasmic encrustation and enzymatic Fe(II) oxidation was first proposed, instead of abiotically catalysed by NO$_2^-$: Additionally, goethite was identified as the Fe(III) biomineral produced (Kappler et al., 2005). A subsequent study using scanning transmission X-ray microscopy (STXM) and high resolution TEM identified amorphous iron phosphates precipitating at a distance from the cells, in the periplasm and markedly accumulated at the cell poles in BoFeN1 (Miot et al., 2009a). In contrast, the photoautotrophic anaerobic Fe(II)-oxidiser *Rhodobacter ferrooxidans* strain SW2, only showed extracellular biomineral precipitation in the form of organic fibers stemming from the cells. The cells and biominerals were analysed with a combination of STXM and X-ray absorption near edge structure (XANES) (Miot et al., 2009b). An additional study determined that the medium and geochemical growth
conditions influence the nature of the biominerals identified, where carbonate and high pH appear to promote the formation of goethite in cultures of BoFeN1 (Larese-Casanova et al., 2010).

The effect of As(III) and As(V) on Fe biomineralisation by Fe(II)-oxidising bacteria has also been tested. BoFeN1 tolerates high concentrations of As (0.5 mM), but when As is present BoFeN1 not only forms goethite but a mixture of goethite and nanocrystalline Fe(III) oxyhydroxides, whereas strain SW2 consistently produces ferrihydrite in the presence or absence of As (Hohmann et al., 2010). Further synchrotron-based studies at the molecular level confirmed the inhibitory effect of As on the formation of goethite in cultures of BoFeN1, and demonstrated that the biogenic amorphous Fe(III) oxyhydroxides have higher affinity for As(V) than for As(III) in these conditions (Hohmann et al., 2011).

As can be noted the majority of studies have concentrated on the biomineralisation products of NDFO. One study with a slightly different focus investigated the relationship between Fe biomineralisation and carbon assimilation in single cells of BoFeN1 using NanoSIMS, in order to evaluate the impact of biomineralisation on cell viability. The authors found heterogeneous $^{13}$C uptake, where highly mineralised cells did not maintain their viability, implying that higher levels of biomineralisation can be deleterious for the cells (Miot et al., 2015). This work posed the intriguing question of why these organisms did not evolve ways to cope with cell encrustation in spite of the prevalence of these bacteria in Fe-rich environments.

Acidovorax sp. strain ST3 is an As(III)-oxidising bacterium isolated from an As-polluted paddy soil (Zhang et al., 2017). This strain contains the periplasmic Aio enzymatic cluster for As(III) oxidation. Initial tests demonstrated that this facultative denitrifying bacteria...
is also a NDFO organism and can use acetate or lactate as organic co-substrates (Fig. 2.8). For these reasons, this NDFO bacterium could be a good candidate to promote the immobilisation of As in its oxidised form, As(V).

2.4 Other environmental factors affecting As mobilisation

The environmental cycling of As is a process that has not been fully elucidated. In previous sections, the microbial processes that affect mobilisation of As were discussed, but there are several environmental factors that will determine the behaviour of As at a given point and time. Reactions such as sorption (for instance on Fe or S minerals), complexation, abiotic oxido-reduction and organic matter interactions will dictate the mobility of As, regardless of its oxidation state (Gorny et al., 2015). All these factors can be present simultaneously and affect the natural behaviour of As in groundwater and sediments, but these are often overlooked under the controlled laboratory conditions where most studies are undertaken.

2.4.1 Redox factors

Bacteria and manganese-(hydr)oxides are able to catalyse oxidation of As(III) at a reasonable rate in the pH range of 5-9; oxygen can oxidise As(III) but at very slow rates, in the order of months to years (Gorny et al., 2015). The iron oxyhydroxide goethite can induce photo-oxidation of As(III) at acidic pH, where humic acids decrease this oxidation rate. Under oxic conditions, magnetite or ferrihydrite can oxidise As(III) in their surface in an Fe(II) mediated reaction (Gorny et al., 2015).

2.4.2 Natural organic matter (NOM)

Natural organic matter (NOM) can influence As behaviour in the environment due to its complex composition containing a wide variety of functional groups that interact with microbes, metals, metalloids and minerals. NOM can directly or indirectly affect the behaviour of As by: (i) mobilising As through competitive desorption, substituting potential As sorption sites on Fe(III) minerals, (ii) complexation on solid organic phases, and (iii) oxidising or reducing As (particularly through the humic acids fraction, as mentioned in section 2.2.5.4). Additionally, many of the NOM functional groups act as electron donors or acceptors, fuelling certain microbial metabolisms (Jiang et al., 2009; Campbell and Nordstrom, 2014).
2.4.3 Minerals in the subsurface

Mineral transformations have been studied widely along with bacterial metabolisms to predict As behaviour in the environment. Field studies confirm the close relationship between Fe minerals and As mobility (Gnanaprakasam et al., 2017). Newly formed Fe(III) minerals have the capability to sorb, sequester and precipitate not only As(V) but also As(III) (Dixit and Hering, 2003). As(III) has largely been thought of as the most mobile and soluble form of As, but it has shown similar sorption rates to common Fe(III)-(oxyhydr)oxide minerals (e.g. ferrihydrite, goethite, hematite) as As(V) at neutral pH, although this sorption is through weakly bound complexes, and as a result, As(III) desorbs more easily than As(V) (Tufano et al., 2008). Additionally to Fe(III)-oxyhydroxides, aluminium hydroxides can adsorb As(V) on their surfaces, with better retention capacities than Mn minerals. Additionally, in all these sorption processes several anions such as CO$_3^{2-}$, NO$_3^-$, HPO$_4^{2-}$ and SO$_4^{2-}$ compete with As(V/III) for sorption sites (Gorny et al., 2015), promoting the mobilisation of As.

2.5 Overview of bacterial transformations of As and Fe

In the previous sections of this literature review a summary of the multiple strategies that bacteria use to conserve energy for their metabolisms using a diversity of molecules available in their environments was presented, where the focus was on Fe and As as electron donors or acceptors. The microbial oxido-reduction of Fe has an effect in the mobilisation or sequestration of trace metalloids, mainly by providing suitable sorption sites, where As is of particular interest, due to its poisonous nature. In consequence, there has been a growing number of investigations using model bacteria that can transform As and Fe in both sides of the oxidation-reduction spectrum. These works are largely supported on electron microscopy techniques, which allow localisation and chemical mapping at high resolution of the minerals and bacteria, whereas DNA-based techniques have been essential for the identification of these organisms. These techniques have been crucial in identifying who is doing what in these environments devoid of oxygen. However, an oversight of these techniques is the capability to spatially correlate metabolically active microbes with the elements and minerals they are transforming. Therefore, the understanding of Fe and As microbial transformations could be expanded by using complementary high resolution chemical imaging techniques, such as secondary ion mass spectrometry (SIMS), a surface chemical
mapping technique that can achieve these two requirements at high spatial resolution. These techniques could contribute to answer not only the who and what but also the how and where in the concomitant Fe and As transformations by bacteria. Thus, two selected SIMS techniques and their operating principles are described in detail in the following sections, explaining why these techniques are appropriate to study the As and Fe transformation by bacteria and their associated (bio)minerals.

2.6 SIMS for the study of the microbe-mineral interface

SIMS is a powerful technique that uses an energetic primary ion beam to bombard and erode a surface, which in consequence produces secondary ions that are detected and analysed in a mass spectrometer. The majority of species emitted are neutral, but this technique focuses on detecting and analysing the secondary ions that are released. Although secondary ion emission was first observed more than 100 years ago (Vickerman, 2009), it wasn’t until the 1950s when this concept started to be exploited and shaped into the development of this analytical technique. By the early 1980s, SIMS had been widely used for depth analysis of materials (McPhail and Dowsett, 2009a) and now it has become a standard instrument in the study of isotopes and trace concentrations of components in the fields of Cosmo and Geochemistry (Hoppe et al., 2013). Moreover, during the last two decades there has been a boom in the analysis of biological samples with SIMS (Vickerman, 2009). These techniques are under constant improvement; new instruments, accessories and tools have been developed since the first SIMS equipment appeared. These improvements have conferred different capabilities to SIMS instruments, as described in the following sections.

2.6.1 General components of SIMS techniques

In SIMS the accelerated primary ion beam transfers its energy to atoms and molecules in the surface, initiating a collision cascade that can deposit its energy down to 30-40 nm, although most of the collected secondary ions originate from the top two layers of atoms. When these species acquire enough momentum, they are released or sputtered from the surface and ionised whereas primary ions are implanted into the upper few nm of the sample surface. This phenomenon translates into the disturbance and damage of the surface of the sample and explains why this technique is considered destructive (Fig. 2.9). The particles emitted can be electrons, neutral species (molecules, atoms), atomic ions and ionised molecules and usually originate from no more than 1 nm below the
surface, and for this reason this technique is considered to be very surface sensitive (see Figure 2.9). SIMS has to be performed under high vacuum conditions, similar to many surface analysis techniques, because the molecules in the air interfere with the analysis by dispersing the electrons and ions that this technique is aiming to detect and analyse (Vickerman, 2009).

2.6.1.1 The basic SIMS equation

The secondary ions emitted (and analysed) are affected by a series of factors described by the basic SIMS equation:

\[ I_m = I_p y_m \alpha^\pm \Theta_m \eta \] (Eq. 2.1)

Where \( I_m \) is the measured secondary ion current of species \( m \), \( I_p \) is the primary ion current, \( y_m \) is the sputter yield, \( \alpha^\pm \) is the ionisation probability (positive or negative ions), \( \Theta_m \) is the fractional mass concentration of species \( m \) and \( \eta \) is the transmission of the instrument (Benninghoven et al., 1987; Vickerman et al., 1989).

The sputter yield (\( y_m \)) is the number of particles emitted from the sample or sputtered atoms (\( N_0 \)) per primary ion bombarded to the surface (\( N_p \)):

\[ y_m = \frac{N_0}{N_p} \] (Eq. 2.2)

The secondary ion yield (\( y_m^\pm \)) is the number of secondary ions (\( N_s \)) per primary ion bombarded to the surface (\( N_p \)):

\[ y_m^\pm = \frac{N_s^\pm}{N_p} \] (Eq. 2.3)

And the ionisation efficiency (\( \beta^\pm \)) is the fraction of secondary ions emitted per total sputtered atoms (\( N_0 \)), therefore its value is less than 1:
\[ \beta = \frac{N_s}{N_0} \quad (\text{Eq. 2.4}) \]

Since \( \Theta_m \) is an intrinsic attribute of the sample and the manipulation of \( I_p \) is limited by the requirements of the primary ion dose, \( y_m, \alpha, \) and \( \eta \) are the parameters that can be improved in order to increase the secondary ionisation efficiency (Vickerman et al., 1989).

### 2.6.2 Stages of SIMS analysis

All SIMS instruments comprise four basic stages: sample ionisation, ion extraction, mass analysis and ion detection (Fig. 2.10). All these steps will be affected by different phenomena which will determine the quality of analysis.

![Schematic of the basic stages in SIMS](Sinha and Hoppe, 2010)

**2.6.2.1 Sample ionisation**

Based on the mechanism of primary ion production the primary beam sources can be classified into four types: i) electron bombardment, ii) plasma, iii) surface ionisation and iv) field ionisation. Each type provides a different sensitivity, spatial resolution, speed of use, etc. (Vickerman, 2009). **Electron bombardment**: the ionisation of the primary ion beam (usually Ar or Xe) is achieved using a high density current of electrons. They are currently used for the bombardment of cluster ion beams like \( \text{C}_6\text{O} \) or \( \text{SF}_5^+ \). **Plasma**: Duoplasmatron for the bombardment of \( \text{O}_2^+ \) is an example of this ionisation method. **Surface ionization**: ions are obtained by warming an adsorbed layer of Cs, for example, on the surface of a metal such as iridium. Electropositive ions such as Cs are produced by this method. **Field ionisation**: liquid metal ion source is the most commonly used in this ionization method, where the primary ion beam can be highly focused, providing high spatial resolution. Liquid metal ion beams based on gold and bismuth generate high yields of cluster ions like \( \text{Au}_3^+, \text{Bi}_3^+, \text{Bi}_5^+ \), etc. (Vickerman, 2009).

**2.6.2.1.1 Primary ion beams**

Static SIMS uses a wide diversity of primary ions. The monoatomic primary ions used include \( \text{Au}^+, \text{Ga}^+, \text{Ar}^+ \), and the polyatomic or cluster primary ions used include \( \text{Au}_2^+, \text{Au}_3^+, \text{SF}_5^+ \) and \( \text{C}_{60}^+ \). Oxygen and caesium are the two primary ion sources used in dynamic
SIMS. In dynamic SIMS an oxygen primary beam (O⁺, O₂⁻ or O₂⁺) can be used to generate either positive or negative secondary ions and a caesium (Cs⁺) primary beam can be used to generate negative secondary ions. The oxygen primary ions in the duoplasmatron ion source are produced by arc discharge and acquire a spread of initial kinetic energy, while Cs⁺ primary ions are produced by surface ionisation, making them essentially monoenergetic, and for this reason, only Cs⁺ ions can be focused into a small spot size (Sinha and Hoppe, 2010). The latest radio frequency plasma oxygen primary ion source for NanoSIMS allows the bombardment of oxygen ions for the collection of positive SI at high sensitivity and a spatial resolution of ~50 nm (Malherbe et al., 2016).

Cluster ion beams

In the last two decades there has been a growing interest in using cluster ion beams in SIMS (Auₙ⁺, Biₙ⁺, SF₅⁺ and C₆₀⁺) instead of atomic ions, due to the different sputtering mechanisms and nature of the information they can provide. When a cluster ion hits the sample surface, the projectile energy is divided between all the atoms; for instance, each atom from a 20 keV C₆₀⁺ cluster will have 333 eV of energy (Wong et al., 2003). Consequently, these ion beams generate less surface damage than atomic ions, where samples can be analysed beyond the static limit, which in principle allows molecular depth profiling; this attribute makes cluster ion beams suitable for the efficient sputtering of large organic molecules with low fragmentation (Vickerman, J.C., 2003). Additionally, these cluster beams increase sensitivity by generating higher secondary ion yields, due to increased sputter yields (Vickerman, 2009). SIMS molecular depth profiling of organic materials was improved with the introduction of large gas cluster ion beams (GCIB) such as Arₙ⁺ (Ninomiya et al., 2009), where beam-induced damage is lower than with the C₆₀⁺ beam (Rabbani et al., 2011). However, these GCIBs are hard to focus and thus spatial resolution is limited to >10 µm (Fletcher et al., 2013). Nonetheless, high spatial resolution (below 1 µm) in static SIMS imaging can be achieved with liquid metal ion guns (LMIG), which use polyatomic ion beams such as Auₙ⁺ or Biₙ⁺ (Kollmer, 2004; Touboul et al., 2005).

The C₆₀⁺ cluster beam used in ToF-SIMS is of particular interest in this research. This cluster ion beam could favour the sputtering and identification of molecules of interest such as proteins or fragments of proteins associated to the cell surface, that might be playing a role in the respiration of Fe(III) and As(V).
2.6.2.2 Mass analysers

Three types of mass analysers have been commonly used in SIMS: Quadrupole mass analyser (QMA), double focusing magnetic sector and Time-of-Flight (ToF). The first static SIMS configurations utilised a QMA because of their high transmission (ca. 1970s) and compatibility with ultra-high vacuum conditions. However, the subsequent need to expand the type and mass of species analysed limited the use of this analyser; its low mass range would not allow the analysis of larger molecules such as polymers.

Since the 1970s, ToF analysers started to be used, proving its high transmission as well as high mass resolution. The parallel detection of all ions was its major advantage, instead of just one mass at a time (Vickerman, 2009). ToF-SIMS is a SIMS instrument that uses a ToF mass analyser.

NanoSIMS uses a double focusing magnetic sector. These analysers supply high transmission, high mass resolution, but can only detect up to 7 masses simultaneously with the latest generation microprobe instrument, CAMECA NanoSIMS 50L (Hillion et al., 1994; CAMECA, 2012).

2.6.2.3 Ion detection

NanoSIMS uses two types of detectors: electron multipliers (EM) and Faraday cups. In electron multipliers every ion hitting the EM produces an electrical pulse which is then amplified by a series of dynodes, and this amplified pulse is registered. These are usually used in the pulse-counting mode. EMs can measure a wide range of counts, between <1 and \(10^6\) counts per second (cps) (Hoppe et al., 2013). Faraday cups measure a current directly proportional to the number of ions hitting a metal plate in the cup. For this reason, Faraday cups are regarded as more accurate than electron multipliers, although they are not as sensitive as EMs. EMs are used in secondary ion imaging mode and Faraday cups are preferred for accurate isotopic measurements in NanoSIMS, although when using Faraday cups no secondary ion image is produced.

ToF-SIMS uses microchannel plate detectors, which share similarities to EMs. When an ion hits a wall in the microchannel plate, it amplifies its signal by several orders of magnitude. Microchannel plate detectors have a high time resolution to measure ion arrival times, which makes them ideal for ToF-SIMS. However, in order to measure at
high time resolutions, the microchannel plate detector has to be finely aligned in an orthogonal position to the ion beam trajectory (Lyon and Henkel, 2010).

2.6.3 Sputtering regime: static or dynamic

Based on the primary ion dose per sample area, there are two sputtering regimes under which SIMS operates: static SIMS and dynamic SIMS. Static SIMS usually operates with ion doses <10^{13} ions cm^{-2} of sample surface so that total sputtered secondary ions are less than 1 monolayer of the surface (Sinha and Hoppe, 2010). Dynamic SIMS typically employs higher ion currents, >10^{16} ions cm^{-2}.

As mentioned earlier, SIMS is essentially destructive, but the group of Benninghoven, in the 1960s, started testing lower primary ion doses to such a level that they observed no significant sputtering of the surface in their tests, giving birth to the concept of static SIMS, meaning that the integrity of the surface is kept or remains «static». The static limit is considered to be 10^{12}-10^{13} ions cm^{-2} for the primary ion energy (Benninghoven, 1973). The use of very low doses of primary ions resulted in a low yield of secondary ions, imposing the need to improve sensitivity. This issue was addressed with the improvement of transmission of the mass analyser and the use of a single-ion counting technique instead of measuring the current of secondary ions (Benninghoven, 1994b). ToF-SIMS can be operated under both sputtering regimes (Sinha and Hoppe, 2010), while NanoSIMS is a dynamic SIMS instrument.

2.6.4 Attributes of SIMS techniques

Three parameters make SIMS analysis advantageous and the technique of choice: sensitivity, mass resolution and spatial resolution; incidentally, depth resolution is also an advantageous trait for depth profiles.

2.6.4.1 Sensitivity and detection limit

Sensitivity is a term often interchanged with detection limit (Wells et al., 2011). In SIMS the sensitivity depends on the ionisation efficiency of the analysed species and secondary ion yield (SIMS basic equation) on the background signal, on peaks not fully resolved and on the transmission of the instrument. SIMS instruments have the capability to detect down to parts per million (ppm) levels. The CAMECA NanoSIMS 50L can detect ppm in element imaging, and parts per billion for elements with high ionisation yields (CAMECA, 2012).
2.6.4.2 Mass resolution

The mass resolution in a mass spectrometer is the ability to separate and distinguish two isobaric species. A good mass resolution is a desired feature in mass spectrometers, and how much is needed will vary between the mass or isobaric interferences expected and the desired information required from the analysis. Many atoms and molecules can act as isobars with the species desired for analysis; these include hydrides, hydrocarbons and oxides, many of which can originate as environmental contaminants or as remnants in the analysis chamber (hydrides and water are ubiquitous). Matrix components can also contribute with mass interferences (Lyon and Henkel, 2010). For example, when analysing the isotope $^{13}$C a typical isobaric interference is $^{12}$CH. To separate these two peaks, the mass resolution ($m/\Delta m$) has to be at least 2900 (Sinha and Hoppe, 2010).

$$\frac{m}{\Delta m} = \frac{m(^{13}\text{C})}{(m(^{13}\text{C}) - m(^{12}\text{C}^1\text{H}))} = \frac{13 \text{ a.m.u.}}{0.0045 \text{ a.m.u.}} = 2900 \quad (\text{Eq. 2.5})$$

Transmission (%) of the system is the ratio of secondary ions formed at the sample surface to the number of secondary ions detected, which will depend on the mass analyser used. Transmission and sensitivity are reduced when improving mass resolution in magnetic sector SIMS (e.g. NanoSIMS), given that part of the secondary ion beam is cut off and shaped by slits in order to obtain narrow peaks (Hoppe et al., 2013).

2.6.4.3 Spatial resolution

Spatial resolution is the ability to resolve details on the sample surface. Spatial resolution primarily depends on the spot size of the primary ion beam, meaning how fine the ion beam can be focused. The highest spatial resolution in TOF-SIMS is typically between 300-500 nm. The CAMECA NanoSIMS 50L instrument can reach a spatial resolution of 50 nm, with both primary ion beams (Cs+ or O-). However, in order to achieve high spatial resolution NanoSIMS uses lens and slits to produce a small primary ion beam; this reduces the primary ion current where fewer secondary ions are generated, which also reduces sensitivity (Slodzian et al., 1992).

Spatial resolution is manually estimated by drawing a line scan across an ion image and obtaining a plot profile in ImageJ or Fiji (https://fiji.sc/). The spatial resolution is the distance (nm or µm) between the 20 and 80 % intensity in the plot profile (Whitby et al., 2012), see Figure 2.11.
2.6.4.4 Depth resolution

SIMS analysis has the potential to provide fine depth resolution. This characteristic can be used in the construction of 3D images, and it is a trait mostly related to dynamic SIMS, traditionally used for depth profiling (McPhail and Dowsett, 2009b). However, the development of polyatomic ion beams in ToF-SIMS lead to the acquisition of high depth resolutions during depth profiling, where it can be as high as 5-10 nm depending on the sample (Fletcher and Vickerman, 2013).

2.6.5 Effects of improving resolutions

High mass and spatial resolution along with high sensitivity are features greatly desired when performing a SIMS analysis, nonetheless, it is impossible to perform analysis at the highest sensitivity, mass and spatial resolutions simultaneously. Usually, the improvement of one condition compromises the other, and thus, trade-offs must be made in the decision of how high or low the spatial or mass resolution can be for the output of information required. Spatial resolution and sensitivity are mainly affected by the beam diameter and by the amount of ions in a single pulse of the primary ion source, in the case of static SIMS (Benninghoven, 1994b). For example, when spatial resolution is increased there is a loss in sensitivity, because the improvement in spatial resolution implies the reduction of the analysis area or pixel diameter, hence, a significant amount of material to be analysed is lost. If the choice is to work at high spatial resolution, the analyst has to make sure that the analyte is at an acceptable concentration in the sample to be detected (Passarelli and Ewing, 2013).
2.6.6 Artefacts in SIMS

There are several artefacts that affect the secondary ion signal and complicate quantification in SIMS. The most important artefacts are preferential sputtering, matrix effect, topography and sample charging. The two latter are closely related to the sample preparation (Hoppe et al., 2013).

2.6.6.1 Preferential sputtering

SIMS analysis depends on the secondary ions collected, and in order to collect secondary ions we first need to ionise and sputter the sample surface with the primary ion. The sputtering and ionisation efficiency depend on the mass and the binding energy of atoms of the analyte and the matrix, and on the incidence angle, energy and mass of the primary ion beam (Sinha and Hoppe, 2010). The secondary ion yield measured in proportion to the sputtered yield is rarely above $10^{-4}$ (Fletcher and Vickerman, 2013). Preferential sputtering is the erosion of the sample surface at different rates. It occurs when there is a chemical difference among the species distributed on the surface (Fletcher and Vickerman, 2013).

2.6.6.2 Sample topography

The intensity of the secondary ion signals will be determined not only by the chemical composition but also by the sample height and inclination of the analysis point on the sample surface. This distribution and structure of the sample on the matrix can block or reduce the signal from regions that are at a different height. In order to avoid this issue, it is desirable to perform the analysis on flat surfaces, avoiding edges, as this will make analysis more reliable (Rangarajan and Tyler, 2006).

2.6.6.3 Sample charging

Some samples analysed in SIMS can be insulating (including bacterial cells, minerals and organic samples). When a sample with this characteristic is bombarded with a positive ion beam the surface potential increases because of the input of positive charge and the release of secondary electrons. The charge can build up very quickly changing the potential of the analytical area resulting in the loss of the signal and the spectrum (Vickerman, 2009). This problem can be solved by coating the samples with a thin layer of a conductive material (e.g. 10 nm of Au or Pt) and the use of the electron flood gun (Herrmann et al., 2007).
2.6.7 Quantification in SIMS

2.6.7.1 Relative sensitivity factors (RSFs)

SIMS is not traditionally thought of as a quantitative analytical technique, although quantification in SIMS is possible. However, this is not a straightforward process because the measurement of the secondary ion current not only depends on the concentration of the analyte species in the sample, but on its sputtering and ionisation efficiency. In this sense, quantification is usually performed by determining parameters known as relative sensitivity factors (RSF), specific to a matrix element of a certain concentration. This implies that a given element may have different RSFs in different matrices. For this reason, quantification in SIMS needs reference materials that are well characterized and with a composition similar to that of the analyte (Sinha and Hoppe, 2010). The following equation gives the basis for the quantification, where RSF_A is the relative sensitivity factor for element A, I_A and I_M are the secondary ion intensities for element A and reference element M, respectively, and C_A and C_M are the concentrations of elements A and M, respectively:

\[
C_A = \frac{1}{\text{RSF}_A} \times I_A \times \frac{C_M}{I_M}
\]  
(Eq. 2.6)

Where the difference in RSFs between different elements can be different orders of magnitude.

2.6.8 Types of analysis in SIMS

SIMS instruments can perform a variety of analyses, these include the acquisition of a mass spectrum, ion imaging, depth profiling and isotope ratio measurement.

2.6.8.1 Mass spectrum

A mass spectrum of analytes is obtained with SIMS. The secondary ions are separated by their mass to charge (m/z) ratio and sent to the detector. The mass spectrum is a plot of the intensity vs. m/z ratio which can show elements as well as molecules of different sizes. This mode of analysis has been widely used to identify large organic molecules (including amino acids, peptides, drugs, vitamins and pharmaceuticals), and thus is very common in the static SIMS regime. NanoSIMS produces a mass spectrum but only of the masses selected in the detectors.
2.6.8.2 Ion imaging

The high spatial resolution of the technique enables the acquisition of secondary ion images by ‘rastering’ the primary ion beam over the sample surface. Secondary ion images are routinely obtained in NanoSIMS, but these images are also common in ToF-SIMS and is one of the main types of analysis presented in this thesis.

2.6.8.3 Depth profile

A depth profile provides two and three-dimensional information of the sample surface. It is usually performed in a small area of the sample. The destructive sputtering capabilities of SIMS reveal the chemical composition and structures of that area layer by layer, several nm or µm below the surface. High spatial resolution requires high primary ion beam energies and for this reason it is usually performed under dynamic SIMS conditions. A crater can be observed in the analysed area as a result of the depth profiling. Usually only the data obtained at the centre of this crater is used for analysis, to avoid the signal from the edges (crater edge effect). Depth profiling is possible thanks to the depth resolution capabilities in SIMS. However, this parameter is affected by the primary ion beam, the topography on the sample surface, the sputtering rates of the species and radiation effects like the mixing of atoms induced by the ion bombardment (Vickerman, 2009). Depth profiling is also possible in ToF-SIMS but would require a prolonged analysis acquisition. See Figure 2.12 for a schematic representation of depth profiling in a given area of interest.

Figure 2.12. Schematic of the collection of secondary ions to their spatial coordinates on all three dimensions, being the Z axis the direction of interest in depth profiling (Fearn, 2015).
2.6.8.4 Isotope ratio measurements

SIMS has the capability to detect accurately low abundance species and differentiate between two isotopes of the same element. The high mass resolution and high transmission in SIMS are important to achieve precise isotope ratio measurements. SIMS techniques are not especially quantitative methods; hence, a reliable isotope ratio measurement needs a careful tuning of the SIMS equipment in order to avoid mass interferences and other effects, for example, associated with the detector (Fletcher, 2009). Most isotope ratio analysis are performed using NanoSIMS due to its high precision measurement of isotope ratios at high spatial resolution, although some works have used the CAMECA IMS 4f series instrument (Sinha and Hoppe, 2010). Faraday cups are the most precise detectors for isotope ratio measurements in NanoSIMS with a precision of ~0.2 ‰, although no ion images can be collected when using this type of detector. Electron multipliers are less precise (~1-2 ‰), but allow the collection of ion images (Sinha and Hoppe, 2010). Carbon, nitrogen, oxygen and sulfur isotopes are generally studied in biological systems. The second most abundant isotopes of these elements have a natural abundance at the percentage level or close to it (1.11 % for $^{13}$C, 0.36 % for $^{15}$N, 0.2 % for $^{18}$O and 4.2 % for $^{34}$S) (Sinha and Hoppe, 2010; Hoppe et al., 2013). Therefore, simultaneous imaging and isotopic ratio measurements can be achieved at a reasonable precision using electron multipliers. The ion images can then be used to create isotope ratio maps. This function is particularly useful to identify metabolically active cells by their accumulation of $^{13}$C, $^{15}$N, $^{18}$O and/or $^{34}$S.

2.6.9 NanoSIMS in detail

The first NanoSIMS was developed in the 1990s and was originally intended for use in medical biology and materials science (Hillion et al., 1994). The instruments commercially available nowadays were developed by CAMECA in France, in close collaboration with Harvard University. It is now used in a wide range of fields, including Microbiology, Cosmochemistry, Biogeochemistry and Materials Science. To date there are nearly 50 instruments installed worldwide. The distinguishing characteristic of the NanoSIMS is its ability to focus a primary ion beam on the surface of a sample with a small diameter of 50 nm for Cs$^+$ or O$^-$ primary ions (CAMECA, 2012).
2.6.9.1 Design and ion optics of NanoSIMS

In most SIMS instruments the primary ion beam bombards the surface at an angle, typically 45 to 60°. In these conditions, preferential sputtering can occur on the closest side of the ion beam, and in consequence the sputtering will not be equal. Another constraint is that the collection of secondary ions should be closest to the sample in order to collect the greatest possible amount of ions but the primary ion optics prevent this (Slodzian et al., 1992). To overcome these limitations, the CAMECA NanoSIMS 50L introduced a special coaxial design where the primary ion beam hits the surface at 90° and the collection of secondary ions is also acquired at 90° from the sample surface, thanks to a set of lenses and deflectors, part of its unique design (Fig. 2.13 and 2.14). This allows a very short working distance between the extraction lens and the sample (=400 µm), higher collection efficiency and an improvement in ion transmission. Nonetheless, this coaxial design implies that the polarity of the primary and secondary ions has to be opposite (CAMECA, 2012).

Figure 2.13. Schematic of the CAMECA NanoSIMS 50L ion optics (Hoppe et al., 2013).
Steady state is a condition exclusive to dynamic SIMS, because the primary ion dose needed in the process has to be at least $10^{16}$ ions cm$^{-2}$. It is said that the steady state is achieved when the secondary ion counts are stable. To get to that point, the primary ions have to be constantly bombarded on the surface before the main analysis is started, the secondary ion counts increase gradually as a consequence of primary ion implantation and sputtering of species from the sample surface (Slodzian et al., 1992). This state is only reached in dynamic SIMS because the primary ion dose has to be high. Moreover, steady state cannot be reached in all samples, since this will depend on the sample thickness as it is usually reached after sputtering a few nm of the sample, and thus, thinner samples will potentially be sputtered away before reaching this state (McPhail and Dowsett, 2009c). In NanoSIMS, the secondary ion yield depends on the primary ion concentration, thus, the yield increases with increasing primary ion dose, until reaching an equilibrium (see Fig. 2.15) (Guerquin-Kern et al., 2005). In a dynamic SIMS instrument reliable quantification has to be made in the steady state regime. The time needed to achieve this state depends on the sputtering time, the current and the raster area.

Mass analyser and detectors

The NanoSIMS uses a magnetic sector mass spectrometer, in which secondary ions are separated by their mass to charge ratio (m/z), as described by the Lorentz force, as they pass through a magnetic field: ions of equal energy but different m/z trace different radial trajectories, where $z$ is the number of elementary charges (Slodzian et al., 1992).
The secondary ions leave the surface with a low kinetic energy, and they are extracted by an electrostatic field to be transferred to the mass spectrometer (see Fig. 2.16). In NanoSIMS 50L a double focussing sector mass spectrometer in “Mattauch-Herzog” configuration is installed. This geometry uses an electrostatic analyser followed by a magnetic sector. The electrostatic analyser separates ions based on their kinetic energy and the magnetic sector on the basis of their m/z ratio (Slodzian et al., 1992). After mass separation the secondary ions are detected using electron multipliers which are useful to detect low intensity ions. Faraday cups are used for higher intensity signals. In NanoSIMS the mass resolution and the mass spectrometer transmission are high (Slodzian et al., 1992).

Figure 2.15. Schematic of the increase in ion intensity for different species until reaching steady state on thyroid follicle. Image obtained from (Guerquin-Kern et al., 2005).

Figure 2.16. Diagram of a double focusing magnetic sector mass analyser (Hart-Smith and Blanksby, 2012).
The latest generation NanoSIMS 50L has six mobile and one fixed detector, allowing the detection of seven secondary ions simultaneously. Unlike ToF-SIMS, NanoSIMS instruments only detect ions that have been preselected, therefore, a full mass spectrum is not generated at each pixel of the image. In contrast with static SIMS, the continuous primary ion bombardment breaks the molecular bonds and thus, only atomic and small molecular ions are usually produced and analysed in NanoSIMS (Boxer et al., 2009).

2.6.10 ToF-SIMS in detail

2.6.10.1 ToF mass analyser

Time-of-Flight is a type of mass analyser whose mass separation principle is that ions with the same energy but with different masses will travel with different velocities. In this design, an electrostatic field accelerates the secondary ions to a common energy. These accelerated ions drift to a field-free space before arriving to the detector. The lighter ions fly with higher velocity and reach the detector before the heavier ions (Benninghoven, 1994a). If all the secondary ions generated after a single pulse with a given mass to charge (m/z) are passed through a fixed accelerating voltage (Uac), acquiring the same kinetic energy before entering the flight path with length L, the measured time-of-flight (T) will be described by the following equation:

\[
\frac{m}{z} = \frac{2UacT^2}{L^2} \quad \text{Eq. 2.7}
\]

See Figure 2.17 for a schematic of this ToF-SIMS instrument.

The major advantages of ToF over quadrupole and magnetic sector type analysers are the extremely high transmission (facilitating the small yield inherent to the static SIMS regime), the parallel detection of all masses and the unlimited mass range. Additionally, all types of insulating samples can be analysed without the requirement of previous conducting coating. In ToF-SIMS a charge compensation process is possible: between primary ion pulses, electrons are pulsed to counteract any surface charge build-up (Belu et al., 2003).

Unlike NanoSIMS and magnetic sector instruments, it is not necessary to have a previous knowledge of the sample in terms of the masses to be analysed, therefore, ToF-SIMS can be employed in exploratory experiments (Fletcher, 2009).
2.6.10.2 Duty cycle and repetition rate

In contrast with other SIMS approaches, in ToF-SIMS the primary ion is pulsed for a short time (typically between a few nanoseconds up to a few microseconds) in order to guarantee that the secondary ions enter the flight path simultaneously (Benninghoven, 1994a). Secondary ions formed by the primary pulse are dispersed as they travel through the detector where the lighter ions, such as hydrogen, may arrive in a time as short as a few µs. Heavier elements will take more time, tens or hundreds of µs. This principle poses the issue that if the primary ion is pulsed with a frequency > 10kHz, there is the possibility of detecting light elements belonging to a second pulse before all of the heavier secondary ion species from the first pulse have arrived at the detector, confusing the identification of species. The duty cycle refers to the time the ion beam is pulsed (on) divided by the time when it is not (off), and it is usually ~10^{-4}. Therefore, the analysis in ToF-SIMS takes longer compared to the few minutes that can take in dynamic SIMS instruments. A ToF-SIMS instrument can take hours to analyse through a sample, making it very time consuming to acquire depth profiles (Lyon and Henkel, 2010).
2.6.11 Comparison between NanoSIMS and ToF-SIMS

Compared with the currently available chemical and chemical imaging techniques, SIMS provides the greatest spatial resolution and the highest sensitivity, characteristics that are of particular interest for the analysis of single cells. Other methods such as inductively coupled plasma mass spectrometry (ICP-MS), ICP-AES (atomic emission spectrometry) or AAS (atomic absorption spectrometry) also provide high sensitivities (ppm-ppt) and can detect biological molecules, but these techniques lack the capability to link location with chemical composition of such molecules in the cell. Fluorescence microscopy, such as confocal laser scanning microscopy, confers the capability to target specific biomolecules (labelling with dyes) although at a lower spatial resolution and only allowing the analysis of a few biomolecules simultaneously. ToF-SIMS does not require labelling, while producing a full mass spectrum in each pixel, a characteristic that makes this technique suitable for exploratory experiments as well (Fletcher, 2009). Table 2.1 summarizes the main characteristics that distinguish NanoSIMS and ToF-SIMS, the two SIMS approaches that were used in the present work.

Table 2.1. Comparison of the two SIMS techniques that were used in the present work: ToF-SIMS and NanoSIMS. Modified from (Fearn, 2015).

<table>
<thead>
<tr>
<th></th>
<th>ToF-SIMS</th>
<th>NanoSIMS 50L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ion source</strong></td>
<td>Bi⁺, Ga⁺, Au⁺ₙ, C₆₀⁺</td>
<td>Cs⁺, O⁻, O₂⁻ or O₂⁺</td>
</tr>
<tr>
<td><strong>Upper mass limit</strong></td>
<td>Molecules (1500-2500 Da)</td>
<td>Elements (isotopes) and small molecules</td>
</tr>
<tr>
<td><strong>Primary ion dose (ions cm⁻²)</strong></td>
<td>&lt;10¹³</td>
<td>~10¹⁶</td>
</tr>
<tr>
<td><strong>Spatial resolution</strong></td>
<td>≥200 nm</td>
<td>≥50 nm</td>
</tr>
<tr>
<td><strong>Mass detection</strong></td>
<td>All the periodic table simultaneously</td>
<td>Only 7 simultaneously</td>
</tr>
<tr>
<td><strong>Ion optics</strong></td>
<td>Traditional (45-60°)</td>
<td>Co-axial (90°)</td>
</tr>
<tr>
<td><strong>Analysis of insulating samples</strong></td>
<td>No conducting coating needed</td>
<td>Conducting coating needed</td>
</tr>
</tbody>
</table>

2.6.12 Biological SIMS

2.6.12.1 Preparation of biological samples for SIMS analysis

SIMS techniques have a great potential to analyse different types of biological materials, nevertheless, samples have to meet a few characteristics in order to be candidates for SIMS analysis. Given that SIMS methods require operating under ultra-high vacuum (10⁻⁹-10⁻¹⁰ mbar) samples have to be stable under such conditions. The sample must be dry and free of volatile components, must be conducting in order to avoid surface charging
effects and the surface has to be flat or with low topography, particularly for NanoSIMS analysis (Rennert et al., 2011). Biological materials are naturally rich in water and removing this water without disrupting the biological structure is the greatest challenge when analysing biological materials in SIMS. Sample preparation plays a critical role in preserving the in vivo chemical composition for SIMS analysis and may widely vary between the diverse sample types of biological systems and the type of instrument used for analysis. The growth media components of the samples interfere in the direct analysis of cells in cultures with SIMS. The nutrient medium can be washed out, but this can disturb the intracellular composition (Chandra, 2008). The samples prepared in this project fall in the category of geomicrobiological samples, where the main components are bacterial cells and minerals. Cells have a high content of water and are soft, in contrast to minerals, which are harder materials where water is not an essential component. Therefore, the selected method should preserve the cells closest to their native hydrated state without altering the chemical composition of both cells and minerals. Cryogenic methods and chemical fixation are some of the preferred methods for preserving the structure of biological samples, as explained below.

**Chemical fixation**

Chemical fixation is a simple method ideal for structure preservation, although the distribution of mobile ions such as Na⁺, K⁺ or Ca²⁺ is not maintained, therefore, this method is only recommended when the analyte is fixed to a cell structure (Robinson and Castner, 2013; Fearn, 2015). This method usually involves immersing the samples in solutions of glutaraldehyde, formaldehyde and osmium tetroxide, where glutaraldehyde and formaldehyde cross-link proteins and osmium tetroxide cross-links lipids, preventing autolysis (Murtey and Ramasamy, 2016). Only formaldehyde cross-linking is reversible. Glutaraldehyde is the best fixative and is normally used at 2-4 % in buffer solutions, such as phosphate saline buffer (PBS) to keep a stable pH and osmolarity. The time that the samples are left in this solution depends on the sample thickness, but the rule is “1 hour per 1 mm”; for microbial cells this time can be as low as 30 seconds to 30 min, at room or refrigerator temperatures (4 °C) (Murtey and Ramasamy, 2016). After chemical fixation, cells are dehydrated with solvents (ethanol, isopropanol or acetone) and finally air dried, resin embedded or dried in a critical point drier (Schädler et al., 2008). Prior to chemical fixation a rinsing step with ammonium
formate or ammonium acetate is recommended, in order to remove excess salts from the growth media, which interfere with the secondary ion yield in SIMS, particularly ToF-SIMS (Robinson and Castner, 2013).

**Critical point drying**

This technique is used to completely dry the samples after chemical fixation, and a critical point drying instrument is required. This method involves a series of steps at varying pressure and temperatures: i) the solvent in the samples is initially exchanged by CO₂ at high pressure, ii) the pressure and temperature are raised until reaching the supercritical point of CO₂ — when the boundary between its liquid and gaseous phases are eliminated — CO₂ fills the chamber as a gas but presents a density as a liquid (and therefore surface tension effects are removed), finally iii) the pressure is slowly decreased releasing CO₂ and leaving the sample dry (Schädler et al., 2008). See Figure 2.18 for an example of bacterial cells preserved by this method.

![Image of bacterial cells](image)

**Figure 2.18.** Strain BoFeN1 cells well preserved by (a) critical point drying and (b) dried with hexamethyldisilazane (HDMS), while non-mineralised cells collapsed in (c) air drying and (d) freeze drying. Scale bar is 1 µm (Zetvogel et al., 2017).

**Resin embedding**

Resin embedding is a method extensively used in the preparation of eukaryotic samples, particularly plant cells (Moore et al., 2012b; Bell et al., 2013). Its use is very limited in the preparation of microbial cells, however, this method can be favourable to preserve vacuoles in bacteria, which usually collapse during dehydration (Schädler et al., 2008). In this method the samples are infiltrated with commercial epoxy resins (Araldite® is a
commonly used brand), the resin is polymerised by heat or by ultraviolet light producing a resin block, which can then be sectioned in order to produce thin slices of the specimen.

**Cryogenic techniques**

Cryogenic techniques are recognised as one of the best methods to preserve structure and composition for biological samples, including multicellular and unicellular organisms. In these methods the key is to rapidly freeze the specimens in order to avoid growth of ice crystals, which can damage the cells (McDonald and Auer, 2006). These procedures are usually performed by cryo-immobilisation, freeze drying or plunge freezing, and further imaging can be performed in instruments with cryo-stages such as cryo-SEM or cryo-TEM, to preserve the freezing conditions for frozen-hydrated specimens.

**Cryo-immobilisation**

In this method the specimen molecules are immobilised through ultrarapid freezing. This process is performed at high pressure (≈2000 atm) where heat is removed at a high speed and water forms amorphous ice (non-crystalline). This method is excellent at preserving the cells close to their native state, however it requires a specialised high-pressure freezing instrument, which is costly and thus not widely available in many research laboratories (McDonald and Auer, 2006).

**Freeze drying**

This technique relies on the principle of sublimation of water in the samples at high vacuum, evading the liquid to gas phase change, where surface tension forces greatly affect the structure. This method requires a freeze dryer, which is accessible, easy to use and available in many chemistry laboratories. The samples can be loaded in aluminium planchettes and these planchettes can be directly loaded into a SEM stage (Murtey and Ramasamy, 2016).

**2.6.12.2 Stable isotope probing (SIP)**

Classic microbiology techniques have provided the means to isolate a vast amount of microorganisms in nature. Unfortunately, it is well known that the majority of microorganisms in the environment (>90 %) are uncultivable by traditional laboratory
methods, obstructing their study. The introduction of genomic and proteomic approaches has allowed the identification of bacterial groups in different environments, that otherwise would not have been identified nor studied. However, the presence of these microorganisms does not necessarily imply that they are performing key transformations or playing an ecological role. During the last two decades molecular tools (e.g. metatranscriptomics, metaproteomics) have been established to access the metabolically active portion of the microbial community, supporting the connection between bacteria identification and function (Héry et al., 2008; Wagner, 2009).

Stable isotope probing (SIP) is a molecular tool that has proven its capabilities to directly link a defined metabolic process to specific members of a microbial community. In this technique microbial cultures are incubated in media containing stable isotope labelled components, typically $^{13}$C or $^{15}$N-labelled organics. Microorganisms actively assimilate these substrates and incorporate the $^{13}$C or $^{15}$N into their cellular material, including DNA, RNA and proteins (Musat et al., 2012). The labelled material can then be studied in ion imaging with SIMS techniques (Héry et al., 2008). Sensitivity is a limitation in the SIP technique, and SIMS has shown to be a good approach to tackle this issue as it is suitable for the analysis of isotopes with good sensitivity (Musat et al., 2012; Musat et al., 2016).

### 2.6.12.3 NanoSIMS applications

NanoSIMS has a high sensitivity, high spatial resolution and capability to analyse isotope ratios accurately, features that make it suitable for the study of trace elements in biological samples. Biological studies using NanoSIMS as the main or complementary technique have flourished in the past decade. NanoSIMS has been used to study the natural distribution of the elements of interest in eukaryotic and prokaryotic cells (Eybe et al., 2008; Moore et al., 2010) or by spiking/incubating the cells with isotopically labelled compounds to study the isotopic accumulation, which can be used to calculate the metabolic uptake of nutrients or to infer active metabolisms (Biesemeier et al., 2016; Giardina et al., 2018). Fluorescent in-situ hybridization (FISH) on its own or in combination with catalysed reporter deposition (CARD-FISH) are complementary techniques that have been applied to trace microbial activities from uncultured microbes in complex environmental samples (Musat et al., 2014; McGlynn et al., 2015; Stryhanyuk et al., 2018). *S. oneidensis* MR-1 was grown with $^{13}$C-acetate and $^{15}$N-
ammonium chloride to assess metabolic effects upon addition of riboflavin (as an extracellular electron transfer (EET) rate enhancer) when grown on electrodes. These cells were analysed in NanoSIMS and it was determined that the enhanced EET rate was not coupled to higher metabolic activity, as shown by the homogeneous $^{15}\text{N}$ accumulation, but it was linked to higher electron donor (lactate) oxidation (Fig. 2.19). This simple and yet relevant study exposed how a higher EET rate is generally assumed to accelerate metabolism in metal-reducing bacteria, but this EET rate can be unfavourable for the cells, as they have to deal with the increasingly oxidising cytoplasm conditions for the oxidation of lactate and the faster electron yield required. This finding is of relevance in microbial fuel cells applications in pollutant removal or metal recovery, which depend on enzymatic processes (Saito et al., 2017).

![Figure 2.19. NanoSIMS images of S. oneidensis MR-1 cells grown on electrodes with and without added riboflavin (RF) showing the similar $^{15}\text{N}$-ammonium chloride assimilation regardless of the addition of RF, suggesting that increased electron transfer is not linked to metabolic activity (Saito et al., 2017).](image)

In a CARD-FISH/NanoSIMS approach, the enriched KS microbial culture was incubated under autotrophic and heterotrophic conditions in order to elucidate the metabolic interactions of the two community members (Gallionellaceae sp. and Bradyrhizobium sp.), This microbial culture is an autotrophic Fe(II)-oxidiser nitrate-reducer consortium, where Gallionellaceae sp. is assumed to be the autotrophic organism accompanied by a heterotrophic community; metagenomics attempts trying to identify these heterotrophs have been made, but there is no consensus in the microorganisms identified. Therefore, CARD-FISH was used to trace the two main members and NanoSIMS was used to estimate the $^{13}\text{C}$ assimilation. The capability of Gallionellaceae sp. to fix CO$_2$ was confirmed, but surprisingly its heterotrophic metabolism was also detected, moreover, the heterotrophic community showed no metabolic gain from the autotrophs during autotrophic growth, therefore the question of how these heterotrophs survive when no organic carbon is available still remains (Fig. 2.20). This work highlights the importance of using complementary analysis for studying complex microbial consortia and evidences the importance of communities in the survival of
microorganisms, where it is clear that most microorganisms have remained uncultured because they rely on the metabolic benefits of an intricate microbial community (Tominski et al., 2018).

In a complementary NanoSIMS, ToF-SIMS and elemental analyser-isotope ratio mass spectrometry (EA-IRMS) study, the $^{15}$N assimilation by the marine cyanobacteria Synechococcus was estimated. The different techniques used estimated dissimilar levels of N assimilation, where NanoSIMS detected the lowest $^{15}$N accumulation, despite using the same sample preparation in ToF-SIMS. However, only NanoSIMS allowed the $^{15}$N accumulation estimation at the single-cell level (Fig. 2.21), which highlights the complementarity of multi-technique approaches. Moreover, metabolic heterogeneity was identified with NanoSIMS, which is impossible to detect with bulk analysis such as EA-IRMS. The importance of this study lies in its implications with the ocean biogeochemical cycles, where these and other N$_2$-fixing cyanobacteria are one of the main drivers of N transformations, therefore contributing to the global N cycle (Giardina et al., 2018).
All these studies have proved the effectiveness of NanoSIMS as a key instrument to address metabolic, ecologic and environmental microbiology problems, particularly at the single-cell level, although it is important to keep in mind the necessity of complementary tools for a comprehensive analysis.

2.6.12.4 ToF-SIMS applications

ToF-SIMS is suitable for the study of large secondary ion fragments. The study of large molecules such as lipids and proteins is often desired in biological studies. Eukaryotic cells predominate the ToF-SIMS biological studies, particularly for applications in medicine and pharmacology (Vickerman, 2009). Prokaryotic cells, such as bacteria, have been less studied by this technique, mainly due to the low spatial resolution (in the order of microns), that may not be able to resolve molecules at the subcellular level in single-prokaryotic cells, typically 1-2 µm in length. Some examples of applications of ToF-SIMS in the analysis of bacteria have focused on examining antimicrobials (Davies et al., 2017) or characteristic molecules (Ding et al., 2016) of bacterial biofilms. In this last study, ToF-SIMS was used to analyse molecular signals from *S. oneidensis* MR-1 biofilms dispersion after stressing the cells with Cr(VI). This work implied the design of a vacuum compatible microfluidic reactor, in order to image biofilms *in situ* (in their hydrated state), more representative of the conditions in which these processes occur. Moreover, multiple strategies were used for accurate peak allocation in the mass spectra, including extensive tests of reference materials, and these allowed the semiquantification of
biomarkers, such as riboflavin and quinolone (a quorum sensing molecule), additional to a loss of signals of polysaccharides, fatty acids and proteins, characteristic of EPS in biofilms (Fig. 2.22). This remarkable work showed the chemical characterisation capabilities of ToF-SIMS even in hydrated biological samples, with the big drawback of loss of spatial resolution to distinguish single cells (Ding et al., 2016).

One study by Cliff et al. traced the isotopic uptake of N and C by bacteria and fungal hyphae in a soil matrix (Cliff et al., 2002). In this work a spatial resolution of approximately 200 nm was reached, allowing the imaging of single bacterial cells (Fig. 2.23), but only for the identification of small molecules ($^{12}\text{C}^{14}\text{N}, ^{13}\text{C}^{14}\text{N}$ and $^{12}\text{C}^{15}\text{N}$). In this analysis it was determined that ToF-SIMS underestimated the isotopic $^{13}\text{C}$ and $^{15}\text{N}$ enrichment. The authors accepted this trade-off, as this error was acceptable for the purposes of the investigation, but advised that for higher precision isotope ratio measurement the analysis had to be maintained for longer (to improve counting statistics) (Cliff et al., 2002). This work demonstrates a general principle in ToF-SIMS (and really in all SIMS techniques): it is not possible to work at the highest sensitivity, mass resolution and spatial resolution simultaneously, because the improvement of one compromises the other. Additionally, biological ToF-SIMS studies reiterate how complex and challenging the molecular identification can be, and these reasons illustrate why ToF-SIMS has not become a routine technique in the analysis of microbial cells.
2.7 Overview of SIMS for the study of microbial As and Fe transformations

SIMS is a powerful technique that could be applied in the study of metal respiring bacteria interacting with minerals due to its high sensitivity, spatial resolution and accurate isotopic ratio measurements. These instruments present inherent artefacts that could severely distort the analysis, and to lessen this effect, an adequate sample preparation and preservation must be planned. NanoSIMS has been successfully used in the analysis of single bacterial cells from diverse environments, where even trace elements can be detected at the single-cell level. ToF-SIMS, on the other hand, has been successfully used to identify molecular components relevant in bacterial biofilms during metal respiration, although the spatial localisation of such molecular fragments at the single bacterial-cell level has yet to be demonstrated.

2.8 References


Lovley, D.R. (2014). Visualization of char...


Chapter 3: Methodology

In the work described in this thesis, cultures of environmental bacteria were used to inoculate the pertinent experiments. The advantage of using pure bacteria cultures over natural sediments is the simplified analysis in surface-sensitive techniques such as SIMS. Moreover, the components (bacteria, minerals, etc.) are more concentrated than in sediments, and therefore, are easier to locate and characterize, optimising the time allocated for the use in the instruments. The experiments can be classified in two types: the As(V)-Fe(III) reduction and the As(III)-Fe(II) oxidation experiments. In general terms, two phases of the samples were analysed, the aqueous and the solid phases. The aqueous phase analysis included the ferrozine assay (for Fe(II) quantification) and the following chromatography approaches: ICP-AES (for total As and Fe), IC-ICP-MS (for As species), IC (for acetate, nitrate and nitrite) and HPLC (for flavins quantification). The analysis of the solid phase used different imaging and spectroscopic techniques such as NanoSIMS and ToF-SIMS and complementary SEM, XRD and TEM. All these techniques, including their principles (except for SIMS) and the type of information they produce, will be explained in the following sections.

3.1 Bacteria strains and culture

The bacteria strains selected for this work have been isolated, characterised and are amenable to grow in laboratory conditions.

For the As(V) and Fe(III) reduction studies the strains used were:

- *Shewanella* sp. strain ANA-3, denominated wild-type (WT), the facultative Fe(III) and As(V)-respiring bacterium strain, isolated from an As-treated wooded pier in Massachusetts and kindly donated by Prof Newman (Saltikov et al., 2003).
- *S. ANA-3 ARRA3*, an *arrA* deleted mutant strain, that does not respire As(V) but maintains its Fe(III) respiring capability (also kindly donated by Prof Newman) (Saltikov and Newman, 2003).
- *Geobacter sulfurreducens*, a strictly anaerobic Fe(III)-reducing bacteria. Strain obtained from the Geomicrobiology research group microbial culture collection at the University of Manchester.
The results of these As(V)-Fe(III) reduction experiments are presented in Chapters 4, 5 and 6.

For the As(III) and Fe(II) oxidation studies the strains used were:

- **Acidovorax** sp. strain ST3, a facultative heterotrophic denitrifier and As(III)-oxidiser bacterium, a nitrate-dependent iron-oxidising (NDFO) organism, isolated from an As-polluted paddy soil in China (Zhang et al., 2017) and kindly donated by Prof Fang-jie Zhao (Nanjing Agricultural University, Nanjing, China). The results of these experiments are presented in Chapter 7.

- **Paracoccus** sp. strain QY30, a facultative autotrophic denitrifier and As(III)-oxidiser bacterium, also a NDFO organism, isolated from an As-polluted paddy soil in China (Zhang et al., 2015) and kindly donated by Prof Fang-jie Zhao. The results of these experiments are presented in Appendix A.

### 3.1.1 Inoculum preparation

All the chemicals used had >99% purity and deionised water (18 MΩ) was used in all the sample handling and to prepare all solutions, growth media, etc. All the materials and solutions that were in contact with the cells were autoclaved (121°C/15 psi/15 min), unless indicated otherwise.

All the bacteria strains, except for G. sulfurreducens, were first cultured under aerobic conditions to promote the growth and formation of biomass. All these steps were executed under sterile conditions.

Both strains of ANA-3 and the strain ST3 were first re-inoculated from frozen stocks and streaked on plates of the nutritionally rich lysogeny broth (LB) (in g L⁻¹: peptone 10, yeast extract 5, NaCl 5, agar 12). S. ANA-3 strains were incubated for 24 h at 30°C, whereas ST3 was incubated at 28°C for 48 h in the dark. QY30 was re-inoculated from frozen stocks and streaked on aerobic plates of R2A medium (in g L⁻¹: yeast extract 0.5, proteose peptone 0.5, casamino acids 0.5, glucose 0.5, soluble starch 0.5, K₂HPO₄ 0.3, MgSO₄ ·7H₂O 0.05, sodium pyruvate 0.3 and agar 15) and incubated at 28°C for 48 h in the dark.

After incubation, single-isolated colonies were picked up from the plates with a sterile loop and suspended in Erlenmeyer flasks containing the same liquid media (no agar) for each strain, in order to obtain a liquid inoculum. The flasks were incubated in a shaker incubator at 150 rpm for 24 h at the same temperature. This liquid media was used to
generate enough biomass prior to switching to anaerobic conditions to form the final inoculum used in the reducing or oxidising experiments.

The ANA-3 strains were transferred to 100 mL serum bottles containing anaerobic minimal medium (AMM). See AMM composition in table 3.1 and vitamins and trace elements solution preparation in tables 3.2 and 3.3, respectively. AMM bottles were incubated statically at 30°C for 36-48 h until a pink colour was observed in the cultures (indicating the lag-phase growth stage of the cells). ST3 was transferred to 100 mL serum bottles containing anaerobic low-phosphate medium (LP) (table 3.4) whereas QY30 was transferred to 100 mL serum bottles containing minimal salts medium (MSM) (table 3.5). ST3 was incubated at 28°C and QY30 at 30°C for 48 h until a light pink colour appeared. These two strains generate less biomass than S. ANA-3 or G. sulfurreducens.

G. sulfurreducens was kept in anaerobic conditions throughout until obtaining the inoculum. This strain is stored in liquid NBAF medium. See NBAF composition in table 3.6 and the salts and mineral solutions used to prepare this medium in tables 3.7 and 3.8, respectively. To prepare fresh inoculum, 10 mL of the stock cells were inoculated in 100 mL NBAF serum bottles and incubated at 30°C for 24-48 h until a bright pink colour was observed.

3.1.1.1 Cell harvesting

After the incubation of the inoculum, the culture medium was removed to assure that only cells were introduced in the experiments, with no additional nutrients than the ones in the conditions tested. This washing step was performed in anaerobic conditions with anaerobic buffers. The cultures in the serum bottles were transferred to 50 mL centrifuge tubes and centrifuged for 30 min/2469 g. The supernatant was discarded, and the pellet obtained was re-suspended in 20 mL of buffer by sparging N₂. 20 mM PIPES [piperazine- N,N’-bis (2-ethanesulfonic acid)] buffer was used for ST3 and QY30 and 30 mM bicarbonate buffer was used to wash ANA-3 and G. sulfurreducens cells. This suspension was centrifuged again (30 min/2469 g). The supernatant was discarded, and the pellet was re-suspended in a small volume of buffer (≈5 mL) in order to obtain a concentrated inoculum of cells. The concentration of cells was estimated spectrophotometrically by measuring the absorbance at a wavelength λ=600 nm in a Jenway 6715 UV/Vis spectrophotometer, using dH₂O as blank. Cells were harvested the same day before starting the experiments.
Table 3.1. Anaerobic minimal medium composition, in g L\(^{-1}\), unless stated otherwise. pH= 6.8-7.0 (Saltikov et al., 2003).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_2HPO_4)</td>
<td>0.225</td>
</tr>
<tr>
<td>(KH_2PO_4)</td>
<td>0.225</td>
</tr>
<tr>
<td>(NaCl)</td>
<td>0.46</td>
</tr>
<tr>
<td>((NH_4)_2SO_4)</td>
<td>0.225</td>
</tr>
<tr>
<td>(MgSO_4\cdot7\ H_2O)</td>
<td>0.117</td>
</tr>
<tr>
<td>(NaHCO_3)</td>
<td>4.2</td>
</tr>
<tr>
<td>Fumaric acid (40 mM)</td>
<td>4.64</td>
</tr>
<tr>
<td>Sodium lactate (20 mM)</td>
<td>2.59</td>
</tr>
<tr>
<td>Trace elements (SL 10)</td>
<td>5 mL L(^{-1})</td>
</tr>
<tr>
<td>Vitamins solution</td>
<td>5 mL L(^{-1})</td>
</tr>
</tbody>
</table>

Table 3.2. Vitamins solution (DL) composition, in mg L\(^{-1}\) (Saltikov et al., 2003).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-aminobenzoic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Biotin</td>
<td>5.0</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.0</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>5.0</td>
</tr>
<tr>
<td>Thiamine</td>
<td>5.0</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Thiotic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin B(_{12})</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 3.3. Trace elements solution (SL-10) composition, in mg L\(^{-1}\), unless stated otherwise (Saltikov et al., 2003).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl (25 % v/v)</td>
<td>10 mL</td>
</tr>
<tr>
<td>FeCl(_2\cdot4)H(_2)O)</td>
<td>1.50 g L(^{-1})</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>70.0</td>
</tr>
<tr>
<td>MnCl(_2\cdot4)H(_2)O)</td>
<td>100.0</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>6.0</td>
</tr>
<tr>
<td>CoCl(_2\cdot6)H(_2)O)</td>
<td>190.0</td>
</tr>
<tr>
<td>CuCl(_2\cdot2)H(_2)O)</td>
<td>2.0</td>
</tr>
<tr>
<td>NiCl(_2\cdot6)H(_2)O)</td>
<td>24.0</td>
</tr>
<tr>
<td>Na(_2)MoO(_4)\cdot2)H(_2)O)</td>
<td>36.0</td>
</tr>
</tbody>
</table>

Table 3.4. Low-phosphate (LP) medium composition for the culture of Acidovorax sp. strain ST3 in g L\(^{-1}\), unless stated otherwise. pH= 6.8 (Hegler et al., 2008).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(KH_2PO_4)</td>
<td>0.6</td>
</tr>
<tr>
<td>(NH_4Cl)</td>
<td>0.3</td>
</tr>
<tr>
<td>(MgSO_4\cdot7\ H_2O)</td>
<td>0.5</td>
</tr>
<tr>
<td>(CaCl_2\cdot2H_2O)</td>
<td>0.10</td>
</tr>
<tr>
<td>Trace elements (SL 10)</td>
<td>1.0 mL L(^{-1})</td>
</tr>
<tr>
<td>Vitamins solution</td>
<td>1.0 mL L(^{-1})</td>
</tr>
<tr>
<td>(NaHCO_3)</td>
<td>0.42</td>
</tr>
<tr>
<td>Selenite tungstate solution ((0.5\ g\ of\ NaOH, 3\ mg\ of\ Na_2SeO_3\cdot5H_2O\ \text{and}\ 4\ mg\ of\ Na_2WO_4\cdot2H_2O\ \text{in} 1\ L\ of\ 0.5\ H_2O))</td>
<td>1.0 mL L(^{-1})</td>
</tr>
</tbody>
</table>

Table 3.5. Mineral salts medium (MSM) composition for the culture of Paracoccus sp. strain QY30 in g L\(^{-1}\), unless stated otherwise. pH= 7.2 (Zhang et al., 2015).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Na_2HPO_4\ 2H_2O)</td>
<td>7.9</td>
</tr>
<tr>
<td>(KH_2PO_4)</td>
<td>1.5</td>
</tr>
<tr>
<td>(NH_4Cl)</td>
<td>0.3</td>
</tr>
<tr>
<td>(MgSO_4\cdot7\ H_2O)</td>
<td>0.1</td>
</tr>
<tr>
<td>(NaHCO_3)</td>
<td>0.8401</td>
</tr>
<tr>
<td>Vitamins solution</td>
<td>10 mL L(^{-1})</td>
</tr>
<tr>
<td>Trace elements (SL 10)</td>
<td>5 mL L(^{-1})</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.8303 ((^{13}C)-acetate) 0.821 ((\text{regular}))</td>
</tr>
</tbody>
</table>

Table 3.6. Trace elements solution (SL-10) composition in mg L\(^{-1}\) (Saltikov et al., 2003).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl (25 % v/v)</td>
<td>10 mL</td>
</tr>
<tr>
<td>FeCl(_2\cdot4)H(_2)O)</td>
<td>1.50 g L(^{-1})</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>70.0</td>
</tr>
<tr>
<td>MnCl(_2\cdot4)H(_2)O)</td>
<td>100.0</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>6.0</td>
</tr>
<tr>
<td>CoCl(_2\cdot6)H(_2)O)</td>
<td>190.0</td>
</tr>
<tr>
<td>CuCl(_2\cdot2)H(_2)O)</td>
<td>2.0</td>
</tr>
<tr>
<td>NiCl(_2\cdot6)H(_2)O)</td>
<td>24.0</td>
</tr>
<tr>
<td>Na(_2)MoO(_4)\cdot2)H(_2)O)</td>
<td>36.0</td>
</tr>
</tbody>
</table>
Table 3.6. NBAF medium composition, in g L\(^{-1}\), unless stated otherwise, pH= 7.0 (Lovley and Phillips, 1988).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl(_2)·2H(_2)O</td>
<td>0.04</td>
</tr>
<tr>
<td>MgSO(_4)·7 H(_2)O</td>
<td>0.1</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>1.8</td>
</tr>
<tr>
<td>Na(_2)CO(_3)·10 H(_2)O</td>
<td>1.15</td>
</tr>
<tr>
<td>Sodium acetate (20 mM)</td>
<td>1.23</td>
</tr>
<tr>
<td>Fumaric acid (40 mM)</td>
<td>4.64</td>
</tr>
<tr>
<td>Na(_2)SeO(_4)</td>
<td>0.19</td>
</tr>
<tr>
<td>Salts solution</td>
<td>10 mL L(^{-1})</td>
</tr>
<tr>
<td>Vitamins solution</td>
<td>15 mL L(^{-1})</td>
</tr>
<tr>
<td>Mineral elixir</td>
<td>10 mL L(^{-1})</td>
</tr>
</tbody>
</table>

Table 3.7. 100x NB salts solution composition in g L\(^{-1}\) (Lovley and Phillips, 1988).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH(_2)PO(_4)</td>
<td>42.0</td>
</tr>
<tr>
<td>K(_2)PO(_4)</td>
<td>22.0</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>20.0</td>
</tr>
<tr>
<td>KCl</td>
<td>38.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>36.0</td>
</tr>
</tbody>
</table>

Table 3.8. NB mineral elixir solution composition in g L\(^{-1}\) (Lovley and Phillips, 1988).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrilotriacetic acid</td>
<td>2.14</td>
</tr>
<tr>
<td>MnCl(_2)·4H(_2)O</td>
<td>0.1</td>
</tr>
<tr>
<td>FeSO(_4)·7H(_2)O</td>
<td>0.3</td>
</tr>
<tr>
<td>CoCl(_2·6H(_2)O</td>
<td>0.17</td>
</tr>
<tr>
<td>ZnSO(_4·7H(_2)O</td>
<td>0.2</td>
</tr>
<tr>
<td>CuCl(_2·2H(_2)O</td>
<td>0.03</td>
</tr>
<tr>
<td>AIZ(SO(_4))(_2·12H(_2)O</td>
<td>0.005</td>
</tr>
<tr>
<td>H(_2)BO(_3)</td>
<td>0.005</td>
</tr>
<tr>
<td>Na(_2)MoO(_4·2H(_2)O</td>
<td>0.09</td>
</tr>
<tr>
<td>NiSO(_4·6H(_2)O</td>
<td>0.11</td>
</tr>
<tr>
<td>NaWO(_4·2H(_2)O</td>
<td>0.02</td>
</tr>
</tbody>
</table>
3.2 As(V) and Fe(III)-reducing experiments

In the As(V) and Fe(III)-reducing experiments a synthetic amorphous Fe(III)-oxyhydroxide (ferrihydrite) was prepared with sorbed arsenate in order to produce a thin film on a silicon wafer, to support cell colonisation on the surface, where bacteria used the Fe(III) and As(V) as terminal electron acceptors. For this reason, only biofilm growth was assessed in these samples. This thin film was flat and suitable for analysis in SIMS instruments.

3.2.1 Arsenical Fe(III)-(oxyhydr)oxide thin film

The ferrihydrite was prepared following a standard procedure by dissolving 16.2 g of FeCl₃ in 500 mL of deionised water. The pH was raised to 7.0 by adding drop by drop a 10 M NaOH solution under constant stirring, the pH was kept at 7.0 for an hour adjusting with 10 M NaOH. The resulting solution was centrifuged (2688 g/20 min) and washed with 12H₂O x6 times (Schwertmann and Cornell, 2000). The final concentration of biologically available Fe in the ferrihydrite solution was determined with the ferrozine analysis (explained in detail afterwards). A solution of sodium arsenate (Na₂HAsO₄ ·7H₂O, Sigma-Aldrich®) was prepared to give a final concentration of 12 % mol/mol As/Fe when mixed with the ferrihydrite solution. This mixed solution was stirred in a roller shaker for 24 h to favour the sorption of As(V) on the Fe(III) mineral surface sites. Thin films of arsenical Fe(III)-(oxyhydr)oxide were prepared by mixing 2 mL of the As/Fe solution with 0.5 mL of methanol (2:0.5) to improve the adhesion of the mineral grains on the silicon surface. 40 µL of this solution were pipetted on acetone-cleaned boron doped silicon wafers (7 x 7 x 0.5 mm) and let dry overnight (Fig. 3.1).

Figure 3.1. Silicon wafer with dry arsenical Fe(III)-(oxyhydr)oxide thin film coating. Photo by the author.
3.2.2 Pure culture experiments

The pure culture experiments for NanoSIMS and ToF-SIMS were prepared in 15 mL anaerobic serum bottles and this set up was carried out under anaerobic and sterile conditions. In these experiments the arsenical Fe(III)-(oxyhydr)oxide was used as the sole terminal electron acceptor and $^{13}$C-labelled lactate (for S. ANA-3) and $^{13}$C-labelled acetate (for G. sulfurreducens) were used as the electron donors. The $^{13}$C is expected to be accumulated by the cells, allowing the identification of cells that were metabolically active during the experiment as their $^{13}$C/$^{12}$C ratio would be above natural abundance. The thin film coated Si wafers were placed vertically in a plastic holder that was fixed to the bottom of the bottle with silicon grease (to avoid disturbance of the thin film). A modified fresh water medium (table 3.9) (Wilkins et al., 2007) with 20 mM $^{13}$C-labelled sodium lactate or acetate was used and 7 mL of this medium were slowly added to the serum bottles. The harvested-washed cells of ANA-3 suspended in bicarbonate buffer were inoculated to give a final OD$_{600}$ of 0.5 and a OD$_{600}$ of 0.3 was used for G. sulfurreducens. The bottles were static incubated at 30°C in the dark for 11 days. Control experiments were also prepared, S.ANA-3 WT and G. sulfurreducens with no electron donor added (“no donor”) and uninoculated FWM (“no cells”). Supernatant samples were withdrawn at intervals of 3 days to measure aqueous Fe and As species and flavins (if applicable). Total aqueous Fe and As were measured only at day 11. All manipulation of these bottles was very gentle in order to keep the Si wafers vertical and to not disturb the thin film.

Table 3.9. Fresh water medium (FWM) composition with Fe(III)-oxyhydroxide as electron acceptor, in g L$^{-1}$, unless stated otherwise. pH= 6.8 (Lovley and Phillips, 1988).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO$_3$</td>
<td>2.5</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>0.25</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$H$_2$O</td>
<td>0.6</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Trace elements (SL-10)</td>
<td>10 mL L$^{-1}$</td>
</tr>
<tr>
<td>Vitamins solution (DL)</td>
<td>10 mL L$^{-1}$</td>
</tr>
<tr>
<td>$^{13}$C-sodium lactate (20 mM)</td>
<td>0.8303</td>
</tr>
</tbody>
</table>
3.3 As(III) and Fe(II)-oxidising experiments

In these experiments biofilm and planktonic growth cultures were prepared. For these tests LP or MSM media (tables 3.4 and 3.5, respectively) were used and amended with sodium arsenite (0.5 mM for ST3 and 1 mM for QY30), 10 mM FeCl$_2$·4H$_2$O and 10 mM $^{13}$C-labelled. The FeCl$_2$·4H$_2$O was added to the medium first forming a green-whitish precipitate, potentially vivianite ($\text{Fe}^{2+}\text{Fe}^{2+}(\text{PO}_4)_{2}·8\text{H}_2\text{O}$), due to the reaction of Fe(II) with PO$_4$. This precipitate was removed by filter-sterilising the medium (0.22 µm), keeping anaerobic conditions, and the medium was not autoclaved. Afterwards, filter-sterilised As(III) and $^{13}$C-labelled acetate were added. Anaerobic conditions were crucial for the manipulation of these media, given that Fe(II) is spontaneously oxidised by oxygen, therefore, all the serum bottles in these experiments were degassed with a N$_2$:CO$_2$ (80:20) mix for 1 h.

3.3.1 Planktonic growth

Planktonic growth experiments were performed in 30 mL serum bottles containing 20 mL of the media LP or MSM media. Three bottles (replicates) were inoculated with ST3 or QY30 cells using the harvested cells suspended in PIPES buffer to give a final OD$_{600}$ of 0.15. See Figure 3.2B.

3.3.2 Biofilm growth

Biofilm samples were obtained by introducing two clean Si wafers in 15 mL serum bottles which were held vertically in plastic holders which were glued with silicon grease to the bottom of the bottle (analogous to the reducing experiments). 7 mL of the growth medium was slowly added (3 replicates) and cells were also incubated to give a final

![Figure 3.2. Serum bottles at the start of the As(III) and Fe(II)-oxidising experiments. A) Biofilm growth (note the Si wafers at the bottom of the bottle) and B) planktonic growth.](image)
OD$_{600}$ of 0.15. The Si wafer supported cell’s growth in biofilm and precipitated biominerals. See Fig. 3.2A.

3.4 Aqueous geochemical analysis

3.4.1 pH
The pH in solutions, growth media and in supernatants of the samples was measured with a Jenway 3520 pH/Eh meter using a Fisherbrand® FB68801 electrode. The electrode was stored in a buffer solution at pH 4, and before use it was rinsed with $\mathrm{dH}_2\mathrm{O}$, excess water was dried with a paper towel and the electrode tip was submerged in the sample. The pH was annotated when the reading in the display was stable. The pH meter was regularly calibrated using Honeywell Fluka™ buffer solutions at pH 4.0, 7.0 and 10.0.

3.4.2 Ferrozine assay for biologically available Fe(II) quantification
The Fe(II) and total biologically available Fe(II) were quantified in filtered supernatants (0.22µm) of the samples (to remove solids), following a standard methodology (Lovley and Phillips, 1986). The principle of the ferrozine assay is that the ferrozine molecule binds ferrous iron, forming a stable purple complex whose intensity is proportional to the Fe(II) concentration. 100 µL of supernatant were digested in 4.9 mL of 0.5 M HCl solution and left to digest for 1 h, in order to solubilise all the Fe in the samples. 50 µL of this digestion were reacted with 2.45 mL of a ferrozine solution (1 g ferrozine and 11.96 g HEPES in 1 L of $\mathrm{dH}_2\mathrm{O}$, pH=7.0) in a plastic macrocuvette for 1 min and the absorbance was measured at 562 nm in a Jenway 6850 UV/Vis spectrophotometer. To measure total biologically available Fe(II), 200 µL of the reductant hydroxylamine hydrochloride (6.25 M) were added to the digested sample and left to react for a further 1 h, in order to reduce any Fe(III) to Fe(II). Total biologically available Fe(II) was measured similarly to Fe(II). The ferrozine solution was used as blank for these measurements. Calibration curves were obtained every time the ferrozine assay was performed, using FeCl$_2$ solutions (0.5, 1.0, 5, 10, 20 and 50 mM) as reference and measured following the same procedure as the samples.
3.4.3 Quantification of As and Fe

Arsenic species were quantified by ion chromatography-inductively coupled plasma mass spectrometry (IC-ICP-MS). In this technique the sample is passed through an ion exchange column (IC) to separate the ionic species and ICP-MS detects and quantifies the analytes at trace levels (high sensitivity). These samples were diluted in $\text{dH}_2\text{O}$ to bring the samples to the detection range (1-100 µg L$^{-1}$). These analyses were performed by Mr Paul Lythgoe.

Total Fe and total As were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using a Perkin–Elmer Optima 5300 DV. In ICP-AES the sample is nebulised (to form a mist) and introduced in a chamber with argon plasma. As the sample comes into contact with the plasma it is broken down to charged ions, and the sample loses electrons generating light (photons) of a wavelength specific for each element ionised. The intensity of the light emitted is proportional to the atoms ionised and this is compared to previous measurements (reference) to estimate the concentration. The samples are dissolved in 2 % v/v HNO$_3$ in order to solubilise all the Fe and As and brought to a concentration in the range of 1-10 ppm prior to analysis. These analyses were performed by Mr Paul Lythgoe.

3.4.4 Quantification of flavins

Flavin mononucleotide (FMN) and riboflavin were measured in the supernatants of the ANA-3 experiments using high performance liquid chromatography (HPLC) with a fluorescence detector. In HPLC samples are mixed with a solvent (methanol) called the mobile phase and passed through an adsorbent column (stationary phase). The sample components interact or are retained by the stationary phase, eluting at different flow rates, which allows the separation and identification of the components. The HPLC used was a Thermo Scientific® Dionex BioLC with a GP50 pump using a Dionex Utimate 3000 fluorescence detector set at 450 nm (excitation) and 520 nm (emission), using a C18 column with a mobile phase of 40% v/v methanol at a flow rate of 1 mL min$^{-1}$. Riboflavin and FMN solutions (0.1-1.0 µM) were used as reference to generate a calibration curve. These analyses were performed by Mr Alastair Bewsher.
3.4.5 Quantification of nitrate, nitrite and acetate
Nitrate, nitrite and acetate were simultaneously measured in the experiments with the As(III)-oxidisers ST3 and QY30 by ion chromatography (IC). In IC the sample is injected and passed through an ionic exchange column, the analytes interact with the column components and are eluted at different flow rates. Ions are detected by their elution time, forming a spectrum, and the intensity of the peak is proportional to the ion concentration. These samples were filtered (0.22 µm) and dissolved in deionised water to keep the samples in the detection range (NO₂/NO₃ 0.1-30 mg L⁻¹ and acetate 0.03-3 mg L⁻¹). The equipment used was a Dionex ICS 5000 equipped with an AS11HC 0.4 mm capillary ion exchange column or an AS18 2 mm microbore ion exchange column. These samples were analysed by Mr Alastair Bewsher.

3.5 Sample preservation
3.5.1 Sample preservation tests for scanning electron microscopy (SEM) and SIMS
Sample preparation is a crucial step for the successful analysis of biological samples in SEM, NanoSIMS and any technique that works under high vacuum. SEM and SIMS operate at high vacuum conditions in order to remove air molecules that could scatter the incident electrons or ions (Vickerman et al., 1989). Biological samples have a high-water content, and under vacuum the cells can collapse and shrink. The success of SIMS techniques is directly related to good sample preparation. For this reason, at the start of my PhD project I dedicated some time testing sample preservation techniques for SEM-SIMS analysis. These tests were performed on samples prepared with the As(V) and Fe(III)-reducer *Shewanella* sp. strain ANA-3 grown on the arsenical Fe(III)-oxyhydroxide mineral and ^13^C-lactate, as detailed previously. This novel sample set-up was sturdy but proved to be fragile after constant manipulations (mainly chipping of the Si wafer), therefore the sample preparation had to be a technique that required limited manipulation but preserved the structure and integrity of the cells along with the chemical composition (Chandra and Morrison, 1992). Moreover, given that the aim of preparing these samples was to analyse them in NanoSIMS/ToF-SIMS, which are very
surface sensitive techniques, the sample preparation had to remove any unwanted residues coating the cells and minerals.

Cryogenic techniques are usually the preferred approaches for structure preservation and minimal disturbance in biological and geomicrobiological samples (Edelmann, 2002; Schädler et al., 2008). These kinds of samples have a mixture of components, including cells (softer & hydrated) and minerals (harder). There were no cryogenic instruments solely dedicated to preserve samples for electron microscopy and high vacuum analysis, for instance, critical point dryer or cryo-SEM available for this work. In addition, these cryogenic methods might not be suitable for further study in NanoSIMS/ToF-SIMS, as the cryogenic conditions would not be conserved during SIMS analysis (due to the lack of a cryo-stage). For this reason, chemical fixation and dehydration (a standard sample preservation method) and a number of freezing-conditions approaches were devised, in order to resemble the cryogenic conditions analogous to sample preparation in dedicated cryogenic instruments.

3.5.2 Methodology

Five sample preservation methods were tested with the Fe(III)/As(V) reduction experiment samples in order to find the best preservation method. At day 11 of incubation the Si wafers were preserved by these five methods:

1) **Chemical fixation and dehydration.** The wafers were submerged in solutions of decreasing concentrations of glutaraldehyde for cell fixation and solutions of increasing ethanol concentration for dehydration. For cell fixation, solutions of glutaraldehyde (2.5, 1.5 and 0.75 % v/v) in phosphate buffered saline (PBS in g/L NaCl 8.0, KCl 0.2, Na₂HPO₄ 1.42 and KH₂PO₄ 0.27) were used. The wafers were first immersed in the 2.5 % glutaraldehyde solution for 4 h and then for 30 min in the 1.5 and 0.75 % v/v solutions. Afterwards, glutaraldehyde was exchanged for dH₂O and incubated for 30 min. After cell fixation, the samples were dehydrated by exchanging the dH₂O for ethanol solutions of increasing concentrations (25, 30, 40, 50, 60, 70, 80, 90 and 100 % v/v) and incubated for 30 min in each solution.
This process was performed in an anaerobic cabinet, and once the wafers were removed from the ethanol, they were air dried in the cabinet overnight.

2) Freeze drying. For freezing the serum bottles were opened, keeping the Si wafers in the plastic holder, and the bottle was directly placed in the freeze drier with pierced aluminium foil, replacing the rubber cap, to allow the removal of water. The samples were freeze dried overnight (> 12 h) at -40°C for primary drying only, while the chamber pressure was 60 mTorr. No secondary drying was performed. These conditions were the same in the rest of the freeze-drying modifications.

3) Liquid nitrogen freezing plus freeze drying. For this modified freeze drying the Si wafers were first removed from the bottle and submerged in liquid N₂ for a few seconds. Afterwards, the wafers were taken back to an empty serum bottle covered with pierced aluminium foil where they were freeze dried overnight (-40°C/60 mTorr).

4) Glycerol preservation plus freeze drying. The Si wafers were first removed from the serum bottle and 40 µL of sterile glycerol (25 % v/v) were deposited on the Si wafers (to act as cryo-protector). Then, the wafers were taken back to an empty serum bottle covered with pierced aluminium foil where they were freeze dried overnight (-40°C/60 mTorr).

5) Cryogenic spray plus freeze drying. A commercial cryogenic aerosol was sprayed on the Si wafer as a proxy of cryogenic methods. The Si wafers were removed from the serum bottle, sprayed with the cryogenic aerosol and freeze-dried overnight in a serum bottle covered with pierced aluminium foil (-40°C/60 mTorr).

Once dry, all the Si wafers were coated with 10 nm of Pt with a sputter coater in order to improve conductivity of the samples. The samples were imaged in SEM and selected samples were also imaged in NanoSIMS.
3.5.3 Results of the sample preservation tests

3.5.3.1 Chemical fixation and dehydration

This sample preparation method preserved the structure and integrity of cells (Fig. 3.3). The sample surface looked free of residues (e.g. growth medium and organic material). These samples were further imaged in NanoSIMS, where the chemical fixation-dehydration proved to be adequate for cell’s preservation and analysis, allowing the imaging of single cells without any residues or interferences (Fig. 3.4). In the samples the cells accumulated up to 8% of $^{13}\text{C}/^{12}\text{C}$. Nonetheless, this method implies the immersion of the samples in many solutions, which alters their chemistry (Edelmann, 2002). Chemical fixation particularly alters the salt ions distribution ($\text{Na}^+$, $\text{K}^+$, $\text{Cl}^-$) and is only recommended when the analyte of interest is fixed to a cell structure (Robinson and Castner, 2013). Additionally, high salt ions concentrations in biological samples can produce high secondary ion signals and produce an unreliable secondary ion yield in ToF-SIMS, in which case rinsing the samples with ammonium formate is recommended (Fearn, 2015); this step is compatible with chemical fixation. Moreover, glutaraldehyde fixation is an oxygen consuming reaction, therefore, oxygen sensitive samples might not be suitable for this method (Schädler et al., 2008).

![Figure 3.3. SEM images of S. ANA-3 cells preserved by chemical fixation and dehydration.](image)
These samples showed very few cells and numerous imprints of the cells’ putative location, additional to a residue coating the surface (Fig. 3.5), possibly composed of the salts in the growth medium and other organic materials. This sample was further imaged in NanoSIMS (Fig. 3.6) and although most of the cells were removed from the sample, there were still a few cells buried in the residual layer, which were enriched with up to 25 % of $^{13}\text{C}/^{12}\text{C}$. Unfortunately, the residual layer also showed accumulation of $^{13}\text{C}$, implying that this residue was biogenic, possibly composed of extracellular polymeric substances (EPS) supporting biofilm growth of cells. This residual layer was undesirable for surface sensitive SIMS analysis.

**3.5.3.2 Freeze drying**

Figure 3.4. NanoSIMS images of S. ANA-3 cells preserved by chemical fixation and dehydration. Notice that many active cells accumulating up to 8 % $^{13}\text{C}$ were imaged and no residual layer was present, allowing the imaging of single cells.

Figure 3.5. SEM images of areas preserved by direct freeze drying. The blue arrows in the first panel are pointing at mineral surfaces devoid of cells, in contrast to the Si wafer, fully coated with cell’s imprints. A thick coating is visible in both panels with the imprint of where the cells were putatively located.
As it can be noted, direct freeze-drying was the least invasive approach given that it involves almost no manipulation of the samples or introduction of external chemicals, and this was the method that conserved the samples closest to their native state. Therefore, the biomass abundance and distribution could be considered the closest to the original hydrated conditions, and the same applies for the $^{13}\text{C}$ accumulation, which did not suffer from any dilution or washing steps. It has been determined that chemical fixation has a diluting effect on $^{13}\text{C}$ accumulation and stable isotopic labelling in general (Musat et al., 2014; Stryhanyuk et al., 2018), which could be significant in quantitatively focused investigations, considering that the chemical fixation samples (Fig. 3.4) showed up to 8 % against the 25 % of $^{13}\text{C}$ accumulation in freeze-dried samples (Fig. 3.6). However, the $^{13}\text{C}$ accumulation was used to distinguish active from inactive cells in these experiments, and not to calculate absolute $^{13}\text{C}$ uptake values.

![Figure 3.6. NanoSIMS images of an area preserved by direct freeze drying. The first panel is the secondary electron (SE) image showing imprints of the cells on the residual layer, the second panel is an overlap of $^{56}\text{Fe}^{16}\text{O}$ in red, $^{13}\text{C}$ in green and $^{75}\text{As}$ in blue, and the last panel is the $^{13}\text{C}/^{12}\text{C}$ ratio, showing a few cells with up to 25 % $^{13}\text{C}$ accumulation.](image1)

### 3.5.3.3 Liquid nitrogen and freeze drying

The structure of the cells was preserved in some cases using a combination of liquid nitrogen and freeze drying, while other cells looked flat and disturbed (Fig. 3.7). Moreover, this technique left a residue on the sample surface.
3.5.3.4 Glycerol and freeze drying

This method left a visible residue on the Si wafer surface, possibly the glycerol solution; therefore, no SEM or NanoSIMS images were collected and this approach was automatically discarded.

3.5.3.5 Cryogenic aerosol and freeze drying

This technique disrupted the cells as can be seen in Figure 3.8, and also left residual particles on the surface. Therefore, this method was also discarded.

All the techniques tested that tried to recreate cryogenic conditions (methods 2-5), only confirmed that the maintenance of the freezing conditions throughout the whole process is critical, as well as rapid freezing (Kashi et al., 2014), in order to prevent the
cells from collapsing and to guarantee undisturbed morphologies of the biological samples. Unfortunately, this is hard to achieve without instruments with controlled conditions of temperature and pressure.

3.5.3.6 Rinsing and air-drying of Acidovorax sp. strain ST3

SEM imaging allowed the comparison of the effect of sample preparation on the biogenic minerals and the structure of strain ST3 cells. Only the chemical fixation-dehydration technique preserved the integrity of cells (Fig. 3.9C) but rendered a morphology of biominerals different to that observed in the rinsed-air dried samples (Fig. 3.9A), suggesting the possible formation of secondary minerals during the chemical fixation-dehydration process. This is important to acknowledge, given that all the samples for further biomineral characterisation (XRD, STEM) were rinsed-air dried and nanoparticles were only identified in these samples. Nevertheless, the rinsed-air dried samples were the closest to their native state because no additional solvents or chemicals were

![Figure 3.9. SEM images of strain ST3 cells preserved by (A-B) rinsing-air drying, and (C-D) chemical fixation-dehydration. Many cells appeared disturbed in the rinsing-air drying approach (yellow arrows in panel A) and a thick layer coating the surface could also be observed in some regions (orange arrows in panel B). In contrast, chemically fixed cells appeared well preserved, although the mineral at the cell surface was possibly altered. The yellow arrows are pointing at disturbed cells, which were very few in the chemical fixation method (panel D).](image-url)
introduced. All the samples were handled in anaerobic conditions, therefore, further oxidation that could have altered the morphology of the biogenic minerals is unlikely.

3.5.4 Selection of preservation method

These initial sample preparation tests allowed the assessment of the effect on cells and ionic distribution of different sample preservation methods. It is clear that the method chosen would influence the results and even the $^{13}$C/$^{12}$C accumulation detected. Chemical fixation and dehydration was finally selected because it preserved the structure of the cells, allowed the imaging of single cells and left no residues on the surface. Additionally, the amorphous Fe(III)-oxyhydroxide structure was also preserved, and could be probed by the ion beam in NanoSIMS, as there were no residues on the surface interfering with the ionisation of the mineral. It is noteworthy that chemical fixation-dehydration removes some of the biomass, when compared to direct freeze-drying, as the samples are washed in many solutions, although it doesn’t seem to transport or re-locate the cells to different areas on the Si wafer. Furthermore, chemical fixation alters the distribution of salts, although these ions were not of interest in the experiments, and also dilutes the $^{13}$C levels accumulated by cells; all these artefacts are acknowledged. Finally, chemical fixation-dehydration is a time-consuming method, but is a simple technique that doesn’t require specialised equipment, such as critical point drying, which are not always available in research laboratories.

The SEM and NanoSIMS samples of the As(III) and Fe(II)-oxidising Acidovorax sp. strain ST3 and Paracoccus sp. strain QY30 were directly preserved by chemical fixation and dehydration.

3.5.5 Sample preservation for Transmission electron microscopy (TEM)

Planktonic samples of strains ST3 and QY30 were analysed in TEM with all its related analysis (HAADF, EDS, SAED). These samples were initially handled in aerobic conditions (for exploratory TEM) and afterwards, under anaerobic conditions (for STEM). The cultures were transferred to 15 mL centrifuge tubes at days 1, 3, 7 and 10 and centrifuged (2469 g/30 min). The supernatant was saved for aqueous geochemical analysis. The precipitates were re-suspended in 5 mL of $^4$H$_2$O and centrifuged/washed twice. Once
rinsed, the precipitates were re-suspended in $\textsuperscript{6}$$\text{H}_2\text{O}$, using the same initial volume, and diluted 10 or 100 times. 1.5 µL of the concentrated re-suspension and the two dilutions were pipetted on copper TEM holey carbon films 400 mesh grid (Agar Scientific®) and dried in an anaerobic cabinet. The S/TEM results are presented in Chapter 6.

Given that these TEM samples were only rinsed with $\textsuperscript{6}$$\text{H}_2\text{O}$, and no further structure preservation methods were used, wafers of the biofilm samples of strain ST3 were “preserved” by only rinsing in $\textsuperscript{6}$$\text{H}_2\text{O}$ (twice) in order to assess the impact on the structure of the cells and biominerals. The wafers were coated with 10 nm Pt and analysed in SEM in order to compare the structures of biominerals and cells.

### 3.6 Solid phase analysis: imaging and spectroscopy techniques

A series of imaging techniques were used for characterisation of the samples. Scanning electron microscopy (SEM) along with NanoSIMS were routinely used to analyse all biofilm samples of both the As-Fe reductive and oxidative systems. Only S. ANA-3 WT biofilm samples were analysed in ToF-SIMS. The planktonic samples of strains ST3 and QY30 were analysed in transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDS). Strain ST3 was further analysed in scanning TEM (STEM) and the related high angle annular dark-field (HAADF). This section starts with an explanation of the electron microscopies and related analysis, followed by complementary techniques (X-ray diffraction), finalising with the SIMS techniques settings. Figures 3.10 and 3.11 show a schematic of the steps that comprise the experimental section of this project, from sample preparation to their analysis, focusing only on the solid phase techniques.

![Sample preparation](image)

**Figure 3.10.** Simplified schematic showing the sequential experimental procedure to prepare and analyse the *Shewanella* sp. strain ANA-3 samples for the study of As(V)/Fe(III) reduction.
Figure 3.11. Simplified schematic showing the sequential experimental procedure to prepare and analyse the Acidovorax sp. strain ST3 and Paracoccus sp. strain QY30 samples for the study of As(III)/Fe(II) oxidation. All these techniques were used to analyse the ST3 samples, while * indicates the techniques used for QY30 samples.

3.6.1 Electron microscopy

3.6.1.1 Transmission electron microscopy

In transmission electron microscopy (TEM) a beam of electrons is passed through a thin sample where the transmitted electrons are analysed. These electrons interact with other electrons or the nucleus of an atom in a specimen, causing electron scattering, the phenomenon that makes the TEM and other electron microscopy techniques possible (Williams and Carter, 2009). Electrons have a particle and wavelike nature. Scattering is a phenomenon that applies more to the particle nature of electrons, whereas diffraction applies more to their wavelike nature (Williams and Carter, 2009).

Diffraction

In TEM, electrons are emitted from a heated filament under vacuum and accelerated through an acceleration potential. Here the wavelength ($\lambda$) will follow the equation:

$$\lambda = \frac{h}{mv} \quad \text{(Eq. 3.1)}$$
Where $h$ is the Planck constant ($h = 6.626 \times 10^{-34}$ J·s), $m$ and $v$ are the mass and speed of the electron. Therefore, if the acceleration potential is increased (speed of electrons), the wavelength is smaller and these higher-energy electrons can penetrate several microns of a solid sample (Egerton, 2005). As a consequence, it is possible to obtain a transmission electron diffraction pattern, where electrons are diffracted by the atomic planes of the sample, and if these transmitted electrons are focused, an image can be formed with a spatial resolution much higher than in optical microscopes (Egerton, 2005). With high acceleration voltages (in the order of kV) the wavelength is smaller, the spatial resolution is higher, and the depth of penetration is also higher, but beam damage occurs. Therefore, the accelerating voltage selected should be the highest the specimen can resist (Williams and Carter, 2009).

Most TEMs routinely work at an electron accelerating voltage of 100-300 kV, which has allowed atomic spatial resolution. TEM has been valuable for biological studies at the subcellular level from plants and animals to bacterial cells and even viruses (Egerton, 2005).

Exploratory TEM work was performed by Dr Heath Bagshaw using a FEI Tecnai F30 operating at an acceleration voltage of 200 kV. Bright-field (BF) and dark-field (DF) images, energy-dispersive X-ray spectroscopy (EDS) and selected area diffraction patterns (SAED) were collected in cells and biominerals to characterise their composition. The results were processed with INCA Energy software (Oxford instruments®). These results are presented in Appendix A.

**Electron diffraction pattern**

The electron diffraction pattern can be used to analyse the crystallinity of a specimen with the selected area electron diffraction (SAED) analysis performed in S/TEM. The crystal orientation in a crystalline sample scatters the electrons in an angular distribution that can be observed as bright spots for single crystal specimens and as concentric sharp rings for polycrystalline specimens, amorphous samples show more diffuse concentric rings (Egerton, 2005). This analysis was used to characterise extracellular biominerals or
on the cell surface in TEM, and were identified using a free access library of electron diffraction patterns for known minerals. This analysis was performed by Dr Heath Bagshaw using a FEI Tecnai F30 instrument and these results are presented in Appendix A.

**Energy-dispersive X-ray spectroscopy (EDS)**

Electron microscopes can provide chemical information of a specimen, as well as producing images and revealing structural details. The primary electrons hitting a sample in electron microscopes interact with the sample and cause the emission of X-rays, and the analysis of the wavelengths or photon energies allows accurate elemental identification, given that these wavelengths are characteristic for each element, particularly for elements of higher atomic numbers (Z) (Egerton, 2005). EDS spectra were collected in TEM and STEM, and acquired by Dr Heath Bagshaw and Dr Simon Fairclough, respectively. These results are presented in Chapter 7 and Appendix A.

**3.6.1.2 Scanning transmission electron microscopy (STEM)**

TEMs can be operated as a scanning transmission electron microscope (STEM) or a dedicated STEM instrument can be used but operating under STEM mode requires additional scanning coils and detectors. In STEM a fine probe (small diameter) is used in scanning mode. The probe is focused to sub-nm dimensions and can provide single-atom resolution. Most STEM images are collected in dark-field mode. The TEM used in STEM mode in this research project used a high angle annular dark-field detector (HAADF), see Fig. 3.12. In this detector the electrons transmitted and scattered are collected at a larger angle, collecting more scattered electrons. This design provides a contrast directly related to the atomic-number (Z-contrast) and high collection efficiency. Elements with higher atomic numbers scatter more electrons at higher angles, therefore, HAADF imaging mode was of particular interest for samples containing arsenic (Egerton, 2005). Additionally, the main beam can directly pass to an electron energy loss spectroscopy (EELS) detector, and both analyses can be performed simultaneously. A FEI Titan G2 80-200 S/TEM ChemiSTEM instrument was used running at 200 kV, using a 180 pA beam.
current and data was collected using Gatan Microscopy Suite® software to analyse ST3 samples. The data acquisition and analysis were performed by Dr. Simon Fairclough. These results are presented in Chapter 7.

**STEM data analysis**

Anaerobic STEM-EDS data was processed using HyperSpy (an open source Python library). Quantification of the EDS used a background correction and Cliff Lorimer factors. Aerobic STEM-EDS data was processed using Bruker® ESPRIT software. Quantification of the EDS used a background correction, Bayes deconvolution and Cliff Lorimer factors. All this analysis was performed by Dr Simon Fairclough.

![Figure 3.12. The FEI Titan G2 80-200 S/TEM ChemiSTEM instrument at the University of Manchester. Photo by the author.](image)

**3.6.1.3 Scanning electron microscopy (SEM)**

The main limitation with TEM is the sample thickness. Thin samples are needed otherwise the electrons are absorbed or scattered. Scanning electron microscopes (SEM) were invented as an answer to this need to analyse “bulk” or thicker samples. From “bulk” samples electrons can be reflected (backscattered) or the primary electrons
bombarded can produce secondary electrons (SE) from the sample surface. In order to obtain a focused image, the primary electrons are focused to a small-diameter probe and scanned across the sample surface, and this scanning principle allows the formation of focused images. Modern SEMs can reach spatial resolutions between 1 and 10 nm, much better than an optical microscope but not as high as TEMs (Egerton, 2005).

SEM analysis of samples was performed routinely prior to NanoSIMS or ToF-SIMS, in order to locate suitable areas with cells and minerals. A FEI Quanta 650 FEG SEM with a 15 kV beam was routinely used and image acquisition was limited to large fields of view (>40 µm), in order to reduce sample damage upon exposure to the primary electron beam, but beam damage prior to SIMS analysis cannot be discounted. Dr Heath Bagshaw provided training and assistance with the SEM, and these results are presented in Chapters 4, 5, 6, 7 and Appendix A.

3.6.2 Powder X-ray diffraction (XRD)

Powder X-ray diffraction was performed in bulk samples of the biominerals produced by ST3 and QY30, and also to confirm that Fe(III)-oxyhydroxide was synthesised for the ANA3 experiments. In this technique a relatively thick film of the sample is smeared on a glass slide and struck with an incident X-ray beam, these X-rays are diffracted by the crystals in the sample at certain angles, producing peaks and these characteristic peaks correspond to different minerals and materials. Analysis was performed in a Bruker D8 advance X-ray diffractometer, with a 40 kV beam. Analyses were run from 5° to 70° diffraction angles (2θ) at a step size of 0.02°, dwell time of 1 second due to the low crystallinity in most of the samples, giving a total time of analysis of 1 hour per sample to increase the signal detection. Dr John Waters provided training and assistance with this technique. Results of this technique are presented in Chapters 6 and Appendix A.

3.6.3 Secondary ion mass spectrometry (SIMS)

3.6.3.1 NanoSIMS analysis settings

Samples prepared on Si wafers were analysed in a NanoSIMS 50L ion microprobe (CAMECA, France) using the Cs⁺ or O⁻ primary ion beams at 16 keV. The primary ion beam
was scanned over the surface of the samples with varying current, depending on the session. The D1 apertures used for imaging ranged from D1=2-3, for large fields of view (30-50 µm), and D1=4-5 for depth profiling of single cells (detailed in section 3.6.3.3). The working spatial resolution obtained was between 300 and 400 nm (using apertures D1=3-2).

Silicon wafer, iron metal and gallium arsenide were used as reference materials to align the detectors, improve mass resolution and avoid peak overlaps from molecular interferences. The CAMECA mass resolving power (MRP) was 5500 for detector 2 (mass 13) to resolve $^{13}\text{C}$ from the hydride $^{12}\text{C}_2\text{H}$, and MRP for detector 7 (mass 75) was 7000, in order to resolve an isobaric interference, detailed in section 3.6.4.2. The slits ES and AS that shape the secondary ion beam were routinely used at ES=3 and AS=2 to improve mass resolution.

The following negative secondary ions (SI) were collected using the Cs$^+$ source: $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{28}\text{Si}^-$, $^{31}\text{P}^-$, $^{56}\text{Fe}^{12}\text{C}^-$, $^{56}\text{Fe}^{16}\text{O}^-$ and $^{75}\text{As}^-$. The following positive SI were analysed using the O$^-$ source: $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{28}\text{Si}^+$, $^{31}\text{P}^+$, $^{39}\text{K}^+$, $^{44}\text{Ca}^+$, $^{56}\text{Fe}^+$, $^{75}\text{As}^+$ and $^{98}\text{Mo}^+$, although only 7 masses could be analysed simultaneously. Notice that $^{56}\text{Fe}^{12}\text{C}^-$ or $^{56}\text{Fe}^{16}\text{O}^-$ were used as proxies of $^{56}\text{Fe}$ in negative SI mode, as the ionisation efficiency for Fe$^-$ is too low.

![Figure 3.13. Schematic representation of NanoSIMS analysis showing 7 masses selected for detection in the mass analyser. Modified from: http://nano.bwh.harvard.edu/mims.](image)
to be analysed using the Cs\(^+\) ion beam. In contrast, \(^{56}\text{Fe}\) has a high ionisation efficiency under the O\(^-\) ion beam and can be directly collected as \(^{56}\text{Fe}\). See Figure 3.13 for a schematic representation of these masses at each detector.

Images were usually collected at a dwell time of 5000 \(\mu\text{s px}^{-1}\) and pixel size was usually 256 x 256. Additionally, an ion-induced secondary electron (SE) image was obtained. In the negative SI mode \(^{12}\text{C}\), \(^{13}\text{C}\) and \(^{12}\text{C}^{14}\text{N}\) signals were used to identify the biomass, and the \(^{13}\text{C}/^{12}\text{C}\) ratio was used to identify active cells by their \(^{13}\text{C}\) accumulation (above the 1.11 \% natural \(^{13}\text{C}/^{12}\text{C}\) abundance). \(^{28}\text{Si}\)\(^-\) was used to image the wafer surface. \(^{56}\text{Fe}^{12}\text{C}\) and \(^{56}\text{Fe}^{16}\text{O}\) were used as \(^{56}\text{Fe}\) proxies, and along with \(^{75}\text{As}\)\(^-\), these SI were used to map the arsenical Fe(III)-(oxyhydr)oxide. Implantation of Cs\(^+\) or O\(^-\) primary ions at a dose of \(1\times10^{17}\) ions cm\(^{-2}\) was used to remove the Pt coating and reach steady state on the sample surface before collecting chemical images (Guerquin-Kern et al., 2005). For standard secondary ion image acquisition, 10-20 planes were typically collected per area of analysis (AOI). NanoSIMS ion images are presented in Chapters 4, 5, 7 and Appendix A.

### 3.6.3.2 Isobaric interference at mass 75

An important mass interference at mass 75 (used to measure \(^{75}\text{As}\)), was detected during the second NanoSIMS session. It was important to tackle this mass interference to assess its contribution to the total signal detected at mass 75, which would interfere with \(^{75}\text{As}\) measurements. High mass resolution mode (HMR) is a mass spectrum routinely collected in NanoSIMS. HMR displays the shape of the peak of interest and the mass resolving power. Therefore, HMR is used as a reference when improving mass resolution. HMR spectra are used to align the detectors at the right mass and highest counts intensity possible, avoiding isobaric interferences. When the Fe standard was run at mass 75, the HMR showed a peak co-locating with the As standard (GaAs) peak, suggesting that the isobaric interference was an Fe molecular interference (see Figure 3.14).

The possible molecular interference was \(^{56}\text{Fe}^{19}\text{F}\) (74.9333 a.m.u.), although \(^{28}\text{Si}^{12}\text{C}^{35}\text{Cl}\) (74.9457 a.m.u.) was an additional potential interference, given that the samples were also rich in silicon, carbon and chlorine. The samples did not contain fluorine, but it could
originate from the residual vacuum in the analysis chamber. For this reason, experiments were designed to assess the contribution of the different sample components to this mass interference.

Two kinds of ferrihydrite were prepared, one using FeCl₃, abundant in ³⁵Cl, and one using Fe(NO₃)₃, with no ³⁵Cl. These ferrihydrite solutions were mixed with arsenate (12 % mol/mol As/Fe). Thin films of both types of ferrihydrite with and without As(V) were deposited on Si wafers and on plastic coverslips (Nunc™ Thermanox™ coverslips, Thermo Fisher Scientific). The plastic coverslips allow assessment of the Si wafer contribution (table 3.10). These thin films were analysed in NanoSIMS and HMR spectra were collected and compared with the As and Fe standards, using the same instrument settings. ES=5 and AS=1 were used in these experiments to improve mass resolution.

The full results of these experiments are not presented, but it was determined that the mass interference was ⁵⁶Fe¹⁹F⁻, with ⁵⁶Fe originating from the Fe(III)-(oxyhydr)oxide and ¹⁹F⁻ likely originating from the residual vacuum in the NanoSIMS analysis chamber.
Table 3.10. Samples tested in the isobaric interference investigation experiments.

<table>
<thead>
<tr>
<th>Type of ferrihydrite</th>
<th>Sample</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrihydrite from FeCl₃</td>
<td>1</td>
<td>No As on Si wafer</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+ As on Si wafer</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>No As on plastic coverslip (no Si)</td>
</tr>
<tr>
<td>Ferrihydrite from Fe(NO₃)₃</td>
<td>4</td>
<td>No As on Si wafer</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+ As on Si wafer</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>No As on plastic coverslip (no Si)</td>
</tr>
</tbody>
</table>

Additionally, the plastic coverslip proved to be not suitable for these samples, given that it was flimsy and failed to keep the ferrihydrite thin film intact. The theoretical mass resolution needed to separate the $^{75}$As$^-$ and $^{56}$Fe$^{19}$F$^-$ peaks was 6377. Therefore, to avoid the mass interference the mass resolving power was improved above 6377 by changing ES=3 and AS=2 and by using the optimal Q and LF4. Q and LF4 shape the secondary ion beam and their optimal value was manually altered until finding the maximum MRP in the HMR scans for each detector. Additionally, the detector was aligned at the left edge of the peak in the HMR scan (vertical red dashed line in Fig. 3.15).

![HMR mass 75](image)

Figure 3.15. HMR spectrum showing the superposition of the GaAs standard and sample 2 (arsenical ferrihydrite on Si wafer) peaks after mass resolution improvement. The red dashed vertical line is where the detector was set after the mass interference experiments. Note that the deflector is not set on flat part of the peak but on the edge of the GaAs standard peak, compromising the intensity of As counts (note HMR scan is presented on a log scale). MRP=7330.
Alignment on the edge of the peak compromised the intensity of $^{75}$As counts in further NanoSIMS analysis but ensured the isobaric interference did not contribute to the analysis (see Figure 3.15). This mass interference was absent in positive secondary ion mode.

### 3.6.3.3 NanoSIMS single-cell depth profiles

After collecting images at large fov (30-50 µm), single active cells were selected in order to collect depth profiles in a smaller fov of 3-5 µm width. Only cells located on the Si wafer and more than 3 µm away from the Fe(III) mineral grains were selected for depth profile analysis, in order to avoid potential re-deposition of As and Fe from the mineral. The spatial resolution was improved by using $D_1=4$ or 5 and in some cases $L_1$ was modified to 3000-7500 V (a smaller $D_1$ aperture and higher voltage in $L_1$ produce a smaller probe diameter). In these cases the spatial resolution was 100-150 nm. Pixel sizes were 128x128 or 256x256, and dwell time varied from 5,000-20,000 µs px$^{-1}$. The number of planes collected were in the range of 50-100, but the scanning was prolonged in these depth profiles until the $^{12}$C or $^{12}$C$^{14}$N$^+$ signal dropped, indicating the complete sputtering of the bacterial cell and rendering chemical information of the whole cell. In positive SI mode, no $^{12}$C was imaged, but the drop in $^{56}$Fe$^+$ signal was monitored as indicator of complete cell sputtering.

### NanoSIMS data analysis

NanoSIMS ion images were processed with L’IMAGE software (Larry Nittler, Carnegie Institution of Washington). Hue-saturation-intensity (HSI) maps of $^{13}$C/$^{12}$C ratios and overlay images were obtained with ImageJ using the OpenMIMS plugin (MIMS, Harvard University; www.nrims.harvard.edu).

To compensate the period during which ions hitting the detector are not registered, a dead time correction of 44 ns was applied to all files.

Regions of interest (ROI) were hand-drawn in every ion image collected in L’IMAGE, and classified in four types: active cells, whether they were located on the wafer surface, on the mineral or adjoining the mineral (“active cells”), mineral in samples with active cells
(“mineral”), mineral in control experiments (“mineral control”) and the wafer background with no mineral nor cells (“background”). For the mineral ROIs flat areas away from the edge of the mineral were selected to avoid topographic effects.

The counts of the relevant masses in each ROI were normalized to the current and dose on each day of analysis, considering the image size, pixel size, dwell time and number of planes acquired, which affect the total dose in each ion image. Box plots for the NanoSIMS counts were produced in OriginPro® software.

3D reconstructions of the depth profiles were created using Thermo Scientific™ Avizo™ Software 9.7.0. Stack data of $^{12}$C$^-$, $^{56}$Fe$^{12}$C$^-$, $^{56}$Fe$^{16}$O$^-$ and $^{75}$As$^-$ (for the reducing experiments) and $^{56}$Fe$^-$ and $^{75}$As$^-$ (for the oxidising experiments) were first extracted with ImageJ and saved in the “.raw” format file. Afterwards, these .raw files were loaded into Avizo™. The Z depth was compressed to 15-20 %. In the reducing experiment files, the $^{12}$C counts were used to generate the bacteria surface by averaging the signal over 2-3 pixels, then, the “generate surface” and “show surface” commands were used sequentially, and finally, the “transparent” display was selected with a transparency of 80 %. The $^{56}$Fe$^{12}$C$^-$, $^{56}$Fe$^{16}$O$^-$ and $^{75}$As$^-$ ion counts were averaged to 1 pixel because of the lower ion counts and overlapped on the $^{12}$C$^-$ surface by displaying as “points”, to enable their visualisation.

In the oxidising experiments, the $^{56}$Fe$^-$ counts were used to generate the bacteria surface by smoothing the signal over 2-3 pixels. The “generate surface” and “show surface” commands were used sequentially, and the “transparent” display with a transparency of 80 % was selected. The $^{75}$As$^-$ ion counts were averaged to 1 pixel because of the lower ion counts and overlapped on the $^{56}$Fe$^-$ surface by displaying as “points” to enable their visualisation. These single-cell depth profile 3D reconstructions are presented in Chapters 5 and 7.
3.6.3.4 ToF-SIMS analysis

Two ToF-SIMS instruments were used in this project, one equipped with a C$_{60}^+$ primary ion gun and one with a Au$_n^+$ liquid metal ion gun (LMIG) termed IDLE-3 (Interstellar Dust Laser Explorer-3) (Henkel et al., 2007).

Reference materials

In contrast to NanoSIMS, in ToF-SIMS each pixel of a secondary ion image provides a full mass spectrum in an unlimited mass range, and in consequence, the data analysis is challenging for complex samples. Therefore, a series of tests were performed prior to sample analysis with the aim of predicting the mass spectrum and molecular ions produced by the specific sample components on their own (the Fe(III) mineral, the cells and the Si wafer). Additionally, mass spectra of reference materials were also obtained. Riboflavin and flavin mononucleotide (FMN) solutions (1 µM) were tested as a reference for flavins. 10 µL of these solutions were pipetted on a Si wafer and let dry overnight in
a fume hood. Poly-L-lysine is a charge enhancer solution typically used to bind cells to solid surfaces in biological samples, but this solution was used as an amino acid reference, in order to analyse the back bone of amino acids, composed of the characteristic amine and carboxylic acid functional groups in proteins. To prepare this sample 40 µL of a poly-L-lysine 0.01 % w/v solution (Sigma Aldrich®) was pipetted on a Si wafer and left to dry overnight. Fe(III)-oxyhydroxide (ferrihydrite) was assessed as well, where 40 µL of a ferrihydrite solution (≈100 mM total Fe) were pipetted on a Si wafer and let dry overnight.

Additionally, bulk S. ANA-3 WT cells were analysed to collect mass spectra from cells without Fe(III) and As(V). The cells were grown in anaerobic minimal medium, washed in bicarbonate buffer, re-suspended in the same buffer (as detailed in sections 3.1.1 and 3.1.1.1 from this Chapter) and 40 µL of this suspension were pipetted on a Si wafer.

**Fe(III) respiration samples**

Samples of S. ANA-3 WT growing as a biofilm on the Fe(III)-oxyhydroxide thin film were analysed. These samples were prepared as detailed in sections 3.2.1 and 3.2.2, although this Fe(III) mineral was not doped with As, to simplify the exploratory analysis, and regular lactate was used as electron donor (no 13C-labelling). Prior to chemical fixation, these samples were rinsed in a 20 mM ammonium formate solution (pH=7.2) for 1 min to remove excess salts (Fearn, 2015). Unlike NanoSIMS samples, the ToF-SIMS samples were not sputter coated.

**ToF-SIMS settings**

The samples and reference materials were analysed using the C$_{60}^+$ ion gun at 40 kV, while the analysis in the IDLE-3 was made using an Au$_n^+$ LMIG which was run at 25 kV for Au$^+$, Au$^{2+}$ and the cluster ions Au$_{2}^+$ and Au$_{3}^+$. The fields of view were typically around 100 µm for both the reference materials and samples. Secondary ions were collected in positive SI mode, although some tests were run in negative SI mode. Pixel size was 128 x 128 or 256 x 256. In contrast to NanoSIMS, more planes need to be collected in ToF-SIMS to improve counting statistics, whereby the typical number of planes ranged from 30-60 for each AOI.
3.6.3.5 ToF-SIMS data analysis

The ToF-SIMS raw data was analysed using a combination of two softwares: the ToFCmd (developed by Drs Detlef Rost and Torsten Henkel, University of Manchester) and TSTSpec (developed by Dr Thomas Stephan, University of Chicago). The mass spectra were first loaded in the ToFCmd software and aligned to correct stage drift, to remove planes with sample charging or with no mass spectra recorded. A detector dead time correction of 40 ns was applied to each file. These corrected files were then loaded in the TSTSpec software where the masses of each peak of interest were manually assigned. Each peak provides an associated image which was obtained by selecting the peak in TSTSpec and then processed again in ToFCmd. Static SIMS library, a peak search tool, was additionally used to identify peaks (Vickerman et al., 1999). These results are presented in Chapter 6.

3.7 References


Chapter 4- NanoSIMS Imaging of Extracellular Electron Transport Processes During Microbial Iron(III) Reduction (research article)


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RESEARCH ARTICLE

NanoSIMS imaging of extracellular electron transport processes during microbial iron(III) reduction

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One sentence summary: NanoSIMS imaging shows that metabolically-active cells of Geobacter sulfurreducens were co-located with an iron(III)-(oxyhydr)oxide surface, consistent with this bacterium requiring direct contact with an extracellular electron acceptor.

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ABSTRACT

Microbial iron(III) reduction can have a profound effect on the fate of contaminants in natural and engineered environments. Different mechanisms of extracellular electron transport are used by Geobacter and Shewanella spp. to reduce insoluble Fe(III) minerals. Here we prepared a thin film of iron(III)-(oxyhydr)oxide doped with arsenic, and allowed the mineral coating to be colonised by Geobacter sulfurreducens or Shewanella ANA3 labelled with 13C from organic electron donors. This preserved the spatial relationship between metabolically active Fe(III)-reducing bacteria and the iron(III)-(oxyhydr)oxide surface and were significantly more 13C-enriched compared to cells located away from the mineral, consistent with Geobacter species requiring direct contact with an extracellular electron acceptor to support growth. There was no such intimate relationship between 13C-enriched S. ANA3 and the iron(III)-(oxyhydr)oxide surface, consistent with Shewanella species being able to reduce Fe(III) indirectly using a secreted endogenous mediator. Some differences were observed in the amount of As relative to Fe in the local environment of G. sulfurreducens compared to the bulk mineral, highlighting the usefulness of this type of analysis for probing interactions between microbial cells and Fe-trace metal distributions in biogeochemical experiments.

Keywords: electron transport; Geobacter; Shewanella; 13C labelling; arsenic; NanoSIMS

INTRODUCTION

The reduction of Fe(III) in minerals can have profound consequences on the mineral composition and aqueous geochemistry of the subsurface environment. One of the outcomes of the bioreduction of ferric minerals, such as ferricydrite, is the reductive dissolution of Fe(III), which forms soluble Fe(II) and is likely to cause the release of other incorporated or sorbed elements such as arsenic (Coker et al. 2008; Cutting et al. 2009). Soluble Fe(II) can in turn recrystallise to form new Fe(II)-bearing minerals such as magnetite, siderite or vivianite depending on the pH, temperature and the other geochemical species present...
These processes can also affect the mobility of arsenic and other toxic metals and radionuclides (Lloyd et al. 2000; Smelley and Kimbrough 2002; Newsome, Morris and Lloyd 2014; Watts et al. 2015). Focusing on the release of arsenic to groundwater as a high profile example, this is likely caused by the development of reducing biogeochemical conditions linked to subsurface microbial activities (Islam et al. 2004a,b). However, As can also be re-sequestered by newly forming minerals such as vivianite and siderite (Islam et al. 2005; Muehe et al. 2016), and magnetite also was found to incorporate As(V) and create surface complexes with As(III) (Islam et al. 2005; Coker et al. 2006; Wang et al. 2014). Such interactions with As during the mineralisation of Fe(II)/Fe(III) minerals could potentially alter the distribution of As on the micro scale and delay its release into aquifers used to supply drinking waters on a macro scale. It is therefore crucial to understand the mechanism of microbial Fe(III) reduction in order to assess its impact on the release (and capture) of metals and metalloids. Given that the contamination of drinking water with arsenic is a major health concern that threatens the lives of more than 140 million people worldwide (Singh et al. 2015), this area warrants particular attention.

Fe(III) is an electron acceptor commonly used in anaerobic microbial respiration. A diverse range of bacteria and archaea covering a wide range of phylogenetic groups has been identified that enzymatically reduce Fe(III) (Lovley, Holmes and Nevin 2004). Fe(III) mostly exists in the natural environment in the solid form as oxides such as ferrihydrite, goethite and haematite, except in strongly acidic conditions where it is soluble. This means that unlike the more energetically favourable electron acceptors oxygen and nitrate, insoluble Fe(III) is unable to diffuse or be transported into the cell to be respired. As the lipid-rich cell membrane is impervious to electrons, Fe(III)-reducing microorganisms have had to evolve mechanisms for extracellular electron transfer from the surface of the cell, in order to be able to generate a proton motive force and hence conserve energy for growth. Most of the studies that underpin our knowledge of extracellular electron transport have been performed on two model organisms, the Gram-negative bacteria Geobacter sulfurreducens and Shewanella oneidensis MR1 (formerly Alteromonas putrefaciens), that both gain energy from reducing Fe(III) to Fe(II). These genera of bacteria were first shown to respire Fe(III) in the late 1980s (Lovley et al. 1987; Myers and Nealson 1988). Both Geobacter and Shewanella species use outer membrane porin-cytochrome complexes to transport electrons to the outer membrane, but the proteins involved are phylogenetically distinct and are considered to have evolved independently (Lovley, Holmes and Nevin 2004; White et al. 2016). They also use different mechanisms of transporting electrons from the surface of the outer membrane to the extracellular Fe(III) mineral electron acceptor, as described below.

Geobacter species are obligate anaerobes that couple the reduction of Fe(III) to the oxidation of acetate and other organic substrates (Lovley et al. 1987; Lovley and Phillips 1988). They are common in the Fe(III)-reducing zone of sediments (Lovley, Holmes and Nevin 2004), and have been found to dominate the subsurface microbial community following biostimulation of the subsurface by acetate additions during targeted uranium bioremediation programmes (Anderson et al. 2003; Wilkins et al. 2009; Kerkhof et al. 2011; Williams et al. 2011). The current consensus is that Geobacter species require direct physical contact with an insoluble electron acceptor in order to transfer electrons (Nevin and Lovley 2000; Lovley, Holmes and Nevin 2004), and this occurs via an electron transport chain that involves both c-type cytochromes and pili. Geobacter sulfurreducens uses multiple and parallel electron transport pathways from the inner membrane quinone pool to the OmcB cytochrome, which is partially exposed to the extracellular environment (Leang, Coppin and Lovley 2003; Malvankar et al. 2011; Liu et al. 2015; Levar et al. 2016). From OmcB the electrons are transported to Omce, then to pili, and then to OmcS cytochromes that pass the electrons to the electron acceptor; without each of these cytochromes and pili, electron transport to a solid electron acceptor is compromised (Childers, Ciufo and Lovley 2002; Mehta et al. 2005; Reguera et al. 2005; Leang et al. 2010; Malvankar et al. 2011). Electron transport along the pili of Geobacter species has been proposed to occur via metallic-like conductivity (Malvankar et al. 2011; Lovley 2012; Malvankar, Tuominen and Lovley 2012; 2017). An early study did suggest that G. sulfurreducens could produce extracellular cytochromes (Seeliger, Cord-Ruwisch and Schink 1998), but this was later proven to be incorrect (Lloyd, Blunt-Harris and Lovley 1999; Straub and Schink 2003). More recently it appears that a pili-deficient mutant was able to restore its ability to reduce electron acceptors by releasing an extracellular c-type cytochrome (PgcA), but the authors note that this adaptation is likely a result of the highly controlled laboratory conditions and is unlikely to occur in the environment (Smith et al. 2014).

Shewanella species are facultative anaerobes that can couple reduction of Fe(III) to the oxidation of organics such as lactate, but not acetate (Myers and Nealson 1988; Lovley, Phillips and Lonergan 1989; Lovley, Holmes and Nevin 2004; Brutinel and Gralnick 2012). They are widespread in the environment in sediments where organic matter is being degraded and are thought to be adapted to the oxic/anoxic transition zone (Fredrickson et al. 2008; Edwards et al. 2015). It is likely that electron transport in S. oneidensis occurs primarily via an electron transport chain that includes flavin-cytochrome complexes on the outer membrane. Transport of electrons from the inner membrane quinone pool to the outer membrane occurs via the MtrCA porin-cytochrome complex, the MtrC cytochrome is partially exposed at the cell surface and from there the electrons can be transferred to the extracellular electron acceptor or to the outer membrane cytochrome OmcA (White et al. 2016). Long-range electron transport is possible in S. oneidensis by the extension of the outer membrane into micrometre long nanowires (Pirbadian et al. 2014), along which electron transport may occur via an electron hopping mechanism (Okamoto, Hashimoto and Nakamura 2012; White et al. 2016), or by direct electron transfer accompanied by intermediate diffusive events (Subramanian et al. 2017). As well as being able to transport electrons via direct contact with an electron acceptor, Shewanella species can also secrete soluble extracellular electron shuttles to support an indirect reduction mechanism (Nevin and Lovley 2000; Marsili et al. 2008; von Canstein et al. 2008). Indeed, flavin-mediated electron transport has been shown to be responsible for up to 75% of electron-acceptor reduction in S. oneidensis (Kotloski and Gralnick 2013). The secreted flavins can also form flavo-cytochrome complexes with MtrC under anaerobic conditions (Okamoto et al. 2014; Edwards et al. 2015), and it has been argued that these bound complexes increase the rate of extracellular electron transport at the cell surface, rather than through electron shuttling via the free soluble flavin shuttles (Xu, Jangir and El-Naggar 2016). An in vivo study with Mn(IV)-oxides found that many S. oneidensis cells were planktonic and made occasional contact with the mineral surface, which could suggest electron transport via direct contact was more important than the secretion of extracellular flavins (Harris, El-Naggar and Nealson 2012). Nevertheless the role of secreted flavins in extracellular metal reduction remains controversial. Although Shewanella species can secrete...
extracellular flavins to reduce electron acceptors that were inaccessible to cells (Lies et al. 2005; Jiang et al. 2010), the likelihood of this mechanism occurring outside carefully controlled laboratory experiments has been questioned (Lovley, Holmes and Nevin 2004; Smith et al. 2014). Potential loss of the flavin mediator to planktonic cultures is clearly a key challenge, but the role of extracellular flavins in mediating Fe(III) and Mn(IV) reduction in biofilms remains poorly constrained.

A range of metals and metalloids can be associated with Fe(III) minerals, and one of the most intensively studied is arsenic. Like iron, arsenic is also redox active, with As(V) sorbing more strongly to Fe(III) oxyhydroxides present in aquifers than As(III) (Islam et al. 2004a). Arsenic can be released to solution when bacteria reduce the Fe(III) oxyhydroxides hosting the arsenic, to aqueous Fe(II), or it can be released via microbial reduction of As(V) to As(III), via dissimilatory As(V) reduction that is mediated by a periplasmic arsenate reductase encoded by the arr operon (Saltikov and Newman 2003; Saltikov et al. 2003). Although certain species of Geobacter and Shewanella are able to respire As(V), such as G. uranireducens and S. ANA3 (Saltikov, Wildman and Newman 2005; Giloteaux et al. 2013), neither G. sulfurreducens or S. oneidensis MR1 are capable of catalysing this process as they do not possess the arr operon (Islam et al. 2005; Lloyd et al. 2011; Jiang et al. 2013), although both strains have been reported to have putative arsenic resistance genes (which can reduce As(III) within the cytoplasm prior to efflux of the As(III) as part of detoxification process) (Islam et al. 2005; Jiang et al. 2009; 2013; Dang et al. 2017).

Most previous studies have focussed on the bulk chemical effects of microbial activity, but do not provide the critical spatial information on the effect of microbial activity that is essential for determining mechanistic information. NanoSIMS is a surface based secondary ion mass spectrometry (SIMS) technique that is able to image up to seven masses and achieve a spatial resolution down to 50 nm (Herrmann et al. 2007b). It can be used in conjunction with stable isotope labelling (for example 13C and 15N) and is therefore particularly useful for imaging metabolically active cells that have incorporated labelled substrates. NanoSIMS, often in combination with stable isotope labelling, has been used previously to study a range of microorganisms and microbial processes, including benzene degrading communities (Schurig et al. 2015), periplasmic encrustation in nitrate-reducing Fe(II)-oxidising bacteria (Miot et al. 2015), anaerobic phototrophy (Musat et al. 2008), nitrogen fixation in cyanobacteria (Ploug et al. 2010; Woebken et al. 2012) and more recently syntrophic interactions between species (Wu et al. 2013; Gieg, Fowler and Berdugo-Clavijo 2014; Green-Saxena et al. 2014), as well as the location of active microorganisms in soil (Herrmann et al. 2007a) and the nature of organic matter in soil microenvironments (Vogel et al. 2014).

The aim of this study was to observe the colonisation of an As(V)-doped Fe(III)-(oxyhydr)oxide surface by different model Fe(III)-reducing bacteria. Geobacter sulfurreducens was selected as a model Fe(III)-reducing bacterium that requires direct contact with an extracellular electron acceptor. In contrast, Shewanella ANA3 was selected as a model Fe(III)-reducing bacterium that does not require direct contact with an extracellular electron acceptor. The cells were supplied with 13C-labelled substrates to enable the localisation of metabolically active cells in relation to the mineral surface by NanoSIMS, and therefore explore mechanisms of iron reduction. Finally, the role of microbial Fe(III) reduction (and enzymatic As(V) reduction) in controlling the fate of As(V) incorporated within Fe(III)(oxyhydr)oxide was also studied using these two organisms. As G. sulfurreducens is unable to reduce As(V), in this experiment the As(V) served as a tracer to allow observations of the effect of Fe(III)-reduction in the local environment of the cells. This contrasts with Shewanella ANA3, which is also able to reduce As(V) enzymatically.

**MATERIALS AND METHODS**

**Thin film preparation**

To enable visualisation of cell-mineral interfaces by microscopy with limited disturbances, supported films of Fe(III) minerals were prepared. These thin films were made using synthetic ferrihydrite, a model amorphous iron(III)-(oxyhydr)oxide mineral relevant to environmental processes and known to be bioavailable for Fe(III)-reducers. Ferrihydrite was synthesised (Schwertmann and Cornell 2000; Cornell and Schwertmann 2006) with As incorporated via the addition of Na₂HAsO₄ to achieve a 12% mol/mol As/Fe ratio. All reagents were analytical grade. The ferrihydrite-As suspension was added to glass slides (for optical microscopy) or boron doped silicon wafers (for SEM and NanoSIMS) by dropping a suspension onto each wafer and leaving it to dry in air.

**Cell culturing**

Geobacter sulfurreducens was obtained from the laboratory culture collection of the University of Manchester Geomicrobiology group, and grown anaerobically in a modified freshwater enrichment medium (after Lovley and Phillips 1988) at pH 7, with 15 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor. Cultures were grown in the dark at 30°C until the late-logarithmic phase, when they were harvested by centrifugation (5000g, 20 min), washed twice in an anaerobic 30 mM bicarbonate buffer, then suspended into the experimental medium as appropriate.

Shewanella ANA3 was also obtained from our laboratory culture collection and grown aerobically on LB agar plates and then inoculated into an anaerobic modified minimal medium (Saltikov et al. 2003) at pH 7 with 20 mM lactate as the electron donor and 40 mM sodium fumarate as the electron acceptor. Cultures were grown in the dark at 30°C until the late-logarithmic phase, when they were harvested by centrifugation (5000g, 20 min), washed twice in an anaerobic 30 mM bicarbonate buffer, then suspended into the experimental medium as appropriate.

**Thin film colonisation preparatory experiments**

To investigate the colonisation of Fe(III) mineral coatings and associated As mobilisation by a model Fe(III)-reducing bacterium, experiments were set up comprising a glass slide coated with the iron-(oxyhydr)oxide mineral held vertically in a sterile glass serum bottle with the headspace degassed with N₂. This was submerged in a modified anaerobic freshwater minimal medium (after Lovley et al. 1991) at pH 7 containing 30 mM bicarbonate, 4.3 mM phosphate and with 10 mM acetate as the electron donor and the Fe(III) thin film coating as the sole electron acceptor. Cells of G. sulfurreducens were added to achieve an optical density OD₅₀₀ of 0.3. The bottles were incubated in the dark at 30°C. To assess the rate of Fe(III)-reduction and cell colonisation, samples of the medium were obtained using a degassed needle and syringe and monitored for OD₅₀₀. At selected time points the glass slides were harvested and either analysed for cell colonisation by staining with DAPI and counting via optical microscopy (Zeiss Axio Imager A1), or for Fe(III)-reduction by dissolving in 0.5 N HCl to assess Fe(II) content via the Ferrozine
Thin film colonisation experiments for NanoSIMS

The methodology described above for the preparatory experiments was used to prepare samples for NanoSIMS analysis, except that conducting Si wafers were used instead of glass slides and for *G. sulfurreducens* 10 mM 13C-acetate (99% CH313CO2Na, ISOTEC INC) or for S. ANA3 10 mM 13C-lactate (99% 13CH2CH(OH)CO2Na, Sigma Aldrich) were used as electron donors to label the metabolically active cells. Cells of *G. sulfurreducens* were added to an OD600 of 0.3 and S. ANA3 to an OD600 of 0.5. Negative controls contained no added electron donor, and controls with no bacteria (in triplicate) were used to assess the abiotic solubility of Fe and As under the experimental conditions used.

Changes in aqueous geochemistry were monitored at selected time points by removing aliquots of the solution using a degassed needle and syringe, taking care not to disturb the wafer. Aliquots were diluted in 2% HNO3 and monitored for Fe and As by ICP-AES (Perkin–Elmer Optima 5300 DV).

The wafers were harvested after 11 days (as this incubation time was found to be most suitable for obtaining optimal cell colonisation by DAPI staining and cell counting) and one replicate wafer was preserved for SEM and NanoSIMS analysis. The remaining aqueous phase was diluted in 2% HNO3 and monitored for Fe and As by ICP-AES, or diluted in deionised water and analysed for As(V) and As(III) by ion chromatography inductively coupled plasma-mass spectrometry (IC-ICP-MS) (Gault et al. 2005). The coatings on the remaining two wafers were dissolved in concentrated HNO3, and the digest was diluted in deionised water and monitored for total Fe and As content via ICP-AES in order to calculate a mass balance.

To investigate whether flavins were secreted by S. ANA3, the supernatants of these experiments were analysed for the sum of flavins using HPLC with a fluorescence detector (Thermo Scientific Dionex BioLC, GP50 pump, Dionex Ultimate 3000 fluorescence detector set at 450 nm (excitation) and 520 nm (emission), using a C18 column with a mobile phase of 40% v/v methanol at a flow rate of 1 mL min−1).

Preservation of samples for SEM and NanoSIMS

To preserve the cell structure for electron microscopy and NanoSIMS, and to maintain the stability of reduct sensitive mineral phases, the wafer was removed from the experimental bottle in an anaerobic cabinet and placed immediately into an anoxic 2.5% glutaraldehyde solution in phosphate buffered saline (PBS in g/l NaCl 8, KCl 0.2, Na2HPO4 1.42 and KH2PO4 0.27) and left overnight (Schurig et al. 2013). The wafer was then placed into an anaerobic 1.5% glutaraldehyde PBS solution for 1 hour, then a 0.75% glutaraldehyde PBS solution for 1 hour, then dehydrated sequentially with 25%, 40%, 50%, 60%, 70%, 80%, 90% and 100% solutions of acetone or ethanol (Schurig et al. 2013), all under anoxic conditions. The wafer was dried in the anaerobic cabinet then immediately coated with gold using a sputter coater. Electron microscopy was performed after the NanoSIMS analysis using a Phillips XL30 ESEM-FEG operated in high vacuum conditions.

NanoSIMS analysis and data processing

The samples were analysed using a Cameca NanoSIMS 50L at the University of Manchester. A 16 keV Cs+ primary ion beam with a current of 0.7–0.8 pA was scanned over the sample surface to generate negative secondary ions. The instrument was aligned to detect 12C−, 13C−, 16O−, 12C14N−, 13C14N−, 56Fe16O− and 75As− taking care to avoid isobaric interferences. Prior to imaging, each area was implanted with a high current, defocused beam to remove the gold coating and achieve a dose of 1 × 1017 Cs+ ions cm−2. The areas for imaging were selected by identifying flat areas of mineral using the ion induced secondary electron image prior to imaging, in order to avoid topographical effects. A dwell time of 5000 μs/pixel was used with an aperture size of 200 μm (D1 = 3) and multiple image planes were acquired to increase the number of counts.

The OpenMIMS plugin (National Resource for Imaging Mass Spectrometry, Harvard, US) for ImageJ (Schneider, Rasband and Eliceiri 2012) was used to correct each image for dead time (44 ns) and then regions of interest (ROIs) were defined by hand using the summed 12C14N images to identify the boundary of every cell (0.41 μm2 ± 0.15, N = 1873). An example showing the cell ROIs for an area of the *G. sulfurreducens* sample is provided as Fig. S1 (Supporting Information). Areas of the sample that appeared particularly bright in the secondary electron images, such as along the edges of flat areas of mineral, were considered likely to have a significant contribution to the signal from topography, therefore were not included in ROIs. The cell ROIs were manually assigned to groups based on whether the cells were located on the iron–(oxyhydr)oxide mineral surface ‘cells on mineral’, next to the mineral ‘cells adjoining mineral’ or located on the wafer background where no mineral was present ‘cells on wafer’. ROIs were also defined where no cells were present on areas of iron–(oxyhydr)oxide mineral ‘mineral’ and the background of the wafer ‘wafer background’.

Average counts per pixel of 12C, 13C, 16O, 12C14N, 13C14N, 56Fe16O and 75As were extracted for each ROI for the *G. sulfurreducens* experiment, and 12C, 13C, 16O, 12C14N, 28Si, 56Fe16O and 75As for each ROI for the S. ANA3 experiment. All values were tabulated in Microsoft Excel and normalised for the total dose implanted during imaging (Table S1, Supporting Information). Normalising by the dose implanted during imaging accounts for differences in signal intensity that may result from differences in primary beam current, number of image planes and area. For *G. sulfurreducens* the 13C enrichment was calculated by taking a ratio of 13C14N and 12C14N to identify cells that had become enriched in 13C compared to natural background; the 12C14N and 13C14N signals were used as these gave higher counts and brighter images with better contrast compared to using 12C and 13C signals. As 12C14N was not measured for the S. ANA3 experiment, 13C enrichment was calculated as the ratio of 13C to 12C. The ratio of 75As relative to 56Fe16O was calculated to indicate where As may be relatively enriched or depleted compared to Fe. Box and whisker plots were generated to visualise the large dataset, with boxes representing the interquartile range and whiskers the 5th and 95th percentile.

It should be noted that after analysing the *G. sulfurreducens* sample, an interference from 56Fe19F was observed in the region of mass 75. Fluorine was not present in the experimental set up, and there are no known biological interactions between *G. sulfurreducens* and fluorine. However, fluorine is usually present in the analysis chamber of NanoSIMS. Although the detector was positioned on the centre of the peak of the As standard it is possible that both 16Fe19F and 75As contributed to...
Figure 1. (A–C) SEM images showing the wafer colonised with Geobacter sulfurreducens after 11 days incubation with electron donor. Many cells appear to be co-located with the iron oxide coating. Arrows on Fig. 1c show example of cells coated in extracellular filamentous material. (D–E) Far fewer cells were present in the no electron donor controls. (F) The iron oxide mineral in the no cell control.

the signal at this mass for the G. sulfurreducens sample. Given the source of the fluorine was the analysis chamber, it would be expected that the contribution from $^{56}$Fe$^{19}$F$^-$ would be homogeneous across the surface of the iron-(oxyhydr)oxide mineral and therefore any changes in the local environment of the cells would be caused by microbially mediated processes. Hereafter we refer to the mass 75 signal as $^{75}$As but we note that some of the counts are likely to have been from $^{56}$Fe$^{19}$F$^-$ as well as $^{75}$As. After the NanoSIMS analysis, wavelength dispersive scanning was performed to measure the K\textsubscript{\alpha} edges using an electron microprobe (JEOL JXA-8530F FEG-EPMA). The results showed conclusively that As was present but there was no F in the sample. The S. ANA sample was analysed after the identification of this interference and the detectors were aligned to avoid any issues with mass interference.

RESULTS AND DISCUSSION
Colonisation of Fe(III) by Geobacter sulfurreducens

After 11 days incubation with G. sulfurreducens and electron donor, SEM imaging showed the wafer surface to be colonised by cells (Fig. 1a–c). Far fewer cells were visible on the wafer from the no electron donor control experiment (Fig. 1d–e). The texture of the iron-(oxyhydr)oxide mineral in the no cell control was similar to that observed in the experiments with cells (Fig. 1f). Different cell morphologies were visible; some cells were present as well preserved individuals (Fig. 1a–b) while some appeared to be coated in extracellular filamentous material, which may have been involved in biofilm formation (Fig. 1c). Geochemical measurements confirmed that soluble iron was released to solution during the 11 day experiment, presumably as Fe(II), given that Fe(III) is very poorly soluble under these conditions.
Colonisation of Fe(III) by Shewanella ANA3

After 11 days incubation, SEM imaging confirmed that the wafer surface was colonised by cells (Fig. 4a–d); some were in contact with (adjoining) the iron-(oxyhydr)oxide coating while others were located on the wafer background. It is noteworthy that unlike with G. sulfurreducens, almost no S. ANA3 cells were located directly on the iron-(oxyhydr)oxide mineral surface. Very few cells could be observed in the no electron donor control experiment (Fig. 4e–f). The texture of the iron-(oxyhydr)oxide mineral in the no cell control was similar to that observed in the experiments with cells (Fig. 1f). Again, geochemical measurements confirmed Fe(III) reduction occurred during the 11 day experiment (Fig. 2a). A greater quantity of Fe was released to solution with S. ANA3 compared to G. sulfurreducens (Fig. 2b), perhaps due to the higher cell density of the S. ANA3 experiment.

Again, NanoSIMS analysis was used to locate metabolically active cells in relation to the mineral. Three areas of the sample were analysed (Fig. S3, Supporting Information). Cell ROIs were manually assigned to the groups, ‘cells adjoining mineral’ (N = 11), and ‘cells on wafer’ (N = 43). No cells were visible on the mineral surface therefore there were no ROIs for this group. ROIs were also defined for ‘mineral’ (1.92 ± 0.96 μm², N = 31) and the ‘wafer background’ (3.35 ± 3.17 μm², N = 32).

Cells enriched in 13C were present in all of the areas analysed (Fig. 5a–c), with some cells enriched up to seven times the natural background levels. Artefacts associated with sample preparation, including dilution of 13C from chemical fixation (Musat et al. 2014) and potential interference from 11B16O– meant that it was not possible to measure absolute 13C enrichment values. However, this was not necessary for this experiment as the 13C was used to locate the metabolically active cells rather than measure absolute values of 13C enrichment. To see where the enriched cells were located in relation to the mineral substrate, composite images were created using ImageJ for the iron-(oxyhydr)oxide mineral, 13C14N and 12C14N. These clearly showed that overall, the cells that were metabolically active and relatively enriched in 13C tended to be co-located with the iron-(oxyhydr)oxide mineral, whereas the cells that were less enriched in 13C tended to be located on the wafer background (Fig. 3a). There were some exceptions to this, such as in Area 6 where there was a cluster of cells that were highly enriched in 13C to the left of the iron-(oxyhydr)oxide mineral, which may have been redistributed during fixation.

Box and whisker plots were made of all cell ROIs to further display the distribution of 13C enrichment (Fig. 3b). The results showed that cells located on or adjoining the iron-(oxyhydr)oxide mineral were significantly more enriched in 13C (P < 0.001) and therefore metabolically active, compared to cells located on the wafer background away from the iron-(oxyhydr)oxide mineral. The 13C enrichment values for the wafer background and the mineral surface were slightly higher than natural background; this is likely due to sorption of some of the 13C-acetate. Cells on the wafer were not significantly different in 13C enrichment compared to the wafer background (P = 0.89).

Figure 2. (A) Bulk geochemical monitoring of the aqueous phase of the NanoSIMS wafer incubations showing solubilisation of Fe in microbially active incubations. G. sulfurreducens is shown with circle symbols (• average 4 replicates, ○ no electron donor control), S. ANA3 with triangles (▲ average 3 replicates, □ no electron donor control), and the no cell control with squares (■). Controls are shown with dotted lines and open symbols. Error bars are ±1 standard deviation. (B) Bulk geochemical monitoring of Fe on duplicate wafers after 11 days incubation with G. sulfurreducens or S. ANA3 supplied with electron donor compared to a no cell control. Annotations represent the % Fe on the wafer.
Figure 3. (A) Composite images showing the location of G. sulfurreducens cells in relation to mineral (\textsuperscript{56}Fe\textsuperscript{16}O in red, \textsuperscript{13}C\textsuperscript{14}N in green, \textsuperscript{12}C\textsuperscript{14}N blue). Metabolically active cells relatively enriched in \textsuperscript{13}C (in green) tend to be co-located with the iron oxide mineral (red), while less enriched cells tend to be located on the wafer background.

(B) Box and whisker plots of \%\textsuperscript{13}C calculated from \textsuperscript{13}C\textsuperscript{14}N and \textsuperscript{12}C\textsuperscript{14}N counts (\textsuperscript{13}C\textsuperscript{14}N/\textsuperscript{12}C\textsuperscript{14}N \times 100), showing that cells located on or adjoining the iron oxide mineral were significantly more enriched in \textsuperscript{13}C than cells located on the wafer background (\(P < 0.001\), Tukey–Kramer test). The \textsuperscript{13}C enrichment on the wafer background and mineral surface, were not significantly different (\(P > 0.99\), Tukey–Kramer test), nor was the \textsuperscript{13}C enrichment of the cells on the wafer compared to the wafer background (\(P = 0.89\), Tukey–Kramer test). ‘Nat’ shows known value of naturally occurring \textsuperscript{13}C of 1.11\%. 

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Figure 4. (A-D) SEM images showing the wafer colonised with Shewanella ANA3 after 11 days incubation with electron donor. Cells are located next to the iron oxide mineral and also on the uncoated wafer surface. (E-F) Almost no cells were visible present in the no electron donor controls.

not significantly enriched with $^{13}$C compared with the natural background. This clearly demonstrates the different mechanisms of electron transport to extracellular Fe(III) minerals used to support the metabolism of these two bacteria.

**Use of NanoSIMS to infer mechanisms of extracellular electron transport**

Our experimental set up was successful in supporting the growth of cells of *G. sulfurreducens* on a thin film of iron(III)-(oxyhydr)oxide. Cells located on or adjoining the mineral were significantly more enriched in $^{13}$C and therefore metabolically active compared to those located on the wafer surface. This is consistent with *G. sulfurreducens* requiring direct contact with an extracellular electron acceptor (i.e. the Fe(III)-(oxyhydr)oxide mineral surface) to transfer the electrons required for respiration, via outer membrane cytochromes or conductive pili extending from the cells surface (Lovley 2012). We believe this to be the first time that this relationship has been visualised using $^{13}$C labelling on an in situ sample.

In contrast, there were almost no cells of *S. ANA3* located on the iron-(oxyhydr)oxide mineral surface, and the cells adjoining the mineral or on the wafer background were similarly enriched in $^{13}$C. This is consistent with previous results showing that Shewanella species do not require direct contact with an extracellular electron acceptor for electron transport to occur (Lies et al. 2005). Instead, electron transport may occur via flavocytochrome complexes on the outer surface of the cells, which make intermittent contact with the mineral surfaces via chemotaxis (Harris et al. 2010; Harris, El-Naggar and Nealson 2012) or alternatively by secretion of extracellular flavins that acted as an electron shuttle (von Canstein et al. 2008). Compared to the no cell control where riboflavin was not detected, when *S. ANA3* cells were present riboflavin concentrations between 60
and 91 nM were measured, similar to those previously observed for *S. oneidensis* MR1 (von Canstein et al. 2008), although unlike in this previous study no extracellular flavin mononucleotide was detected in this experiment. This suggests that the secretion of extracellular flavins by *S. ANA3* may have contributed to electron transport in this system.

**Impact of microbial Fe(III)-reduction on arsenic solubility and speciation**

To further explore the impact of extracellular electron transfer on redox-active trace elements associated with Fe(III) minerals during bioreduction, the fate of the incorporated As(V) was determined. After 11 days of incubation, As was released to solution (Fig. 6a). Some arsenic was solubilised as As(V) in the no electron donor controls and the no cell control, which indicates that As was partially soluble under these experimental conditions. This may have been due to the experimental set up that comprised a relatively large volume of minimal medium to a small quantity of mineral, with a large surface area presented as a thin film. Slightly more arsenic was released to solution in the microbiologically active incubations suggesting that microbial Fe(III)-reduction had somewhat enhanced the release of arsenic under these experimental conditions.

Speciation of aqueous As (IC-ICP-MS) showed that 99.9% was present as As(V) with *G. sulfurreducens*, and 21% As(V) and 79% As(III) (± 1.6%) with *S. ANA3*, as expected given that *G. sulfurreducens* lacks the operon that encodes for respiratory As(V) reduction. It is noteworthy that after incubation with the model As(V) reducer *S. ANA3*, the majority of As had been reduced to As(III), which suggests that at least some of the aqueous-phase As(V) that was released from the mineral abiotically must have been respired, as well as the As(V) that was likely released during microbial Fe(III)-reduction. Previous work has also noted the co-dependency of the rate of As(V) release from ferricyanide and the rate of formation of As(III) by *Shewanella* species (Revesz, Fortin and Paktunc 2016).

The amount of iron and arsenic present on the wafers after 11 days was measured and compared to the amount that was solubilised to assess whether arsenic had been preferentially released from the mineral matrix to solution compared to Fe(II). Analyses were performed on the two replicate wafers that were
aqueous geochemistry results (Fig. 6a). The values for the cally under these experimental conditions, also reflected in the As (relative to Fe) in the thin films was solubilised abiotically 12 mol/mol starting value, showing that more than 50% of Fe in the no cell control. All values were substantially lower than the 100 nmol Fe with Fe present on the wafer, compared to 4.4 ± 0.1 nmol Fe per 100 nmol Fe with S. ANA3 and 5.3 ± 0.03 nmol Fe per 100 nmol Fe in the no cell control. All values were substantially lower than the 12 mol/mol starting value, showing that more than 50% of the As (relative to Fe) in the thin films was solubilised abiotically under these experimental conditions, also reflected in the aqueous geochemistry results (Fig. 6a). The values for the G. sulfurreducens wafers were significantly different to the no cell controls at the 95% confidence level, confirming that the presence of metabolising G. sulfurreducens cells led to more As being released to solution relative to Fe, over and above the abiotic As solubilisation. The values for the S. ANA3 wafers were not significantly different to the no cell control wafers or the G. sulfurreducens wafers at the 95% confidence level, but this was affected by the higher variation in the duplicate S. ANA3 wafer measurements, suggesting more replicates would be required to conclusively show whether or not the presence of S. ANA3 caused increased mobilisation of As from the wafer relative to Fe.

Using the relationship between As and Fe to observe the effect of microbial Fe(III)-reduction by Geobacter sulfurreducens

Data were extracted from the NanoSIMS analysis (Fig. S2, Supporting Information) to assess whether the distribution of iron and arsenic was different in the local environment of the cells, and whether this was different to the bulk geochemical measurements. Given that S. ANA3 was not co-located with the mineral surface, the G. sulfurreducens data set was used for this analysis, which aimed to determine if NanoSIMS could be used to probe the direct impact of microbial metabolism on trace metals associated with Fe(III)-(oxyhydr)oxides.

The ratio of $^{75}$As to $^{56}$Fe$^{16}$O was calculated for each individual cell ROI and for the ROIs on the mineral surface where no cells were present to assess the impact of the cells on the As/Fe distribution of the mineral (Fig. 7). The box and whisker plots clearly demonstrate that the distribution of $^{75}$As in the mineral surface where no cells were present. There was some heterogeneity between individual areas of the sample (Fig. S4, Supporting Information), highlighting the importance of making multiple measurements even within the same sample.

To hypothesise the processes that could lead to differences in the relative amount of As in the local environment of G. sulfurreducens, we reason that the inoculated cells would first become associated with the wafer surface through diffusion, electrostatic attraction or chemotaxis. The cells that had direct contact with the iron(III)-(oxyhydr)oxide mineral would then begin to respire the Fe(III) coupled to the use of $^{13}$C-acetate. This would lead to the release of aqueous Fe(II) and the consequent release of As(V) to solution, over and above what occurred in the no cell and no electron donor controls, probably due to the microbially-induced dissolution of the Fe(III) mineral via reduction and solubilisation as Fe(II). Here, in the local environment of the cells,
there was proportionally more As present compared to where no cells were located, showing the preferential release of Fe(II) from the mineral, compared to As(V). Although the $^{75}$As relative to $^{56}$Fe$^{16}$O results from the NanoSIMS analysis were supported by similar measurements from bulk geochemical monitoring, care should be exercised when interpreting these findings as the ionisation yields for $^{75}$As$^{-}$ and $^{56}$Fe$^{16}$O$^{-}$ are not the same and will be affected by matrix effects.

**Use of NanoSIMS to assess the impact of microbial metabolism on bulk and trace elements**

Using NanoSIMS was an effective way to visualise the relationship between active Fe(III)-reducing bacteria and a solid Fe(III) electron acceptor substrate. The contrasting results for bacteria that use different mechanisms of extracellular electron transport illustrates the requirement for direct contact with the substrate and consequent mineral attachment with Geobacter sp., compared to the ability of Shewanella sp. to conduct extracellular electron transport without attaching to the mineral surface. This technique could be useful to study a wide range of Fe(III)-reducing microorganisms, alongside biochemical and genetic analyses to fully understand the mechanisms of electron flow to Fe(III) and other insoluble electron acceptors.

Given that the environmental behaviour of many trace elements is controlled by Fe/Mn biogeochemistry, using NanoSIMS could provide new insights into how their fate could be controlled by microbial processes. Our findings using arsenic as an example of a trace element show that there appears to be differences in the proportion of arsenic relative to iron in the local environment surrounding metabolically active cells, compared to bulk geochemical measurements. Further work needs to be done to investigate this effect. It is likely that S. ANA3 reduced the bulk of As(V) to As(III) in the aqueous phase, and again additional work will be required to confirm this. In aquifer systems, the ability to respire As(V) that is mobilised during the reductive dissolution of Fe(III)-(oxyhydr)oxides, will result in the formation of poorly sorbing As(III), which will accumulate in groundwaters once available sorption sites in the aquifer have been saturated (Gnanaprakasam et al. 2017). This work opens the possibility for more detailed studies on Fe-As coupling using model systems and mixed community studies, using synthetic minerals and also aquifer materials. This type of analysis could be highly useful for probing Fe-trace metal distributions when studying a broader range of coupled processes, for example, Mn/Fe/S systems, and their impact on biogeochemical cycling of toxic metals and radionuclides such as Se, I and U.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSEC online and the full dataset is available at Mendeley data (http://dx.doi.org/10.1763/2/gppn7styxx.1).

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**Conflict of interest** None declared.

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### SUPPORTING TABLES

Table S1. Correction factors used to normalise for total image dose

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<th>Area</th>
<th>Number of scans performed</th>
<th>Total dose implanted during imaging (Cs atoms cm(^{-2}))</th>
<th>Dose correction factor applied to dataset *</th>
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*The dose correction factor was obtained by dividing the total dose implanted by \(1 \times 10^{15}\)
Figure S1. (a) Example showing all cell Regions of Interest (ROIs) defined for Area 6 of the *Geobacter sulfurreducens* experiment. (b) ROIs for cells on the mineral surface. (c) ROIs for cells on the wafer background.
Figure S2. Images for the nine areas of the Geobacter sulfurreducens sample that were analysed with NanoSIMS. The first column shows secondary electron intensity distribution maps. $^{12}\text{C}^{14}\text{N}$ shows where biomass was present. $^{56}\text{Fe}^{16}\text{O}$ shows the iron oxide mineral. The $^{75}\text{As}$ images are likely to show some contribution from the $^{56}\text{Fe}^{19}\text{F}$ interference. $^{13}\text{C}$ enrichment was calculated as a ratio of $^{13}\text{C}^{14}\text{N}$ to $^{12}\text{C}^{14}\text{N}$. 
Figure S3. Images for the three areas of the S. ANA-3 sample that were analysed with NanoSIMS. The first column shows secondary electron intensity distribution maps. $^{12}$C$^{14}$N shows where biomass was present. $^{56}$Fe$^{16}$O shows the iron oxide mineral. The $^{75}$As images show co-localization with the mineral and the $^{56}$Fe$^{16}$O signal. $^{13}$C enrichment was calculated as a ratio of $^{13}$C to $^{12}$C.
Figure S4. Box and whisker plots of $^{75}$As counts as a percentage of $^{56}$Fe$^{16}$O counts illustrated for the entire *Geobacter sulfurreducens* dataset (same data as Figure 7, here far left grey/pink) and for the nine areas of the sample analysed (black/red). Black/grey boxes show data for regions of interest (ROIs) on the mineral surface, red/pink boxes are ROIs of the cells located on the mineral. N refers to the number of ROIs. Results clearly demonstrate that in 8 of the 9 areas analysed there was a higher proportion of $^{75}$As relative to $^{56}$Fe$^{16}$O in the local environment of the cells compared to the un-colonised mineral surface ($P < 0.001$, Tukey-Kramer test).
Figure S5. Normalised count data for the entire *Geobacter sulfurreducens* dataset.

(a) $^{12}$C$^{14}$N. (b) $^{13}$C$^{14}$N. (c) $^{56}$Fe$^{16}$O. (d) $^{75}$As. (e) $^{16}$O.
Chapter 5- A Single-cell Approach to Elucidate the Mobilisation Mechanism of Adsorbed Arsenate in *Shewanella* sp. strain ANA-3

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Keywords

Arsenate, arsenite, arsenic mobilisation, iron(III)-oxyhydroxide, *Shewanella* ANA-3, NanoSIMS, flavins, extracellular electron transfer

5.1 Abstract

In anoxic environments bacteria are thought to play a controlling role in arsenic mobilisation from sediments through dissimilatory metal reduction. Fe(III) minerals are natural sinks for As, and the Fe(III) reduction by subsurface bacteria can stimulate the desorption of this toxic element as arsenate-As(V); this arsenate can additionally be mobilised as arsenite-As(III) by As(V)-respiring bacteria. This investigation focuses on the study of the reductive dissolution of As(V) sorbed onto Fe(III)-(oxyhydr)oxide by model Fe(III)- and As(V)-reducing bacteria, to broaden the understanding of the mechanisms used in this extracellular electron transfer process. Axenic cultures of *Shewanella* sp. ANA-3 wild type (WT) cells (able to respire both Fe(III) and As(V)) were grown using \(^{13}\)C-labelled lactate, on a silicon wafer coated with arsenical Fe(III)-(oxyhydr)oxide, and after colonisation, the distribution of Fe and As in the solid phase was assessed using high
resolution nanoscale secondary ion mass spectrometry (NanoSIMS), complemented with analyses of the aqueous Fe, As and flavins. In addition, parallel experiments were conducted using an arrA mutant that was unable to respire As(V) (but could still respire Fe(III)). The majority of active cells (that were labelled with $^{13}$C) were not in direct contact with the Fe(III) mineral, and flavins were released by both strains, supporting the electron shuttle mechanism as the main mediator in Fe(III)-(oxyhydr)oxide respiration by Shewanella species. Our findings also show that cell-secreted flavins are not facilitators of As(V) reduction, only of Fe(III) reduction. Single-cell NanoSIMS depth profiles revealed As and Fe are re-sorbed to the cell surface in the WT samples, whereas the arrA mutant sample (which only solubilised As(V)), showed Fe but not As sorbed to the cell surface. It therefore appears that Shewanella ANA-3 cells respire As(V) in a multistep process: cells first reduce Fe(III), leading to reductive dissolution of the Fe mineral and release of As(V), and once in solution, As(V) is respired by the cells to As(III), which is more soluble in these experiments, and by inference in environmental systems.

5.2 Introduction

In environments where oxygen is absent or depleted, bacteria and archaea have developed mechanisms to harness other molecules as terminal electron acceptors to conserve energy (Weber et al., 2006). Fe(III) respiration by dissimilatory metal-reducing bacteria is a well-known of these anaerobic processes (Lee and Newman, 2003; Lloyd, 2003). Iron is a necessary element for most life forms, although it is usually required in trace amounts. Physicochemical conditions dictate the chemical form in which iron is found in the environment, where Fe$^{2+}$ and Fe$^{3+}$ are the most common forms (Hedrich et al., 2011). In anoxic conditions, ferrous iron (Fe$^{2+}$) predominates, while in oxygen rich environments ferric iron (Fe$^{3+}$) is the predominant species. Fe(III) minerals are strong sorbents of metals and metalloids, and their reduction could solubilise the minerals along with potentially toxic sorbed molecules, for example arsenate. Arsenic is a poisonous element that occurs naturally in rocks, sediments and groundwater; its concentration and incidence in soils and fresh waters greatly depends on host geology and also anthropological contributions, for example through mining or pesticide use in
agriculture (Bowell et al., 2014). The World Health Organisation (WHO) has set a limit of 10 µg L⁻¹ of As in drinking water. The most common forms of arsenic are inorganic trivalent arsenite [As(III)] and pentavalent arsenate [As(V)] oxyanions (Cullen and Reimer, 1989; Bowell et al., 2014). Arsenate and arsenite have different sorption behaviours to iron surfaces; they can both sorb to common Fe(III) minerals (e.g. ferrihydrite, goethite, hematite) although As(III) is sorbed through weakly bound complexes, desorbing more easily than As(V) (Tufano et al., 2008). Therefore, arsenite is regarded as the most soluble and mobile of these oxyanions, and more toxic than As(V) (Cullen and Reimer, 1989; Shen et al., 2013).

Underpinning work on bacterial As respiration was undertaken by studying soda lakes sediments, which has helped elucidate the role of bacteria in As mobilisation, mainly through bacterial As(V) dissimilatory reduction (Dowdle et al., 1996; Oremland et al., 2000; Oremland et al., 2002; Oremland and Stolz, 2003; Hoeft et al., 2004; Oremland et al., 2004). Field studies in As-impacted aquifers and lakes have shown that As(V) predominates near the surface whereas As(III) predominates as depth increases (Oremland and Stolz, 2003; Gnanaprakasam et al., 2017; Podgorski et al., 2017). In this sense, the occurrence of As(V) usually matches that of amorphous Fe(III) minerals (such as ferrihydrite and goethite), which are reduced as depth increases, implying a decrease in the number of As sorption sites. In Bangladesh and West Bengal, a widely studied area impacted with natural high levels of As in groundwater (reaching above 50 µg L⁻¹ of As in some cases), As is thought to be desorbed and reduced as arsenate from Fe(III) minerals (Bowell et al., 2014; Gnanaprakasam et al., 2017). In subsurface conditions As could be mobilised through different mechanisms, where more than one mechanism might be at play through a diversity of bacterial metabolisms (Zobrist et al., 2000). For example (i) Fe(III)-respiring bacteria could desorb As(V) via reductive dissolution of Fe(III) minerals, (ii) dissimilatory arsenate-reducing bacteria (DARB) could directly reduce sorbed As(V), or (iii) As could be mobilised by a sequential process where dissimilatory Fe(III) and As(V)-respiring bacteria first desorb As(V) from Fe(III) minerals and then respire soluble As(V)
(Zobrist et al., 2000). The exact mechanism elucidating the spatial and temporal As(V) reduction and accumulation of As(III) is still unclear.

*Shewanella* is a genus of metabolically flexible facultative anaerobes capable of respiring a variety of terminal electron acceptors including nitrate, fumarate, Fe(III) minerals, Mn(IV)/(III) minerals, As(V) and radionuclides such as U(VI) and Tc(VII) (Hau and Gralnick, 2007; Fredrickson et al., 2008). This respiratory diversity appears to be advantageous for cells inhabiting interfacial environments (Hau and Gralnick, 2007). *Shewanella* species unlike other Fe(III)-reducing species such as *Geobacter* do not require direct contact with the Fe(III) mineral they are respiring. In anoxic environments Fe(III) minerals are usually poorly soluble, posing a challenge for uptake and utilisation by the cellular electron transport system. The four proposed mechanisms for the reduction of poorly soluble extracellular Fe(III) oxide minerals by *Shewanella* species are: (i) use of an endogenous (e.g. flavins) or exogenous (e.g. humic acids) electron shuttle, (ii) through chelators, (iii) via direct contact with outer membrane heme-containing c-type cytochromes and (iv) by using electrically conductive nanowires in the form of outer membrane protein extensions (Weber et al., 2006). Until recently, flavin mononucleotide (FMN) and riboflavin were identified as the electron shuttles secreted by different *Shewanella* strains, whereas flavin adenine dinucleotide (FAD) is produced intracellularly but not released to the medium (von Canstein et al., 2008). Additionally, when *S. oneidensis* MR-1 is growing in biofilms using electrodes as terminal electron acceptors, flavins can bind to outer membrane cytochromes, forming flavocytochrome complexes that can accelerate extracellular electron transfer (Okamoto et al., 2013; Edwards et al., 2015; Xu et al., 2016). This electron shuttling mechanism could be advantageous when growing in biofilms, where there are higher microbial densities, or when cells cannot get in direct contact with an insoluble electron acceptor, such as in the clay pore space in sediments (Brutinel and Gralnick, 2012). Humic substances (HS) are a complex heavy molecular weight material present in aquifers and soils with the capability to actively transfer electrons in the environment in interactions with bacteria and simple organic molecules (Lovley et al., 1996; Lovley et al., 1998; Scott et al., 1998). A large amount of evidence
supports the hypothesis that the quinones fraction in HS are the sites for electron transfer where bacteria reduce HS and the reduced HS shuttle electrons to insoluble Fe(III) oxides in an energy conserving mechanism (Lovley et al., 1998; Scott et al., 1998). One of the proposed models for long-range electron transfer from Shewanella cells was thought to be through electron conductive nanowires, analogous to Geobacter (Malvankar et al., 2014; Pirbadian et al., 2014; Adhikari et al., 2016; Reguera, 2018). This theory has been recently challenged as the cytochromes in these extensions may not be tightly packed enough to support electron transfer or electron hopping from cytochrome to cytochrome. Alternatively, it has been suggested that a combination of direct electron hopping and diffusion with electron shuttles could explain the electron transfer observed (Subramanian et al., 2018).

The mechanism of electron flow from organic electron donors (such as lactate, pyruvate or amino acids) in Shewanella cells involves the Mtr pathway, where six multihaem c-type cytochromes (CymA, Fcc3, MtrA, MtrC, OmcA and STC) and a porin-like outer membrane protein (MtrB) transport electrons from the menaquinone pool in the cytoplasmic membrane to the cell surface. Thus electrons are transported from the cytoplasm to the outer cell surface through the Mtr pathway, and from the cell surface electrons are carried by flavins, hopping from flavin molecule to molecule until reaching the surface of an insoluble electron acceptor (Shi et al., 2012; Shi et al., 2016).

In contrast to Fe(III) mineral reduction, As(V) reduction is not mediated via the complex network of c-type cytochromes present in Fe(III)-reducing bacteria. The ability to reduce arsenate to arsenite by microorganisms is, however, widespread in bacteria and yeast (Cullen and Reimer, 1989; Oremland and Stolz, 2003). In most cases this reduction is part of a resistance or detoxification process, mediated through the well-studied ArsC arsenate reductase system (Oremland and Stolz, 2003; Héry et al., 2008), where the arsenate-resistant microbes do not gain energy from the process. A smaller group of microorganisms, the dissimilatory arsenate-reducing bacteria, reduce arsenate to conserve energy (Oremland and Stolz, 2003). Shewanella sp. strain ANA-3 possesses both the As(V) detoxification and respiratory systems. The As(V) detoxification system is
encoded in the *ars*DABC operon, located in the cytoplasm and active in both aerobic and anaerobic conditions (Saltikov et al., 2003). The *S*. ANA-3 As(V) respiration system, encoded in the two-gene cluster *arrAB*, contains an As(V) reductase that anaerobically scavenges As(V) and couples its reduction to the oxidation of an electron donor. Both gene subunits are essential for As(V) respiration (Saltikov and Newman, 2003). Moreover, the X-ray crystal structure of the Arr arsenate reductase complex of *S*. ANA-3 was recently elucidated in an impressive work by Glasser et al. (2018b), where phosphate was predicted to mainly affect as an arsenate desorption enhancer, by competing with arsenate for sorption sites, rather than enzymatically inhibiting it, given that the arsenate reductase also binds phosphate but preferably binds arsenate.

Metagenomic DNA-based approaches have been used to initially characterise the microbial communities inhabiting As-impacted aquifers (Desoeuvre et al., 2016; Gnanaprakasam et al., 2017), although these methods are limited for targeting active microorganisms *in situ*. Transcriptomic and proteomic based approaches have been proposed for the identification of the transcripts and proteins expressed, complementary to metagenomic studies. Additional to the geochemical, genetic and enzymatic identification of key role players in Fe and As transformations in the environment, routine imaging techniques have been used to study these microorganisms and their interactions with the mineral they are transforming. NanoSIMS is a high resolution imaging technique that allows the identification of metabolically active microorganisms —linking function and identity—and the semi-quantification of trace element abundances at the nanoscale (Li et al., 2008). These characteristics can be exploited to investigate the fate of As and Fe in the solid phase during bacterial respiration. In NanoSIMS, a solid surface is bombarded by an energetic primary ion beam, eroding it and generating secondary ions (SI) that are detected and analysed in a mass spectrometer. The spatial resolution of NanoSIMS, down to 50 nm (Hoppe et al., 2013) make this technique suitable to study microbial cells, even at the subcellular level. For instance, NanoSIMS has been applied to study cyanobacteria within hypersaline microbial mats (Fike et al., 2008), phototrophic bacteria to infer C and N cycling in lake...
Cadagno sediments (Musat et al., 2008), to image cyanobacteria and map the uptake of halogens (Eybe et al., 2008), to distinguish active from inactive Fe(II)-oxidising bacteria (Miot et al., 2015) or to study methane-oxidising microbial consortia to elucidate syntrophy via electron transfer between archaea and bacteria (McGlynn et al., 2015).

The aim of this work was to study the reduction of adsorbed As(V) onto an Fe(III)-(oxyhydr)oxide by two Shewanella sp. ANA-3 strains using NanoSIMS, a high resolution imaging technique. NanoSIMS was used to image bulk and trace As and Fe, including during single-cell depth profiles, allowing the subcellular localisation of these two elements. The active fraction of the cells was identified by $^{13}$C accumulation above natural abundance. The S. ANA-3 arrA deletion mutant made it possible to contrast the contributions of Fe(III) reductive dissolution and the ars As(V) detoxifying system on As mobilisation, with the S. ANA-3 WT strain additionally expressing the arr As(V) respiratory system. The effect of flavins in As(V) reduction, as an example of secreted electron shuttles, was also assessed in this work. NanoSIMS was therefore used to address the question of how As is mobilised by bacteria in subsurface conditions by studying the fate of As in the solid phase, complementary to the study of As in the aqueous phase through geochemical analyses. This model system could advance the understanding of long-term persistence of arsenite in subsurface environments under unaltered redox conditions.

5.3 Materials and methods

5.3.1 Bacteria strains and culture

Shewanella sp. ANA-3 strain wild type (WT) and the non As(V)-respiring arrA deletion mutant, S. ANA-3 strain ARRA3, were used for these experiments. The bacteria strains were kindly donated by Prof Dianne Newman. Both strains were grown in aerobic lysogeny broth (LB) agar plates at 30°C for 24 h. Isolated colonies were used to inoculate aerobic LB broth in Erlenmeyer flasks (to generate biomass) and incubated at 30°C/150 rpm for 24 h. This aerobic culture was used to produce the anaerobic inoculum for the experiments, using 5 mL of this aerobic culture to inoculate serum bottles with 100 mL
of anaerobic minimal medium containing in g/L: K$_2$HPO$_4$ 0.225, KH$_2$PO$_4$ 0.225, NaCl 0.46, (NH$_4$)$_2$SO$_4$ 0.225, MgSO$_4$ 7H$_2$O 0.117, NaHCO$_3$ 4.2, fumaric acid 4.64, in mL/L: sodium lactate 2.59, trace elements and vitamin solutions 10 mL each (Saltikov et al., 2003). These bottles were incubated statically in the dark at 30°C. After 48 h the cells were washed with anaerobic 30 mM bicarbonate buffer by centrifuging at /30 min (twice) and the resulting pellet was re-suspended in a small volume of the same buffer, keeping the anaerobic conditions throughout this washing process. The concentration of cells was measured as OD$_{600}$ in a Jenway 6715 UV/Vis spectrophotometer and used to inoculate the pure culture experiments.

5.3.2 Arsenical Fe(III)-(oxyhydr)oxide thin film

An amorphous Fe(III)-(oxy)hydroxide (ferrihydrite) was prepared by dissolving 16.2 g of FeCl$_3$ in 500 mL of deionised water (dH$_2$O), the pH was raised to 7.0 by adding a 10 M NaOH solution under constant stirring, and the resulting solution was washed with dH$_2$O and centrifuged 6 times (4000 rpm/20 min) (Schwertmann and Cornell, 2000). The final concentration of biologically available Fe in the ferrihydrite solution was 140 mM. A solution of sodium arsenate—As(V) (Na$_2$HAsO$_4$·7H$_2$O, Sigma-Aldrich®)—was prepared to give a final concentration of 12 % mol/mol As/Fe when mixed with the ferrihydrite solution, and this mixed solution was stirred in a roller shaker for 24 h to favour the sorption of As(V) on the Fe(III) mineral surface sites. Thin films of arsenical Fe(III)-(oxyhydr)oxide were prepared by depositing small grains through precipitation on boron doped silicon wafers (7 x 7 x 0.5 mm) by pipetting 40 µL of the 12 % mol/mol As/Fe solution and let dry overnight.

5.3.3 Preparation of microbial samples for NanoSIMS analysis

Microbial incubation samples for NanoSIMS analysis were prepared in 15 mL anaerobic serum bottles. The thin film coated Si wafers were placed vertically in a plastic holder that was fixed to the bottom of the bottle with silicon grease (to avoid disturbance of the thin film). Seven mL of fresh water medium (FWM in g/L: NaHCO$_3$ 2.5, NH$_4$Cl 0.25, NaH$_2$PO$_4$·H$_2$O 0.6, KCl 0.1; vitamin mix and mineral mix 10 mL each (Wilkins et al., 2007) with 20 mM $^{13}$C-labelled sodium lactate (Sigma-Aldrich®) were slowly added to the
serum bottles. The arsenaic Fe(III)-(oxyhydr)oxide was used as the sole terminal electron acceptor and $^{13}$C-labelled lactate was used as the electron donor, where $^{13}$C is expected to be accumulated by the cells, allowing the identification of cells that were metabolically active during the experiment. Cells of either *Shewanella* strain were inoculated to give a final OD$_{600}$ of 0.5, following a procedure previously reported (Newsome et al., 2018). This set up was carried out under anaerobic and sterile conditions. The bottles were incubated at 30°C in the dark for 11 days. Two control experiments were prepared, S.ANA-3 WT with no electron donor added (“no donor”) and uninoculated FWM (“no cells”). Supernatant samples were withdrawn at intervals of 3 days to measure aqueous Fe and As species and flavins. Total aqueous Fe ($Fe_{total}$) and As ($As_{total}$) were measured only at day 11.

5.3.4 Analytical methods
The HCl-extractable Fe(II) and total biologically available Fe(II) were quantified by reacting the samples with a ferrozine solution and measuring the absorbance at 562 nm, following a standard methodology (Lovley and Phillips, 1986). As(III) and As(V) species in solution were quantified by dissolving the samples in $d_H_2O$ and analysed by IC-ICP-MS. $Fe_{total}$ and $As_{total}$ were measured by acidifying the samples in 2 % v/v HNO$_3$ and analysed by ICP-AES (Perkin–Elmer Optima 5300 DV). Flavin mononucleotide (FMN) and riboflavin were measured on the supernatants using HPLC with a fluorescence detector, as detailed elsewhere (Newsome et al., 2018). Riboflavin and FMN solutions (0.1-1.0 µM) were used as reference. Geochemical results and statistical analyses were obtained and plotted in OriginPro® 8.5.1.

5.3.5 Sample preservation for scanning electron microscopy (SEM) and NanoSIMS imaging
At day 11 the Si wafers were removed from the serum bottles and preserved by chemical fixation and dehydration in an anaerobic cabinet. The wafers were submerged in solutions of decreasing concentrations of glutaraldehyde (2.5, 1.5 and 0.75 % v/v) in phosphate buffered saline (PBS in g/L NaCl 8.0, KCl 0.2, Na$_2$HPO$_4$ 1.42 and KH$_2$PO$_4$ 0.27) adjusted to pH 7.0. The wafers were left in the 2.5 % glutaraldehyde solution for 4 h and...
for 30 min in the other two solutions, afterwards, glutaraldehyde was exchanged for $d$H$_2$O and incubated for 30 min. After cell fixation, the samples were dehydrated by exchanging the $d$H$_2$O for ethanol solutions of increasing concentrations (25, 30, 40, 50, 60, 70, 80, 90 and 100 % v/v) and left 30 min in each solution. The wafers were removed from the solvent and air dried in the anaerobic cabinet overnight and once dry, coated with 10 nm of Pt with a sputter coater.

5.3.6 Scanning electron microscopy

Scanning Electron Microscopy (SEM) imaging was used to localise areas of interest with cells and mineral, using a FEI Quanta 650 FEG SEM with a 15 kV beam. The SEM image acquisition was kept to large areas in order to reduce beam damage to the cells as much as possible, but beam damage prior to NanoSIMS analysis cannot be discounted.

5.3.7 NanoSIMS imaging

Samples were analysed in a NanoSIMS 50L ion microprobe (CAMECA, France) using a Cs$^+$ primary ion beam at 16 keV. The primary ion beam was scanned over the surface of the samples with a current of 1.08-0.78 pA to obtain a lateral spatial resolution of $\approx$400-100 nm (aperture D1=3-5) and a CAMECA mass resolving power (MRP) of 5500 and 7000 were used (ES=3 AS=2) for detectors 2 (m/z 13) and 7 (m/z 75), respectively. Silicon wafer, iron metal and gallium arsenide were used as reference materials to improve mass resolution and avoid peak overlapping of molecular interferences, such as $^{12}$C$^{14}$H at m/z 13, $^{28}$Si$^{16}$O$^-$ at m/z 72 and $^{56}$Fe$^{19}$F$^-$ at m/z 75 (as detailed in the next section). The following negative secondary ions (SI) were collected: $^{12}$C$,^{13}$C, $^{12}$C$^{14}$N, $^{28}$Si, $^{56}$Fe$^{12}$C$,^{56}$Fe$^{16}$O$^-$ and $^{75}$As$^-$ (from now on $^{12}$C, $^{13}$C, $^{12}$C$^{14}$N, $^{28}$Si, $^{56}$Fe$^{12}$C, $^{56}$Fe$^{16}$O and $^{75}$As) and analysed using a double focusing mass spectrometer. Images were collected at a dwell time of 5000 µs px$^{-1}$ and pixel size was 256 x 256. Additionally, an ion-induced secondary electron (SE) image was obtained. The $^{12}$C, $^{13}$C and $^{12}$C$^{14}$N signals were used to identify the biomass, and the $^{13}$C/$^{12}$C ratio was used to identify active cells by their $^{13}$C accumulation (above the 1.11 % natural $^{13}$C/$^{12}$C abundance); $^{28}$Si was used to image the wafer surface; $^{56}$Fe$^{12}$C and $^{56}$Fe$^{16}$O were used as $^{56}$Fe proxies, and along with $^{75}$As, these secondary ions were used to map the arsenaclal Fe(III)-(oxyhydr)oxide. Implantation of
Cs$^+$ ions at a dose of $1 \times 10^{17}$ ions cm$^{-2}$ was used to remove the Pt coating and reach steady state on the sample surface before collecting chemical images (McPhail and Dowsett, 2009c).

5.3.7.1 Mass interference at m/z 75
An important mass interference at m/z 75 was detected. After performing tests with standards and a non-silicon based sample matrix (Nunc™ Termanox™ coverslips, Thermo fisher Scientific) it was concluded that the mass interference was $^{56}$Fe$^{19}$F, with $^{56}$Fe originating from the Fe(III)-(oxyhydr)oxide and $^{19}$F likely originating from the residual vacuum in the NanoSIMS analysis chamber. This mass interference was approached by improving MRP over 7000 and aligning the detector at the left edge of the peak in the high mass resolution (HMR) scan (Fig. S5.1), which compromised the intensity of $^{75}$As counts, but allowed to circumvent the isobaric interference.

5.3.7.2 NanoSIMS single-cell depth profiles
After collecting bigger field-of-view (fov) images (30-50 µm width) we zoomed into single active cells in order to collect depth profiles (fov=3-5 µm width). To avoid interferences with As and Fe re-deposition, cells on the Si wafer more than 3 µm away from arsenical Fe(III)-(oxyhydr)oxide grains were selected for the depth profile analysis. The spatial resolution was improved to ~100 nm using D1=5 and in some cases L1 was modified to 3000-7500 V (a smaller D1 aperture produces a smaller probe diameter whilst a higher voltage in L1 increases SI yield), pixel sizes were 128x128 or 256x256, and dwell time varied from 5,000-20,000 µs px$^{-1}$. The scans were prolonged in these depth profiles until the $^{12}$C or $^{12}$C$^{14}$N signal dropped, indicating the sputtering of the bacterial cell and rendering chemical information of the whole cell.

5.3.7.3 NanoSIMS data analysis
NanoSIMS images were obtained with L’IMAGE (Larry Nittler, Carnegie Institution of Washington). Hue-saturation-intensity (HSI) maps of $^{13}$C/$^{12}$C ratios and overlay images were obtained with ImageJ using the OpenMIMS plugin (MIMS, Harvard University; www.nrims.harvard.edu).
Regions of interest (ROI) were hand-drawn in every image collected in L’IMAGE. For the mineral ROIs flat areas away from the edge of the mineral were selected to avoid topographic effects. The counts of the relevant masses analysed (\(^{12}\)C, \(^{13}\)C, \(^{12}\)C\(^{14}\)N, \(^{56}\)Fe\(^{12}\)C, \(^{56}\)Fe\(^{16}\)O and \(^{75}\)As) in each ROI were normalized to the current and dose on each day of analysis. Box plots for the NanoSIMS counts were created with OriginPro\textsuperscript{®}. 3D reconstructions of the depth profiles were created with Thermo Scientific™ Avizo™ Software 9.7.0. Stack data of \(^{12}\)C, \(^{56}\)Fe\(^{12}\)C, \(^{56}\)Fe\(^{16}\)O and \(^{75}\)As were first extracted with ImageJ and saved in the “.raw” format file and loaded into Avizo™. The Z depth was compressed to 15-20 %. The \(^{12}\)C counts were used to generate the bacteria surface by smoothing the signal over 2-3 pixels, then, the “generate surface” and “show surface” commands were used sequentially, and the “transparent” display with a transparency of 80 % was selected. The \(^{56}\)Fe\(^{12}\)C, \(^{56}\)Fe\(^{16}\)O and \(^{75}\)As ion counts were averaged to 1 pixel (because of the lower ion counts) and overlapped on the \(^{12}\)C surface by displaying as “points” to enable their visualisation.

5.4 Results

5.4.1 Fe and As in the aqueous phase

The measurement of total aqueous Fe(II) by ferrozine showed that by day 11 insoluble Fe(III) was reduced to soluble Fe(II) by both S. ANA-3 strains incubated with \(^{13}\)C-lactate as the electron donor (26 µM by the WT and 20.6 µM by ARRA3) (Fig. S5.2), although no Fe(II) was detected in the previous days of incubation, potentially due to the lower Fe(II) concentrations present, below the detection limit of the technique. No Fe(II) was detected in the “no donor” and the “no cells” controls by day 11 (Fig. S5.2). Complementary ICP-AES analyses of the supernatants in \(^{13}\)C-incubated samples showed that the amounts of Fe\(_{\text{total}}\) solubilised by day 11 were comparable to the ferrozine results (29.5 µM by the WT and 20.3 µM by ARRA3) (Fig. 5.1A). However, ICP-AES allowed the detection of low amounts of Fe\(_{\text{total}}\) solubilised in the “no donor” control (8.9 µM) and the “no cells” control (1.16 µM) (Fig. 5.1A). The As\(_{\text{total}}\) measurement by ICP-AES showed that the WT solubilised higher amounts of As (71.43 µM), followed by ARRA3 (45.08 µM), the “no donor” control (41.12 µM) and “no cells” control (37.93 µM). The As mobilisation in
the “no cells” control implies that up to 53 % of the As mobilised by the WT could be due to abiotic desorption. The release of As increased with incubation time, therefore, the last day of incubation showed the highest release of As(III), As(V) or both (Fig. S5.3). S. ANA-3 WT released 83 % of the total As in the form of As(III), whereas ARRA3 cells

Figure 5.1. Aqueous geochemistry of the experiments at day 11. (A) Total As and Fe in solution quantified by ICP-AES and (B) As species in solution quantified by IC-ICP-MS, where total As is the sum of As(III) and As(V) quantified by this method. $^{13}$C-incubated S. ANA-3 WT significantly released more Fe ($p<0.05$, Tukey’s test), while its As release was not significantly higher than in the other conditions ($p>0.05$, Tukey’s test). Only the S.ANA-3 WT experiments released As(III), including the “no donor” control, however the $^{13}$C-incubated S. ANA-3 WT significantly produced more As(III) than the WT incubated with no electron donor ($p<0.05$, Tukey’s test). Results are average of 3 replicates and error bars indicate standard deviation.
released negligible (0.17 %) of As(III), as expected (Fig. 5.2B). The “no donor” control released 33 % of As\textsubscript{total} in the form of As(III), potentially due to residual endogenous reduced cofactors in the cells at the start of the experiment (Fig. 5.1B). The abiotically desorbed As from the “no cells” control was 99.9 % As(V) (Fig. S5.2B). There were slight discrepancies in the As\textsubscript{total} quantification (Fig. 5.1A) and the total As estimation by adding the two As species (Fig. 5.1B), potentially due to the sample preparation, where the As\textsubscript{total} by ICP-AES samples were diluted in HNO\textsubscript{3}, solubilising more As, in contrast to the IC-ICP-MS samples that were prepared by only dissolving in \textsubscript{d}H\textsubscript{2}O.

5.4.2 Fe(III) respiration at a distance

In this work the secretion of flavins (FMN and the degraded form, riboflavin), which can act as extracellular electron shuttles to Fe(III) oxides, was monitored. Flavins were detected in all cultures except in the “no cells” control (table 5.1). Surprisingly the “no donor” control released more total flavins (204.7 nM) by day 11, followed by \textsuperscript{13}C-incubated S. ANA-3 WT (185.8 nM) and ARRA3 (185 nM). From the start of the experiment FMN was detected and its concentration decreased over the incubation time (Fig. S5.4A), whereas riboflavin concentrations increased in the supernatants with time (Fig. S5.4B).

Table 5.1. Flavins secretion in all experiments at day 11. Results are average of three measurements. Units: nM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FMN</th>
<th>Riboflavin</th>
<th>Total flavins</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. ANA-3 WT</td>
<td>121.7</td>
<td>64.1</td>
<td>185.8</td>
</tr>
<tr>
<td>S.ANA-3 ARRA3</td>
<td>99.7</td>
<td>85.3</td>
<td>185</td>
</tr>
<tr>
<td>No donor</td>
<td>80</td>
<td>124.7</td>
<td>204.7</td>
</tr>
<tr>
<td>No cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

5.4.3 SEM imaging

Cells of both S. ANA-3 strains incubated with \textsuperscript{13}C-labelled lactate were observed adjoining the Fe(III) mineral, but the majority of the cells (>90 %) were observed microns away (1-20 µm) from the Fe(III) mineral on the Si wafer surface (Fig. S5.5). Very few cells of the WT strain were observed growing on the Fe(III) mineral, and no cells from the ARRA3
(non As(V)-respiring) were found on the Fe(III) mineral surface. The “no donor” control produced much lower biomass than the $^{13}$C incubated WT and ARRA3 samples, as expected, due to the lack of an organic substrate to use for energy yield and as a carbon source (Fig. S5.5E).

**5.4.4 As and Fe on the solid phase imaged by NanoSIMS**

Not all the cells observed using the SEM accumulated $^{13}$C (as observed in NanoSIMS), and these were considered metabolically inactive. However, the majority of the active cells of both the *S. ANA*-3 strains were observed growing at a distance from the Fe(III)-(oxyhydr)oxide mineral on the uncoated Si wafer surface of the nine areas analysed for each strain (N=112 for the WT and N=94 for ARRA3) (Fig. 5.2). A few cells adjoining the Fe(III)-(oxyhydr)oxide mineral were active (N=9 for the WT and N=4 for ARRA3) and very few cells of the WT strain were observed growing on the Fe(III)-(oxyhydr)oxide surface (N=4) (Fig. 5.2C). The mean $^{13}$C/$^{12}$C accumulation of *S. ANA*-3 WT was 6.79 % ± 3.02 for cells on the uncoated Si wafer, 4.47 % ± 1.39 for cells adjoining the mineral and 3.19 % ± 0.81 for cells growing on the Fe(III) mineral (Fig. S5.6A). This implies that WT cells accumulated 3 to 6 times more $^{13}$C than the natural $^{13}$C/$^{12}$C ratio. The ARRA3 cells accumulated a mean $^{13}$C/$^{12}$C of 5.62 % ± 0.71 for cells on the uncoated wafer and 5.28 %

![Figure 5.2. NanoSIMS overlay images of *Shewanella* sp. ANA-3 strains WT (A-D) and ARRA3 (E-H) incubated with $^{13}$C-labelled lactate. Green is $^{13}$C, red is $^{56}$Fe$^{16}$O and blue is $^{75}$As. Notice that most of the cells are on the Si wafer, with the exception of a few WT cells on the Fe(III)-(oxyhydr)oxide mineral surface (C-D).](image-url)
± 0.4 for cells adjoining the Fe(III) mineral, implying an accumulation of up to 5 times the natural $^{13}\text{C}/^{12}\text{C}$ ratio (Fig. S5.6B). All the areas analysed in NanoSIMS can be seen in Figures S5.7 and S5.8 for the WT and ARRA3 strains, respectively.

5.4.5 As and Fe sorption on the cell surface as revealed by NanoSIMS single-cell depth profiles

NanoSIMS analysis in large fields-of-view (30-50 µm) showed trace counts of Fe and As on the cells. Therefore, we selected single active cells to collect NanoSIMS depth profiles at higher spatial resolution and prolonged acquisition in order to image trace Fe and As in cells of both S. ANA-3 strains. Low counts of Fe and very low counts of As in the cells were imaged at the cell surface (Fig. 5.3A & C). In general $^{56}\text{Fe}^{12}\text{C}$ produced a stronger signal than $^{56}\text{Fe}^{16}\text{O}$, potentially due to the naturally higher $^{12}\text{C}$ content in cells. The WT strain showed $^{75}\text{As}$ on the cell surface in contrast to the ARRA3 strain, which showed no $^{75}\text{As}$, only Fe (as $^{56}\text{Fe}^{12}\text{C}$ and/or $^{56}\text{Fe}^{16}\text{O}$) (Fig. 5.3A & B). However, it is noteworthy that the $^{56}\text{Fe}^{12}\text{C}$, $^{56}\text{Fe}^{16}\text{O}$ and $^{75}\text{As}$ counts in or on the cells were much lower than on the Fe(III) mineral surfaces, as evidenced in the NanoSIMS stack images (Fig. S5.7 & S5.8), which show the total sum of these ion counts after the acquisition of several planes. See Figure S5.9 for additional 3D models of single-cell depth profiles of both S. ANA-3 strains.

5.5 Discussion

*Shewanella* species do not require direct contact with the Fe(III) mineral they are respiring, with flavins (endogenous or exogenous) considered as the main mechanism mediating extracellular transfer of electrons (von Canstein et al., 2008; Edwards et al., 2015; Xu et al., 2016). The amount of flavins secreted in all samples in this experiment was in the sub-micromolar level, in accordance to previous reports for other *Shewanella* species (Marsili et al., 2008; von Canstein et al., 2008). In addition, flavins have proved to be effective for reductive Fe(III) dissolution at nanomolar concentrations and it has been observed that above 1 µM the rate of Fe(III) dissolution is not enhanced (Wang et al., 2015). Moreover, the conversion of FMN to riboflavin has been previously observed in cultures of different *Shewanella* strains (von Canstein et al., 2008). The culture medium
Figure 5.3. 3D reconstructions of single-cell depth profiles of S.ANA-3 strain WT (A-B) and ARRA3 (C-D). (A) and (C) are 3D reconstructions, where $^{12}$C is displayed in blue (surface), $^{56}$Fe$^{12}$C is shown as red dots, $^{56}$Fe$^{16}$O as green dots and $^{75}$As as orange dots. (B) and (D) are NanoSIMS stack images of the same cells, (B) and (D) are stacks of 50 planes. The horizontal colour scale bar indicates the $^{13}$C-accumulation percentage in the $^{13}$C/$^{12}$C images. $^{56}$Fe$^{12}$C and $^{56}$Fe$^{16}$O counts were imaged in the cells of both strains, few counts of $^{75}$As were imaged in the WT strain and negligible counts were observed in the ARRA3 strain.
(FWM) contained 61 nM riboflavin as part of the vitamins solution added, but no riboflavin was detected in the “no cells” control, suggesting that all the riboflavin detected in the experiments with cells was produced biotically.

Abiotic As release in the “no cells” control, which accounted for 53 % of the total As mobilised by the $^{13}$C-incubated WT sample, suggests that these experimental conditions supported significant As(V) desorption and, if new mineral phases were formed, these did not promote As(V) re-sequestration. In the present work phosphate (3 mM) was added in higher amounts than typically found in natural environments, to promote bacterial growth. Phosphate usually correlates positively with arsenate in groundwater, and this ion promotes arsenate desorption by competing for sorption sites, for instance on Fe(III)-oxyhydroxide minerals (Biswas et al., 2014). Desorbed arsenate is more bioavailable, and this phosphate-promoted arsenate desorption combined with the arsenate desorbed via reductive dissolution of Fe(III)-oxyhydroxides, are critical steps in As mobilisation in the environment (Biswas et al., 2014; Glasser et al., 2018b). Mineral transformations can also influence As mobilisation in the environment; newly formed Fe(III) minerals have the capability to resorb, re-sequester and re-precipitate not only As(V), but also the more mobile As(III) (Tufano et al., 2008). Solubilised Fe(II) can also affect secondary mineralisation by accelerating the dissolution of amorphous Fe(III)-(oxyhydr)oxides, promoting its transformation to the more crystalline phases goethite or magnetite, in an Fe(II) concentration-dependant mechanism (Hansel et al., 2003).

Unfortunately, given the characteristics of our samples, it was not possible to analyse the secondary minerals produced because the acid extraction would have altered their structure.

Our aqueous As species results show that bacterially produced flavins, as an example of semiquinones, are not involved in As(V) reduction under the conditions tested, only playing a role in Fe(III) respiration, as shown by the negligible As(III) release in the non As(V)-reducer ARRA3. The stoichiometry was possibly unfavourable for this reaction to occur, although flavin molecules are likely recycled (Coursolle et al., 2010). According to the reduction midpoint potential of the ferrihydrite/Fe(II) couple, between -100 to +100
mV (Straub et al., 2000), and the As(III)/As(V) couple, +70 mV (van Lis et al., 2013), the reduction of ferrihydrite would be thermodynamically more favourable than that of As(V) (Saltikov et al., 2005). For this reason, the energy harvested from the reduction of Fe(III) would make it adequate for S. ANA-3 cells to invest energy in producing and releasing flavins (Covington et al., 2010), which in themselves have high reducing capacities with reduction potentials at -208 mV and -219 mV for riboflavin and FMN, respectively (van der Zee, 2002).

NanoSIMS showed that cells of both strains accumulated $^{13}$C at varying levels (%). A small number of active WT cells (N=4) were observed on the Fe(III) surface and the $^{13}$C accumulation was the lowest (3.19 %) in contrast to cells adjoining the mineral (4.47 %) or on the uncoated Si wafer (6.79 %). However, there were no statistically significant differences in $^{13}$C accumulation regarding the S. ANA-3 WT cells’ location on the Si wafer as determined by one-way ANOVA (p>0.05, Tukey’s test). We hypothesized that these $^{13}$C accumulation differences reflect different growth stages. Overall, more active cells (90 % of the total) were imaged at a distance from the Fe(III) mineral, suggesting that the dominant Fe(III)-(oxyhydr)oxide reduction mechanism in S. ANA-3 is not through direct cell-mineral contact but through electron shuttling under the conditions imposed in this experiment. The inactive cells imaged by NanoSIMS were most likely present in the starting inoculum and did not actively grow and oxidise the organic matter analogue, $^{13}$C-labelled lactate. Chemical fixation can dilute the $^{13}$C fraction in cells (Musat et al., 2014), leading to an underestimation of the incorporation rates of isotopically-labelled lactate, although the estimated $^{13}$C/$^{12}$C ratio was appropriate to locate metabolically active cells and the calculation of absolute ratios was not the aim of this work.

These samples, which were abundant in iron, proved challenging for the analysis of $^{75}$As in negative secondary ion mode in NanoSIMS, due to the formation of the undesirable isobaric interference $^{56}$Fe$^{19}$F. Nevertheless, $^{75}$As imaging was possible, although it is advisable to assess the appearance of this mass interference at m/z 75 in samples more abundant in Fe than As, if the goal is to analyse $^{75}$As.
The higher counts of surface Fe and As detected, in contrast to intracellular Fe and As in NanoSIMS single-cell depth profiles, suggest preferential Fe and As sorption on the cell surface, which is likely in a medium with soluble Fe(II) and As(III) (when applicable) (Shiraishi et al., 2018), although NanoSIMS does not discriminate the valence of the elements analysed. Only the WT strain showed counts of $^{75}$As on the cells; in this sample As(III) was detected as the predominant aqueous As species. As(III) has a high affinity for thiol groups in proteins and can bind to lipids, carbohydrates, amines, amides and aromatic groups on the cell wall (Shen et al., 2013). In the ARRA3 strain only aqueous As(V) was detected, which would be expected to preferentially resorb to the Fe(III)- (oxyhydr)oxide mineral.

In one study *S. putrefaciens* strain CN32 accumulated iron in the form of intracellular granules (mixed Fe valence) when growing with hydrous ferric oxides as electron acceptors, but this is the only *Shewanella* strain (and indeed only study) to show these granules (Glasauer et al., 2007). In this work, S. ANA-3 did not contain any structures that resembled intracellular Fe granules in NanoSIMS, rather, the Fe counts imaged were scattered. Membrane-bound c-type cytochromes, that are active during this electron transfer process, could also be contributing to the Fe counts detected on the cell surface.

To the best of our knowledge this is the first time that active As(V) and Fe(III)-respiring bacteria have been studied at the single-cell level. Imaging this close association between bacteria and metal contributes to understand how these microorganisms interact with these metals and ultimately predict the fate of As in subsurface environments, which could be extended to the study of other trace metals and metal-respiring bacteria. Our results also show the capabilities of the technique to image low concentrations of key metals (and other trace elements) at submicron scales, facilitating the identification of cellular location.

Bacterial cells have been proposed as a remediation agent to remove arsenic from aquifers through intracellular As accumulation and As binding on the cell surface (Kostal et al., 2004; Takeuchi et al., 2007; Villadangos et al., 2010; Vishnoi et al., 2014). The NanoSIMS single-cell depth profiles showed that As preferentially associated to the outer
cell membrane or cell surface, although very low counts were detected. Intracellular As was negligible in single-cell depth profiles, and these results suggest that As removal would be favoured through As surface binding to S. ANA-3 cells, but at a questionable efficiency. On the contrary, S. ANA-3 cells contribute to As mobilisation through the desorption of adsorbed arsenate, the respiration of As(V) and accumulation of As(III) in the aqueous phase, an observation that could be extended to other dissimilatory arsenate-respiring bacteria, that not only resist As, but thrive in its presence. A more sensible approach to remove As from aquifers would consist in promoting the formation of Fe(III)-(oxyhydr)oxide or iron sulphide mineral assemblies, which are more effective than bacterial cells for As sequestration (Omoregie et al., 2013).

In this work we imaged two possible mechanisms for insoluble Fe(III) reduction by *Shewanella* sp. ANA-3. In our experimental system, this bacterium predominantly grows at a distance from the Fe(III)-(oxyhydr)oxide, with Fe(III) reduction most likely mediated by flavins acting as electron shuttles, however, a small proportion of cells could grow by direct contact with the Fe(III) mineral. S. ANA-3, like other dissimilatory arsenate-respiring bacteria, has a respiratory As(V) reductase located in the periplasm, and this would make the direct reduction of As(V) sorbed to solid Fe(III)-(oxyhydr)oxides unlikely. Our findings suggest that the majority of S. ANA-3 WT cells did not directly contact the solid phase-As(V), sorbed to the Fe(III)-(oxyhydr)oxide, therefore, the reduction of As(V) must have occurred once it was mobilised to the aqueous phase. This was in accordance to a *Clostridium* strain observed to reduce only soluble arsenate (possibly as a detoxification mechanism) (Langner and Inskeep, 2000), but contrasts with the mechanism observed in another Gram-negative bacterium with the capability to reduce sorbed arsenate through a membrane-bound arsenate reductase (Zobrist et al., 2000).

Even though the characterisation of the spatial and temporal fluctuations of As in impacted ecosystems is challenging, we propose a multistep Fe(III) and As(V) reduction mechanism by S. ANA 3 in the conditions studied: the cells first reduce the insoluble Fe(III)-(oxyhydr)oxide, mainly through flavins used as electron shuttles, this reduction leads to the solubilisation of Fe(II) and desorption of As(V), and once in solution, As(V) is
taken up by the cells, reduced to As(III) in the periplasm, and finally, As(III) is exported from the cells and accumulated in the aqueous phase. We propose that this model could explain the mechanism of As release in reducing aquifers, supported by recent field data (Gnanaprakasam et al., 2017). The geochemical conditions in As impacted sites dictate the ultimate fate of arsenic. At lower depths and reducing conditions there are fewer As sorption sites and, unless As(III)-oxidising bacteria or manganese oxides are present (Gorny et al., 2015), arsenite will likely persist.

5.6 Conclusions

Under the conditions tested, S. ANA-3 cells actively reduce the Fe(III)-oxyhydroxide and consequentially desorb arsenate, this arsenate is taken up by the cells from the aqueous phase and respired, producing arsenite. These stationary environmental conditions sustain arsenite accumulation, which is the case in many As-polluted groundwaters. This arsenite persistence is key in the toxicity of groundwater destined for human consumption, where Fe(III) and As(V)-respiring bacteria play a significant role by actively desorbing arsenate and releasing arsenite.

In the present work NanoSIMS coupled to stable isotope-labelling of active cells contributed to get a comprehensive spatial profile at subcellular resolution of the cells actively respiring iron and arsenic. Further studies stimulating the active microorganisms in sediments from As impacted aquifers are necessary, which could potentially be studied through an *in-situ* hybridisation-NanoSIMS approach, in order to extend our understanding of these processes at the microbial community level. Nonetheless, the application of this technique should warrant the coupling of routine approaches, for instance, metagenomics, transcriptomic and proteomic tools. This holistic approach could be applied in the study of other bacteria transforming elements with relevant geochemical cycles, such as nitrogen, manganese and sulfur, which are also involved in the mobilisation of toxic trace elements in the subsurface.
5.7 Acknowledgments

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5.8 References


5.9 Supporting information

A Single-cell Approach to Elucidate the Mobilisation Mechanism of Adsorbed Arsenate in *Shewanella* sp. strain ANA-3

Figure S5.1. High mass resolution (HMR) spectrum showing the superposition of the GaAs standard peak and the *Shewanella* ANA-3 WT sample peak (black dashed line) after mass resolution was improved. The red dashed vertical line is where the detector was set after the mass interference experiments. Note that the deflector is not set on flat part of the peak but on the edge of the GaAs standard peak, compromising the intensity of As counts (note HMR scan is presented on a log scale). MRP=7330.
Figure S5.2. Total Fe(II) measurement in supernatants through incubation time via the ferrozine method. Notice that only the samples incubated with electron donor solubilised Fe(II). Bars represent the standard deviation, n=3.
Figure S5.3. As species quantification by IC-ICP-MS in the aqueous phase through incubation time. (A) As(III) and (B) As(V). Only the WT samples released As(III), including the “no donor” control. There was considerable abiotic As(V) release, although ARRA3 released more As(V) at day 11. Bars represent the standard deviation, n=3.
Figure S5.4. Flavins secreted through the incubation experiments. (A) Flavin mononucleotide (FMN) and (B) riboflavin. Bars represent the standard deviation, n=3.
Figure S5.5. SEM images of pure cell culture experiments prior to NanoSIMS. (A-B) S. ANA-3 WT, (C-D) S. ANA-3 ARRA3, (E) “no donor” control and (F) “no cells” control. The lime green arrows indicate cells of both strains, primarily located on the Si wafer.
Figure S5.6. Box and whisker plots of the $^{13}\text{C}/^{12}\text{C}$ ratio of active cells, Fe(III) mineral and natural background for comparison. (A) S. ANA-3 WT and (B) S. ANA-3 ARRA3. Dashed line is the natural $^{13}\text{C}/^{12}\text{C}$ abundance of 1.11%.
Fig. S5.7. All nine areas of *S. ANA*-3 strain WT analysed in NanoSIMS. The first panel are the secondary electron (SE) images, followed by $^{12}$C$^{14}$N, $^{56}$Fe$^{16}$O, $^{75}$As and the $^{13}$C/$^{12}$C ratio (%).
Fig. S5.8. All nine areas of S. ANA-3 strain ARRA3 analysed in NanoSIMS. The first panel are the secondary electron (SE) images, followed by $^{12}$C$^{14}$N, $^{56}$Fe$^{16}$O, $^{75}$As and the $^{13}$C/$^{12}$C ratio (%).
Fig. S5.9. Additional 3D reconstructions of single-cell depth profiles of *S. ANA*-3 strain WT (A-B) and ARRA3 (C-D). (A) and (C) are the 3D reconstructions, where $^{12}$C is displayed in blue (surface), $^{56}$Fe$^{12}$C is shown as red dots, $^{56}$Fe$^{16}$O as green dots and $^{75}$As as orange dots. (B) and (D) are NanoSIMS stack images of $^{56}$Fe$^{16}$O, $^{56}$Fe$^{12}$C, $^{75}$As and the $^{13}$C/$^{12}$C ratio of the same cells. (B) is a stack of 95 planes; (D) is a stack of 100 planes. The dashed yellow box in panel $^{13}$C/$^{12}$C in (B) is the 3D modelled cell in (A). The horizontal colour scale bar indicates the $^{13}$C-accumulation percentage in the $^{13}$C/$^{12}$C images. $^{56}$Fe$^{12}$C and $^{56}$Fe$^{16}$O counts were imaged in the cells of both strains, few counts of $^{75}$As were imaged in the WT strain and negligible counts were observed in the ARRA3 strain.
Chapter 6-Molecular SIMS Analysis of the Fe(III)-oxyhydroxide Respiration by *Shewanella* sp. strain ANA-3

6.1 Introduction

Time-of-Flight secondary ion mass spectrometry (ToF-SIMS) is a well-established technique for surface analysis. The principle, discussed in Chapter 2, is similar to other SIMS techniques, where an energetic primary ion beam is bombarded to erode a solid surface, producing, among other species, secondary ions (SI), which are detected and analysed in a mass spectrometer (Sinha and Hoppe, 2010). Time-of-Flight (ToF) is the type of mass analyser used in ToF-SIMS, whose mass separation principle is that ions with same energy but different masses will travel a path with different velocities. In this design, an electrostatic field accelerates the SI to a common energy and the lighter ions fly at higher velocities and reach the detector before the heavier ions. The major advantages of ToF over quadrupole mass analysers are the extremely high transmission, improved sensitivity (by $10^3$-$10^4$), the parallel detection of all masses and the unlimited mass range (Weibel et al., 2003; Lyon and Henkel, 2010). Additionally, insulating samples can be analysed with less tendency to charging than is encountered using direct current ion beam sputtering, although in ToF-SIMS, charge compensation using electron flooding is possible to counteract any surface charge build-up (Belu et al., 2003). In principle, all these characteristics make ToF-SIMS a suitable technique for the analysis of biological materials. The study of large molecules is often desired in biological studies, hence, ToF-SIMS is a suitable technique for the study of large secondary ion fragments. There has been a growing interest in using cluster ion beams in SIMS, due to the different sputtering mechanisms and nature of the information they can provide (Appelhans and Delmore, 1989; Hill and Blenkinsopp, 2004; Rabbani et al., 2011). The $C_{60}^+$ cluster beam is of particular interest in this work, as it promotes the efficient sputtering of large organic molecules with low fragmentation (Wong et al., 2003; Fletcher et al., 2006). This characteristic could favour the identification of molecules of interest, particularly for the identification of molecules or fragments of proteinaceous nature associated to the cell...
surface, such as c-type cytochromes and flavocytochromes involved in Fe(III) respiration
by S. ANA-3.

Nevertheless, ToF-SIMS has not been widely applied in the analysis of microbiological
samples, possibly due to insufficient sensitivity and molecular sputtering at the desired
spatial resolution, typically 2-3 µm for the C60⁺ beam (Hill and Blenkinsopp, 2004), along
with challenges in sample preservation (Chandra, 2008). Additionally, the mass spectra
obtained with ToF-SIMS is complex and peak identification can be challenging, where
more than one strategy has been used to analyse these large datasets, including the use
of commercially available databases and statistical approaches such as multivariate
analysis, particularly principal component analysis (PCA) (Vickerman et al., 1999; Graham
et al., 2006; Klinkert et al., 2007; Vaidyanathan et al., 2008; Xiong et al., 2012; Wehrli et
al., 2014). For instance, PCA has been successfully applied in the screening of pathogenic
bacterial isolates, where a pre-processing step of the spectra and images was included
in order to only extract and analyse the information relevant to the purposes of the
investigation (Vaidyanathan et al., 2008; Vaidyanathan et al., 2009).

The aim of the experimental work summarised in this Chapter was to co-locate molecules
associated to the Fe(III)/As(V) reduction by and S. ANA-3 WT cells using ToF-SIMS. ToF-
SIMS, with its surface sensitivity and low molecular fragmentation, could allow the
analysis of organic molecules on the surface of the cell. As mentioned previously, the
molecules of interest involved in this electron transfer process are flavins, c-type
cytochromes and flavocytochromes, which have characteristic functional groups that
could be detected and analysed with this static SIMS technique. The spatial resolution
may be limiting for the analysis of single bacterial cells, in contrast to that obtained with
NanoSIMS. In order to approach this limitation, two ToF-SIMS instruments were used,
one equipped with the cluster beam C60⁺ and one with a Auₙ⁺ liquid metal ion gun (LMIG).
These are described in Henkel et al. (2007). In order to facilitate peak identification in
the mass spectra, preliminary analysis of reference materials relevant to the samples
were initially acquired.
6.2 Methodology

Reference materials were analysed prior to the analysis of the samples in order to collect mass spectra from each of the sample components and molecules of interest. The reference materials used were: Fe(III)-oxyhydroxide (ferrihydrite), riboflavin, flavin mononucleotide (FMN), poly-L-lysine and bulk *Shewanella* ANA-3 WT cells. All the reference materials were prepared by depositing 10-40 µL of solution with the materials on a silicon wafer (7 mm x 7 mm), as detailed in Chapter 3.

Two different primary ion beams were used to sputter the standard materials to assess how the characteristics of the sputtered secondary ion species were determined by the primary ion species. The primary ion species were 25 kV Au\(_n^+\) LMIG and 40 kV C\(_{60}^+\). Secondary ions were mainly collected in positive secondary ion mode, although some tests were run in negative SI mode.

The ToF-SIMS raw data was analysed using a combination of the softwares ToFCmd and TSTSpec. The mass spectra were first loaded in the ToFCmd software and the files were corrected for detector dead time. These corrected files were then loaded in the TSTSpec software where the masses of each peak of interest were manually assigned. Each peak also provides an associated ion image. The static SIMS library (Vickerman et al., 1999) was also consulted to identify unknown peaks.

As discussed in Chapters 2 and 3, the data analysis of ToF-SIMS spectra can be truly challenging: there is at least one peak per every mass unit, sometimes more than one peak, which leads to thousands of possible masses to identify correctly. In order to simplify this analysis, the study by Ding et al. (2016) was used as reference, given that they analysed a thick biofilm of *S. oneidensis* MR-1.

Mass calibration was performed in order to allocate the masses of interest accurately. Atomic ions and molecular ions have different secondary ion energy spectra and their time focus is slightly different in the time of flight mass spectrometer. Therefore, for the highest mass accuracy when analysing molecular species, known reference molecules (not atoms) were used to calibrate the mass scale (Green et al., 2006).
with which a molecular composition and structure may be assigned to a peak in the mass spectrum depends upon the accuracy with which the mass of that peak may be determined. Mass accuracy across the mass spectrum may be determined by calibrating the measured time delays of peaks of known exact mass and looking at the deviations of those measured times. The mass accuracy given in table 6.1 shows the deviations in calibration for known molecules determined in the mass spectrum of riboflavin. A high mass accuracy for determining peak masses of <5 mmu deviation at masses up to 300 was considered sufficiently good to determine the structure and composition of molecules with masses that are very close in composition. For instance, this mass accuracy allowed the identification of multiple peaks at m/z 72: $^{28}\text{Si}_2\text{O}$ (m/z 71.94877), $^{28}\text{Si}_2\text{CH}_4$ (m/z 71.98518), $^{28}\text{SiC}_3\text{H}_8$, (m/z 72.03957) and $\text{C}_5\text{H}_{12}$ (m/z 72.09396), see Figure 6.1.

<table>
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<tr>
<th>Species</th>
<th>m/z</th>
<th>Deviation calibration (mmu)</th>
<th>Bin error</th>
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<td>0</td>
</tr>
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<td>0</td>
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<td>-2</td>
</tr>
<tr>
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<td>207.0324</td>
<td>-2.99</td>
<td>-2</td>
</tr>
<tr>
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<td>281.0449</td>
<td>+4.47</td>
<td>+3</td>
</tr>
</tbody>
</table>

The masses screened for flavins as reference materials were the riboflavin peak at m/z 376.1383 ($\text{C}_{17}\text{H}_{20}\text{Na}_4\text{O}_{8}^+$), FMN peak at m/z 456.1046 ($\text{C}_{17}\text{H}_{21}\text{N}_4\text{O}_9\text{P}^+$) and riboflavin fragments at m/z 241.0725 ($\text{C}_{12}\text{H}_9\text{Na}_4\text{O}_2^+$) and 243.0882 ($\text{C}_{12}\text{H}_{11}\text{N}_4\text{O}_2^+$).
Across the mass spectra of almost all materials analysed (except for ferrihydrite) there were significant peaks at masses 73.0468, 147.0656 and 281.0449, plus other significant peaks that can be identified as molecular fragments of polydimethylsiloxane (PDMS, \((\text{C}_2\text{H}_6\text{OSi})_n\)). The almost universal presence of these peaks in most materials analysed led to additional confidence in their identification and use in determining the mass calibration.

6.3 Results

The reference materials yielded complex mass spectra, as expected. Sputtering by the \(\text{C}_{60}^+\) ion beam allowed the formation of high mass fragments up to the higher mass end (≈1000 a.m.u.) although with a minimum spot-size of 2-3 µm, whereas the \(\text{Au}_n^+\) LMIG provided a higher spatial resolution, particularly when used in the doubly charged mode (\(\text{Au}^{2+}\)), although this setting did not yield organic mass fragments beyond 400 a.m.u.

Tables 6.2 and 6.3 summarise the most abundant peaks detected in positive (table 6.2) and negative (table 6.3) ion mode in all the reference materials and samples analysed, for both primary ion beams used.
Table 6.2. Peak allocation of key ions in the positive ion mode.

<table>
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<tr>
<th>Theoretical m/z</th>
<th>Measured m/z</th>
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Table 6.3. Peak allocation of key ions in the negative ion mode.

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<td>$^{12}$C$<em>{12}$H$</em>{22}$N$<em>{4}$O$</em>{6}$ $^{31}$P (flavin mononucleotide)</td>
</tr>
<tr>
<td>575.4388</td>
<td>575.43823</td>
<td>$^{56}$Fe$^{16}$O$_{8}$</td>
</tr>
</tbody>
</table>
6.3.1 Fe(III)-oxyhydroxide reference analysis

Analysis using C$_{60}^+$

The most abundant peaks formed by sputtering the C$_{60}^+$ primary beam on the Fe(III) mineral were identified at m/z 56 ($^{56}$Fe$^+$), at 72 with two peaks, $^{56}$Fe$^{16}$O$^+$ and $^{28}$Si$_2^{16}$O$^+$, and at 73 ($^{56}$Fe$^{16}$O$^+$H$^+$). The highest mass peak with reasonable intensity, approximately 18 counts (cts), was at m/z 575.4388 ($^{56}$Fe$_8^{16}$O$_8^+$). In this area of interest (AOI) the estimated spatial resolution was 8 μm. See Figures 6.2 and 6.3.

| Mass (amu) | Fragment | Molecular Formula | Symbol
<table>
<thead>
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<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>82.05312</td>
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<td>histidine</td>
<td>(histidine)</td>
</tr>
<tr>
<td>120.0817</td>
<td>$^{12}$C$<em>8$H$</em>{10}^{14}$N</td>
<td>phenylalanine</td>
<td>(phenylalanine)</td>
</tr>
<tr>
<td>129.1028</td>
<td>$^{12}$C$<em>6$H$</em>{13}^{14}$N$_2^{16}$O</td>
<td>lysine</td>
<td>(lysine)</td>
</tr>
<tr>
<td>130.0657</td>
<td>$^{12}$C$_8$H$_8^{14}$N</td>
<td>tryptophan</td>
<td>(tryptophan)</td>
</tr>
<tr>
<td>137.0926</td>
<td>$^{12}$C$<em>8$H$</em>{13}^{14}$N$_2^{16}$O$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>147.1134</td>
<td>$^{12}$C$<em>6$H$</em>{13}^{14}$N$_2^{16}$O$_2$</td>
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<td>197.1205</td>
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<td></td>
<td>$^{12}$C$<em>{14}$H$</em>{15}^{14}$N</td>
</tr>
<tr>
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<td>241.0720</td>
<td></td>
<td>$^{12}$C$<em>{14}$H$</em>{16}^{14}$N$_4$</td>
</tr>
<tr>
<td>376.1384</td>
<td>376.1377</td>
<td></td>
<td>$^{12}$C$<em>{17}$H$</em>{20}^{14}$N$_4^{16}$O$_6$</td>
</tr>
</tbody>
</table>

Figure 6.2. Ion images of the Fe(III) mineral sputtered with C$_{60}^+$. The vertical colour scales indicate the Si intensities. Field of view (fov)= 200 μm.
Analysis using Au\textsuperscript{2+}

The analysis of the Fe(III) mineral with Au\textsuperscript{2+} yielded a mass spectrum shown in Figure 6.4, where the highest intensity peak was detected at m/z 56 (\textsuperscript{56}Fe\textsuperscript{+}) followed by the \textsuperscript{28}Si\textsuperscript{+} peak. This analysis provided higher spatial resolution for the Fe(III) mineral, estimated at 3 µm (Fig. 6.5), although the highest mass peak was identified at m/z 325 (an unidentified \textsuperscript{28}Si containing peak).
**Analysis using Au$_3^+$**

The analysis of the Fe(III) mineral with Au$_3^+$ yielded a mass spectrum shown in Figure 6.6, where the highest intensity peaks were detected at m/z 41 ($^{12}$C$_3$H$_5^+$) followed by a peak at 91 ($^{12}$C$_7$H$_7^+$) and at 56 ($^{56}$Fe$^+$). This analysis provided a spatial resolution estimated at 15 µm (Fig. 6.7); however, this low spatial resolution was measured on an ion image on the sample and not on a standard or more adequate material (such as a Cu grid). The highest mass peak identified was at m/z 575.4388 ($^{56}$Fe$_8^{16}$O$_8^+$).

![Figure 6.5. Ion images of the Fe(III) mineral sputtered with Au$_2^+$. The vertical colour scales indicate the SI intensities. Fov= 100 µm.](image)

![Figure 6.6. Mass spectrum at the lower mass end of the Fe(III) mineral sputtered with Au$_3^+$.](image)
6.3.2 Riboflavin reference analysis

**Analysis using C\textsubscript{60}\textsuperscript{+}**

The analysis using C\textsubscript{60}\textsuperscript{+} allowed the detection of the riboflavin molecule at m/z 377.146 (C\textsubscript{17}H\textsubscript{20}N\textsubscript{4}O\textsubscript{6}H\textsuperscript{+}) and a characteristic riboflavin fragment at 243.088 (C\textsubscript{12}N\textsubscript{4}O\textsubscript{2}H\textsubscript{11}\textsuperscript{+}), by loss of the ribityl side chain (see Fig. 6.8).

![Riboflavin mass spectrum using the C\textsubscript{60}\textsuperscript{+} primary beam, showing two riboflavin peaks at 377.146 and 378.153 a.m.u. and the riboflavin fragment at 243.088 a.m.u.](image)

Due to the sample preparation method, where 40 µL of the riboflavin solution were pipetted on the Si wafer, ion images are not presented. The same applies to FMN, poly-L-lysine and bulk S. ANA-3 cells reference analysis.

**Analysis using Au\textsubscript{2}\textsuperscript{+} and Au\textsubscript{3}\textsuperscript{+}**

Use of the primary ion beams Au\textsubscript{2}\textsuperscript{+} and Au\textsubscript{3}\textsuperscript{+} has the potential for higher spatial resolution analyses than by using the C\textsubscript{60}\textsuperscript{+} primary ion beam. The riboflavin analysis with Au\textsubscript{2}\textsuperscript{+} and Au\textsubscript{3}\textsuperscript{+} allowed the identification of the riboflavin peak but at m/z 381 (C\textsubscript{17}H\textsubscript{25}N\textsubscript{4}O\textsubscript{6}\textsuperscript{+}) and a
riboflavin fragment at m/z 243 \((C_{12}H_{11}N_{4}O_{3}^+)\) (not shown). The most abundant peaks were at m/z 23 \((^{23}\text{Na}^+)\), 28 \((^{28}\text{Si}^+)\), 29 \((^{28}\text{SiH}^+)\), 41 \((C_3H_5^+)\), 43 \((C_3H_7^+)\) and 73 \((^{28}\text{Si (CH}_3)_3^+)\). Comparable to the analysis with \(C_{60}^+\), several additional hydrocarbon and polymer molecules were detected, where the highest mass species with reasonable abundance \((\sim 15\text{ cts})\) was at m/z 597 (unidentified species).

### 6.3.3 FMN reference analysis

**Analysis using \(C_{60}^+\)**

The tests of flavin mononucleotide (FMN) using the \(C_{60}^+\) ion beam showed a spectrum where the FMN molecule at m/z 456 was detected at low intensity, 20-50 cts (not shown), but the riboflavin molecule (at m/z 376, \(C_{17}H_{21}N_4O_9P^+\)) was detected at higher intensity (Fig. 6.9). The most abundant peaks were at m/z 23 \((^{23}\text{Na}^+)\), 27 \((C_2H_5^+)\), 39 \((^{39}\text{K}^+)\), 41 \((C_3H_5^+)\), 55 \((C_4H_7^+)\) and 73 \((C_3H_7NO^+)\). These areas analysed were heavily coated with the material, hence, the \(^{28}\text{Si}\) signal was not among the most abundant peaks. The highest mass peak was at 981 a.m.u. (unidentified species).

![Figure 6.9. FMN mass spectra using \(C_{60}^+\) with lens 1 at 28 kV. Notice the small FMN peak at 456 a.m.u. and the more significant riboflavin fragment at 376 a.m.u.](image)

The spectrum obtained with the \(Au_2^+\) and \(Au_3^+\) LMIG showed peaks at m/z 456 for FMN \((C_{17}H_{21}N_4O_9P^+)\) with low intensity. The riboflavin fragment at m/z 243 was also observed.
The most abundant peaks were at m/z 23 ($^{23}\text{Na}^+$), 29 ($^{16}\text{C}_2\text{H}_5^+$), 41 ($^{12}\text{C}_3\text{H}_5^+$), 43 ($^{13}\text{C}_3\text{H}_5^+$), 55 ($^{12}\text{C}_4\text{H}_7^+$), 57 ($^{14}\text{C}_4\text{H}_7^+$). The highest mass peak was at 594 a.m.u. (unidentified species).

### 6.3.4 Bulk S. ANA-3 cells reference analysis

**Analysis using $^{129}\text{C}_6\text{O}^+$ (negative SI)**

The analysis in negative SI mode of bulk S. ANA-3 cells using $^{129}\text{C}_6\text{O}^+$ produced a series of peaks relevant to the structure of the cells, such as lipids and proteins, and to the Fe(III) respiration, such as riboflavin. In this reference sample, the most abundant peaks were at m/z: 16 ($^{16}\text{O}^+$), 24 ($^{12}\text{C}_2^+$), 25 ($^{12}\text{C}_2\text{H}^+$), 26 ($^{12}\text{C}_2\text{H}_2\text{O}^+$), 36 ($^{12}\text{C}_3\text{H}^+$), 42 ($^{12}\text{C}_2\text{H}_2\text{O}^+$), 50 ($^{12}\text{C}_4\text{H}_2^+$), 60 ($\text{SiO}_2^+$), 63 ($\text{CH}_4\text{O}^+$), 76 ($^{12}\text{C}_4\text{H}_2\text{Si}^+$), 77 ($\text{C}_3\text{H}_2\text{O}^+$), 79 ($\text{C}_3\text{H}_3\text{O}^+$), 137 ($\text{C}_5\text{H}_4\text{N}_2\text{O}_3^+$) and 197 ($\text{C}_1\text{H}_3\text{N}^+$). Peaks relevant to flavins were observed at m/z 241 ($^{12}\text{C}_1\text{H}_9\text{O}^+$), 376 ($^{12}\text{C}_1\text{H}_2\text{O}^+$), 377 ($^{12}\text{C}_1\text{H}_2\text{O}^+$) and 378 ($^{12}\text{C}_1\text{H}_2\text{O}^+$). Peaks with reasonable abundance were observed at the higher mass end, where even at m/z 997 a peak was observed, although unidentified. Moreover, various amino acid peaks were detected at high abundance ($>10^3$ cts), such as glycine ($\text{CH}_3\text{N}^+$), serine ($\text{C}_2\text{H}_6\text{NO}^+$), proline ($\text{C}_4\text{H}_6\text{N}^+$), threonine ($\text{C}_3\text{H}_8\text{NO}^+$), histidine ($\text{C}_4\text{H}_6\text{N}^+$), phenylalanine ($\text{C}_8\text{H}_10\text{N}^+$), lysine ($\text{C}_6\text{H}_{13}\text{N}_2\text{O}^+$), and tryptophan ($\text{C}_9\text{H}_8\text{N}^+$) (see Figure 6.10).

### 6.3.5 Samples of S. ANA-3 growing on Fe(III)-oxyhydroxide

In all the areas analysed of the S. ANA-3 WT samples one of the most abundant peaks in the positive secondary ion spectrum was $^{23}\text{Na}^+$. This ion is abundant in the growth medium and has a high ionisation rate, but unfortunately it is not one of the peaks of interest. Other abundant peaks were detected at m/z 56, where $^{56}\text{Fe}^+$ and $^{28}\text{Si}^+\text{O}^+$ peaks sit, as well as $^{28}\text{Si}^+$ and $^{28}\text{Si}$ combined with other species, including PDMS.

**Analysis using $^{129}\text{C}_6\text{O}^+$**

The most abundant peaks produced with $^{129}\text{C}_6\text{O}^+$ were at m/z 28 ($^{28}\text{Si}^+$), 56 ($^{56}\text{Fe}^+$), 96 ($^{12}\text{C}_4\text{H}_1\text{Si}^+$) and 112 ($^{56}\text{Fe}_2^+$) (Fig. 6.11). The mass spectrum was dominated by abundant low mass hydrocarbons such as $\text{CH}_3^+$ (15), $\text{C}_2\text{H}^+$ (25), $\text{C}_2\text{H}_2^+$ (26), $\text{C}_2\text{H}_3^+$ (27), $\text{C}_2\text{H}_5^+$ (29), $\text{C}_3\text{H}^+$ (37), $\text{C}_3\text{H}_2^+$ (38), $\text{C}_3\text{H}_3^+$ (39) and $\text{C}_3\text{H}_5^+$ (41). The most abundant nitrogen species were $\text{CHNO}^+$.
and CNO\(^+\) (43 and 42 a.m.u., respectively), although other nitrogen species were also identified at high abundance (>10\(^4\) cts) at m/z 84 (C\(_2\)N\(_2\)O\(_2\)\(^+\)), 128 (C\(_3\)N\(_2\)O\(_4\)\(^+\)), 140 (C\(_5\)H\(_4\)N\(_2\)O\(_3\)\(^+\)) and 196 (C\(_9\)H\(_4\)N\(_6\)\(^+\)). Potential fragments of fatty acids were detected at high abundance (>10\(^4\) cts) at m/z 108 (C\(_6\)H\(_4\)O\(_2\)\(^+\)), 124 (C\(_6\)H\(_4\)O\(_3\)\(^+\)) and 152 (C\(_4\)H\(_8\)O\(_6\)\(^+\)). PO\(_2\)\(^+\) and PO\(_3\)\(^+\) were identified but at low abundance. Amino acids were identified as well, although at much lower abundance (≤ 10\(^3\) cts) in contrast to the bulk cells sample, these included glycine (CH\(_4\)N\(^+\)), proline (C\(_4\)H\(_8\)N\(^+\)), histidine (C\(_4\)H\(_6\)N\(_2\)\(^+\)), leucine (C\(_4\)H\(_8\)NO\(_3\)\(^+\)), arginine (C\(_4\)H\(_{10}\)N\(_3\)\(^+\)), tyrosine (C\(_7\)H\(_7\)O\(^+\)) and tryptophan (C\(_9\)H\(_8\)N\(_3\)\(^+\)). Moreover, the riboflavin

Figure 6.10. Bulk S. ANA-3 cells mass spectrum using the C\(_{60}\)\(^+\) primary beam, highlighting peaks of interest. Notice the different scale on the Y axis in both plots.
fragments at m/z 241 (C\textsubscript{12}N\textsubscript{4}O\textsubscript{2}H\textsubscript{9}\textsuperscript{+}) and 243 (C\textsubscript{12}N\textsubscript{4}O\textsubscript{2}H\textsubscript{11}\textsuperscript{+}) were identified with peak intensities of around 100 cts, the riboflavin peak at 376 (C\textsubscript{17}H\textsubscript{20}N\textsubscript{4}O\textsubscript{9}P\textsuperscript{+}) was identified at lower peak intensity (≈30 cts) and even FMN was detected but at very low intensity (≈7 cts). Indole (C\textsubscript{6}H\textsubscript{8}N\textsuperscript{+}), a physiologically relevant signal molecule was also identified (0.8x10\textsuperscript{3} cts). The highest mass peak with reasonable abundance (>10 cts) was detected at m/z 760 (likely C\textsubscript{60}H\textsubscript{40}\textsuperscript{+}). The spatial resolution was not estimated, but it was likely much lower than in the other settings tested, as no particular features could be distinguished in the AOIs analysed (Fig. 6.12).

Figure 6.11. Mass spectrum of a selected AOI of S. ANA-3 cells using the C\textsubscript{60}\textsuperscript{+} primary beam. The more abundant organic peaks are highlighted.

Figure 6.12. SI images of an AOI of S. ANA-3 WT growing on Fe(III)-oxyhydroxide using the C\textsubscript{60}\textsuperscript{+} ion beam. Rf is riboflavin. The vertical colour scale indicates the SI intensities. Fov= 40 µm. the scale bar is 10 µm.
**Analysis using Au⁺**

The analysis with Au⁺ produced ion images where the Fe(III)-oxyhydroxide grains could be distinguished, although no cells were directly imaged, only organic molecules associated to them. Several fatty acid chains were identified including $\text{C}_{15}\text{H}_{27}\text{O}_2^+$, $\text{C}_{15}\text{H}_{29}\text{O}_2^+$ and $\text{C}_{15}\text{H}_{31}\text{O}_2^+$ at m/z 239, 241 and 243, respectively (Fig. 6.13), additional to a possibly protein-derived peak at 281 ($\text{C}_{11}\text{H}_{29}\text{N}_4\text{O}_4^+$). A few amino acids were identified, glycine ($\text{CH}_4\text{N}^+$) and alanine ($\text{C}_2\text{H}_6\text{N}^+$) at high intensity ($>10^4$ cts), whereas threonine ($\text{C}_3\text{H}_8\text{N}_2\text{O}^+$), histidine ($\text{C}_4\text{H}_6\text{N}_2^+$) and lysine ($\text{C}_5\text{H}_{10}\text{N}^+/(\text{C}_6\text{H}_{13}\text{N}_2\text{O}^+)$) were identified at lower abundance ($<10^3$ cts). All these organic molecules were strongly co-located with the Fe(III) mineral grains, suggesting that these organic residues remain on the surface of the mineral. No peak was allocated to riboflavin, FMN or any of the fragments. The highest mass peak was detected at m/z 505.5 (unidentified). The spatial resolution was estimated to be 9 µm (Fig. 6.14).

![Figure 6.13. Mass spectrum of S. ANA-3 WT growing on Fe(III)-oxyhydroxide using the Au⁺ ion beam. Note the different scale on the Y axis.](image-url)
Analysis using Au$_2^+$

This analysis produced a smaller number of organic peaks in contrast to the other conditions tested. Few amino acids were detected: histidine and leucine with 100-300 cts, lysine with 70 cts and arginine with very few cts (≈7) (Fig. 6.15). No riboflavin, FMN or any of their fragment side chains were identified. One major drawback is that the peaks at higher masses were lost; the highest mass peak with reasonable abundance was at m/z 324 (≈13 cts). However, the estimated spatial resolution was higher, approximately 5 µm (Fig. 6.16).

Figure 6.15. Mass spectrum of S. ANA-3 WT growing on Fe(III)-oxyhydroxide using the Au$_2^+$ primary ion beam.

Figure 6.14. SI images of the selected AOI of S. ANA-3 WT growing on Fe(III)-oxyhydroxide using the Au$^+$ ion beam. The vertical colour scale indicates the SI intensities. Fov= 100 µm.
Analysis using Au$_2^+$

The Au doubly charged (Au$_2^+$) setting produced different organic peaks, additional to the most abundant peaks $^{23}$Na$^+$, $^{28}$Si$^+$ and $^{56}$Fe$^+$ (Fig. 6.17). PO$_2^+$ and PO$_3^+$ were identified as well as abundant low mass hydrocarbons such as CH$_3^+$ (15), C$_2$H$_2^+$ (26), C$_2$H$_3^+$ (27), C$_2$H$_5^+$ (29), C$_3$H$_3^+$ (39) and C$_3$H$_4^+$ (40). The most abundant nitrogen species was CHNO$^+$ (>10$^3$ cts). Six amino acids were identified at reasonable abundance (50-300 cts): serine (C$_2$H$_6$NO$^+$), valine (C$_4$H$_9$N$^+$), threonine, leucine, tyrosine and lysine. Some fatty acids chains were also identified but at low abundance (10-20 cts) such as C$_8$H$_{16}$O$_2^+$, C$_{10}$H$_{20}$O$_2^+$, C$_{13}$H$_{25}$O$_2^+$ and C$_{15}$H$_{27}$O$_2^+$ (m/z 144, 172, 213 and 239, respectively). Unfortunately, the riboflavin and FMN molecules were unidentifiable, and only a riboflavin fragment at m/z 241 (C$_{12}$H$_9$N$_4$O$_2^+$) was identified at very low abundance (=8 cts). The highest mass peak with reasonable abundance (>10 cts) was at m/z 615 ($^{56}$Fe$_{11}^+$). In this analysis the organic molecules were primarily co-located with the Fe(III)-mineral, except PO$_2^+$ and C$_{15}$H$_{29}$O$_2^+$, which were located on the whole AOI. The estimated spatial resolution was the highest measured, at approximately 2.7 µm (Fig. 6.18).
6.3.6 Attempted detection of organic components from bacterial cells

Cells were imaged populating scratches on the silicon wafer near an Fe(III)-oxyhydroxide mineral grain, as shown in the SEM image in Figure 6.19. Attempts were made to detect organic components (mainly flavins) localised with these bacterial cells using a Au$_3^+$ primary ion beam, but were unsuccessful (Fig. 6.20).
6.4 Discussion

6.4.1 Reference materials

One of the most abundant peaks in all the AOIs analysed was $^{28}\text{Si}$, and several peaks of $^{28}\text{Si}$ clusters and $^{28}\text{Si}$ combined with other elements were also observed, indicating that the Si sample matrix produced several secondary peaks. The sample preparation
consisted in a thin film of the Fe(III) mineral grains on the Si wafer and a monolayer of cells growing primarily on the Si wafer, in the vicinity of the mineral grains. For this reason, much of the signal detected in the AOIs stemmed from the Si wafer. This is somewhat undesirable, as it makes the spectra analysis more complex, but Si wafer was chosen because it is resistant, amenable to handle and is also an inert material to grow the cells on. Moreover, the $^{28}$Si signal is useful to distinguish where the Fe(III) mineral and the Si wafer are.

Sodium ($^{23}$Na) was also an abundant peak in all the samples with cells and in the flavins reference materials. This element is abundant in the growth medium and is part of the structure of flavin mononucleotide (crystallised as a sodium salt). Additionally, it has a high ionisation efficiency. Therefore, $^{23}$Na is unavoidable in positive SI mode.

The flavins reference materials were tested at a concentration of 1 µM, a concentration slightly higher than what is biologically secreted by *Shewanella* species (0.1-0.6 µM) (Marsili et al., 2008; von Canstein et al., 2008) and to what was expected to measure in these samples, where the growth medium was removed.

Identifiable riboflavin molecules and molecular fragments were detected when sputtering standard materials of riboflavin and flavin mononucleotide with C$_{60}^+$ and Au$_3^+$ primary ions, but neither yielded sufficient sensitivity to detect these or similar molecules from individual bacterial cells.

The spectra of all reference materials, except ferrihydrite, produced PDMS peaks. The origin of this silicon-based organic polymer in the samples is uncertain. However, the samples were prepared on a silicon matrix and its combination with organic specimens could have promoted the formation of this polymer.

**6.4.2 Bulk S. ANA-3 cells vs biofilm samples**

The bulk S. ANA-3 cells analysis using C$_{60}^+$ showed several biologically relevant peaks including amino acids, fatty acids and flavins. These species were abundant, with peak intensities above $10^3$ cts. This result is directly related to the bulk nature of the sample, given that there were likely thousands of cells in the AOI analysed, in clear contrast to
the biofilm samples of cells growing with Fe(III) minerals, where only tens of cells are observed per AOI. The analysis using \( C_{60}^+ \) in the biofilm samples allowed the identification of some amino acids and fatty acid chains. These lipids are structural membrane components, as well as phosphates, which were identified as \( PO_2^+ \) and \( PO_3^+ \). The \( Au_{n}^+ \) LMIG also allowed the identification of some of these organic molecules, but the fragments yield was limited. Nevertheless, the spatial resolution was the highest using the \( Au^{2+} \) setting, although no riboflavin nor FMN were identified, and this setting produced only lower mass organic molecules (as hydrocarbons, phosphates and some amino acids).

6.4.3 Biomarkers of bacterial cells

The ToF-SIMS ion images of the parallel scratches where cell colonisation was observed (Fig. 6.20) achieved a high spatial resolution, however no single cells could be imaged. Nevertheless, the small hydrocarbon molecules identified along with the cations \( ^{23}Na^+ \), \( ^{24}Mg^+ \), \( ^{39}K^+ \) and \( ^{40}Ca^+ \) are adequate biomarkers of the bacterial cells in the conditions tested, as can be observed by the co-localisation of these secondary ions (Fig. 6.20) and the cell colonisation in SEM (Fig. 6.19).

As it can be noted, it was not possible to work at acceptably high sensitivity, mass and spatial resolution simultaneously. The \( Au^{2+} \) setting provided the highest spatial resolution, however, it did not yield as many organic fragments as \( C_{60}^+ \) and it did not allow the identification of both flavins, which are key biomolecules in the Fe(III) respiration by \( S. \) ANA-3. The \( C_{60}^+ \) beam produced the most biologically relevant mass spectra but at a low spatial resolution.

6.5 Conclusions

The results show that \( C_{60}^+ \) is the most suitable ion beam to yield secondary higher mass organic fragments, including amino acids, fatty acids and flavins, however, the spatial resolution obtained was above 3 \( \mu \)m, making it unsuitable for the spatial correlation of the chemical species identified at the single-cell level. The analysis using the \( Au^{2+} \) LMIG produced the highest spatial resolution, but the trade-off was the loss of higher mass
fragments and important organic molecules, even at the lower mass range, such as amino acids. The $\text{Au}_3^+$ LMIG yielded high mass peaks but at low spatial resolution. $\text{Au}^+$ was possibly the least favourable setting, as it did not yield good mass spectra with biologically relevant peaks and it did not offer a good spatial resolution. For this reason, this setting was not tested in all reference materials and samples. Even so, the results with $\text{C}_60^+$ and the $\text{Au}^{2+}$ LMIG could be considered complementary, and although no single cells were imaged, key molecules of this electron transfer process were detected on the solid surface of the samples, in spite of their low concentration.

The extensive analysis of reference materials and samples under different ToF-SIMS settings, summarised in this chapter, only tested $\text{S. ANA-3}$ WT growing with Fe(III)-oxyhydroxide, in order to remove one additional element ($^{75}\text{As}$) and simplify the mass spectra characterisation. Nevertheless, the aim of this experimental section was to test $\text{Shewanella}$ sp. strain ANA-3 using both Fe(III) and As(V) as terminal electron acceptors, and image this redox process with ToF-SIMS, granting that key biomolecules would be imaged using this technique. Given the results obtained, where no biomolecule could be allocated to single cells, it was decided that no further tests would be done in $\text{S. ANA-3}$ growing with both Fe(III) and As(V). The nature of the samples, where only a few tens of cells were dispersed on the areas selected for each analysis, tested the sensitivity limits of the technique for the detection of molecules of interest such as flavins. A different sample preparation could yield a more positive result, where ideally silicon wafer was substituted by other material. The samples show a thick biofilm prior to the chemical fixation process (see Chapter 3), which includes rinsing with several solutions, a step that appears to remove biomass and therefore analytes. New preservation approaches could be tested, for example cryogenic methods, including a rinsing step with ammonium formate, although this would not guarantee the removal of the thick organic layer that builds up on the sample surface (originating from the growth medium and extracellular polymeric substances-EPS). Under such conditions the analysis could be prolonged to guarantee that the cells and Fe(III) mineral are sputtered and probed. Gas cluster ion beams such as $\text{Ar}_n^+$ could greatly enhance the molecular sputtering and intensities from
these biological samples (Ninomiya et al., 2009), however its use may not be that relevant for the purposes of this work due to its lower spatial resolution (Fletcher et al., 2013), where the interest was to co-locate molecules of interest with single bacterial cells.

It is very likely that the molecules identification in this work omitted many biologically relevant peaks. This issue is recurrent in biological ToF-SIMS, and the scientific community is making efforts in order to make the mass peak allocation more efficient and accurate. One simple way to improve our knowledge and peak identification in these complex samples is studying diverse systems and reference materials, reporting the relevant peaks and storing them in libraries, so that future users have better tools for their analysis. A more refined option is the use of tandem mass spectrometry (MS/MS). Many ToF-SIMS instruments now run in this mode, for example the Ionoptika J105 3D chemical imager operates the MS/MS in a ToF-ToF configuration, which enhances mass resolution and allow accurate peak identification (Fletcher et al., 2008). This configuration is considered crucial for molecular identification above m/z 150 in ToF-SIMS mass spectra. The latest instrument settings offer parallel MS/MS imaging at high spatial resolution while maintaining the high mass resolution (Fisher et al., 2016). For instance, an Ar$_{4000}^+$ gas cluster ion beam was combined with ToF-SIMS tandem MS/MS to increase the sensitivity and peak abundances in the analysis of *Drosophila* brain lipids (Phan et al., 2017). However, and as mentioned earlier, ToF-SIMS instruments have not successfully imaged single bacterial cells at high mass resolution and sensitivity, while generating high mass molecular fragments. NanoSIMS has already achieved single-cell imaging at high mass resolution and sensitivity, and could be used to analyse high mass molecules, but one of its biggest limitation is its capability to only collect 7 masses simultaneously. The current trends suggest that there will be future improvements in ToF-SIMS instruments, but for the time being it can be used as a complementary imaging technique in microbiological studies.
6.6 References


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Chapter 7- Elucidating Iron Biominalisation Patterns in Denitrifying As(III)-Oxidising Bacteria: Implications for Arsenic Immobilisation

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**7.1 Abstract**

Nitrate-dependent iron(II) oxidation is a process widely distributed in neutrophilic heterotrophic bacteria. These bacteria can promote the formation of a variety of Fe minerals of diverse crystallinity which can influence the fate of soil and groundwater pollutants, such as arsenic. In the present study *Acidovorax* sp. strain ST3, an As(III)-oxidiser and denitrifying bacterium, was cultured in order to investigate simultaneous nitrate-dependent Fe(II) and As(III) oxidation. In this system the formation of poorly soluble Fe(III) minerals was expected to promote the immobilisation of arsenic as its least mobile species, As(V). The effect of Fe(III) oxidation and mineralisation on cells of this *Acidovorax* strain is unknown, but was hypothesised to be driven by both biotic and
abiotic processes (including through intermediate N species). $^{13}$C-labelled acetate was used as a co-substrate to label the metabolically active cells for imaging using nanoscale secondary ion mass spectrometry (NanoSIMS). The intracellular As and Fe distribution was also mapped and modelled in 3D through single-cell NanoSIMS depth profiling. Powder XRD was used to identify bulk biominerals formed, with scanning transmission electron microscopy (STEM) used to further characterise the biominerals at high resolution. Metabolic heterogeneity was detected with NanoSIMS, with most cells mineralised and inactive at the end of the incubation, while metabolically active cells showed low levels of biomineralisation. These data suggest that high levels of cell encrustation were deleterious to strain ST3 cells. Heavy cell mineralisation was imaged with STEM from day 1 of incubation, when amorphous nanoparticles were first identified. The periplasm and cell surface were the sites of mineralisation. By day 7 a new “flake-like” crystalline phase was detected. STEM-EDS allowed quantitative elemental mapping of these biominerals, showing that the amorphous nanoparticles were composed of 20-30 % As normalised to Fe and the crystalline flakes only contained 2-10 % As/Fe. Lepidocrocite was identified in bulk. An incubation period of 7 days promoted over 90 % of As removal, suggesting that the biogenic mineral phases were good hosts for As sequestration, but might be susceptible to further transformations under variable redox conditions, potentially re-solubilising As if metal-reducing conditions prevail.

7.2 Introduction
Arsenic is a poisonous element whose solubility and toxicity are driven by speciation. Many regions of the world, particularly Bangladesh and West Bengal, are affected by the presence of elevated concentrations of arsenic in groundwater, posing a health risk for millions of people (Mitchell, 2014). Arsenic is mobilised in the environment through anthropogenic activities, such as mining and agriculture, or through natural processes such as volcanic activity, weathering of As-bearing minerals and through microbial processes (Bowell et al., 2014). Bacteria capable of metabolising arsenic are found in a range of environments and are acknowledged as playing a key role in mobilising As in the environment (Campbell and Nordstrom, 2014; Huang, 2014). Arsenic has a high affinity
for sulfur and iron minerals in the subsurface, therefore, the formation of new mineral phases containing these elements could stimulate the immobilisation of As in the environment (Campbell and Nordstrom, 2014).

Denitrification is a widespread microbial process, where NO$_3^-$ is enzymatically reduced to N$_2$ when oxygen is depleted. Denitrification is achieved through four reductases encoded by the *nar, nir, nor* and *nos* gene clusters (Lycus et al., 2018). In this process bacteria use NO$_3^-$ as a terminal electron acceptor coupled to the oxidation of suitable electron donors, such as arsenite, which is oxidised to As(V), the less toxic and less mobile As oxyanion (Zhu et al., 2014). It is assumed that denitrifying bacteria are inherently able to catalyse Fe(II) oxidisation, in a process known as nitrate-dependent iron(II) oxidation (NDFO), which is thought to be a result of both biotic and indirect biotic effects (Carlson et al., 2013). However, the mechanism of Fe(II) oxidation remains elusive. Moderate cell growth has been observed during NDFO, suggesting Fe(II) oxidation is an energy source for the cells (Muehe et al., 2009; Chakraborty et al., 2011). NO$_2^-$ and NO can abiotically oxidise Fe(II), but these nitrogen species are also produced and accumulated during NO$_3^-$ reduction in the presence of Fe(II) (Kappler et al., 2005), and therefore considered responsible for some of the Fe(II) oxidation observed in cultures of denitrifiers. However, the contribution of NO$_2^-$ or NO in abiotic Fe(II) oxidation has been questioned (Kappler et al., 2005; Klueglein and Kappler, 2013). Equivalent abiotic Fe(II) oxidation is detected at higher NO$_2^-$ concentrations than what is biotically produced, therefore, NO$_2^-$ cannot account for all the Fe(II) oxidation observed, and the contribution of enzymatic Fe(II) oxidation cannot be discarded (Klueglein and Kappler, 2013). In the hypothetical enzymatic pathway, c-type cytochromes, associated to the cell membrane, could potentially be involved in electron transfer from Fe(II) to the quinone pool (Liu et al., 2017), as is the case for aerobic Fe(II) oxidation in acidic conditions in well-studied organisms such as *Acidithiobacillus ferrooxidans* (Ilbert and Bonnefoy, 2013). Moreover, *Acidovorax ebreus* expressed heavy metal efflux pumps possibly as a response to nitrosative/oxidative stress due to nitrogen species and Fe(II) during NDFO (Carlson et al., 2013), and this finding supports the hypothesis that Fe(II) oxidation under denitrifying
conditions is a detoxification mechanism rather than an energy conserving reaction (Ilbert and Bonnefoy, 2013). Organics and particularly extracellular polymeric substances (EPS) have also been proposed as playing a key role in Fe(II) oxidation, and are produced by many NDFO bacteria. EPS contain polysaccharides that are known to complex Fe(II), therefore, EPS have also been proposed as intermediates in the biotic NDFO, as these could be a site for mineral precipitation too (Jamieson et al., 2018).

Abiotic Fe(II) oxidation with nitrate is limited, since it typically requires relatively high pH, high temperature or specific catalysts, which are not commonly found in environmental conditions, and therefore is discarded as a potential Fe(II) oxidation mechanism. In contrast, bacterial-mediated Fe(II) oxidation under denitrifying conditions occurs at circumneutral pH and can produce Fe minerals of varying crystallinity (Smith et al., 2017). Fe(II)-oxidising bacteria have been reported to produce the intermediate oxidation state minerals green rust (Chaudhuri et al., 2001; Jamieson et al., 2018) and magnetite (Chaudhuri et al., 2001; Sun et al., 2016), and Fe(III)-(oxyhydr)oxide minerals such as ferrihydrite (Hohmann et al., 2010), goethite (Kappler and Newman, 2004; Kappler et al., 2005; Larese-Casanova et al., 2010) and lepidocrocite (Kappler and Newman, 2004; Larese-Casanova et al., 2010; Xiu et al., 2016; Li et al., 2017). Additionally, the presence of arsenic (III/V) in the growth culture of Fe(II)-oxidising bacteria did not affect the formation of ferrihydrite (in cultures of strain SW2), although it promoted the formation of a minor Fe(III)-oxyhydroxide phase in cultures of strain BoFeN1, in addition to goethite (Hohmann et al., 2010). As(III) thus seems to alter the crystallinity of the biomineral through the formation of ferrihydrite with a minor proportion of goethite, and in these conditions the amorphous Fe(III) oxyhydroxides had higher affinity for As(V) than for As(III) (Hohmann et al., 2011).

During NDFO, bacteria have different sites for Fe precipitation, including the periplasm and the cell surface (Benzerara et al., 2008; Miot et al., 2009b; Suzuki et al., 2011; Baumgartner et al., 2013; Schmid et al., 2014; Miot et al., 2016a; Miot et al., 2016b). The periplasm is an important cellular compartment with respect to energy generation and the transit of nutrients and waste, therefore, its encrustation is thought to be lethal to
the cells (Miot et al., 2015). Conversely, microaerophilic Fe(II)-oxidising bacteria form extracellular organics in the shape of stalks (Gallionella spp. and Mariprofundus spp.) or sheaths (Leptothrix spp.), that work as templates for Fe precipitation, keeping the cells free of Fe encrustation (Banfield et al., 2000; Chan et al., 2011), whereas Rhodobacter ferrooxidans strain SW2 precipitates biominerals extracellularly in the form of organic fibers stemming from the cells (Miot et al., 2009c).

Acidovorax are facultative bacteria, typically inhabiting soil and water, with some members able to denitrify and includes a number of plant pathogens (Huang et al., 2012). Acidovorax sp. strain BoFeN1 has become a model NDFO organism (Kappler et al., 2005). Numerous imaging and spectroscopic techniques have been used to study the NDFO process and biomineralisation patterns in this bacterium since it was first isolated (Kappler et al., 2005; Miot et al., 2009a; Miot et al., 2009b). These studies have identified the effect of the geochemical composition of the growth medium on the types of biominerals identified, for instance, carbonate as well as high pH seem to favour the formation of goethite in cultures of BoFeN1 (Larese-Casanova et al., 2010).

Stable isotope labelling coupled to NanoSIMS has been used previously to trace, at the single cell level, the active fraction of cultures of strain BoFeN1 (Miot et al., 2015) and a mixed (“KS”) microbial community (Tominski et al., 2018) growing under denitrifying conditions. These works found heterogeneous C or N uptake by the bacteria studied, suggesting that Fe(II) oxidation is not the primary energy source as this process did not promote abundant cell growth, despite the thermodynamically favourable conditions provided by the presence of nitrate. Under these conditions Fe(II) oxidation was considered likely to be a detoxification mechanism (Miot et al., 2015; Tominski et al., 2018). Furthermore, in these studies heavily mineralised cells were metabolically inactive, suggesting that biomineralisation is deleterious for the cells (Miot et al., 2015), posing the intriguing question of why some bacteria have not coped with high Fe(II) concentrations and cell encrustation by Fe(III)-containing minerals.

Acidovorax sp. strain ST3, in contrast to other Acidovorax species, was isolated from a paddy soil polluted with arsenic and isolated due to its capacity to oxidise As(III) upon
addition of NO$_3^-$, where it was assumed that As(III) oxidation was coupled to NO$_3^-$ reduction (Zhang et al., 2017). In this initial work, the periplasmic AioAB oxidase was identified in strain ST3 and this bacterium was also described as a NDFO organism. In the present work, strain ST3 cells were cultured under denitrifying conditions and then used to study the simultaneous oxidation of As(III) and Fe(II) using high resolution imaging techniques (including NanoSIMS and STEM). $^{13}$C-labelled acetate was used to identify active cells through $^{13}$C accumulation, identified using NanoSIMS. NanoSIMS single-cell depth profiles were also collected to study the distribution of Fe and As in the cells, and these data were compared with STEM-EDS images of encrusted cells, in order to determine the site and composition of this encrustation in the cells. This work is not only of fundamental academic interest, but As(III)-oxidising bacteria could be used to remediate As(III) polluted environments by catalysing As(III) oxidation to As(V). This process, coupled to Fe(II) oxidation under denitrifying conditions, may promote the formation of Fe mineral phases that could sequester As. Mixed valence Fe biominerals with different crystallinity and thermodynamic stability can be formed under such conditions; their identification is important to help identify if they are susceptible to further microbial reduction and consequential re-solubilisation of As(III) and As(V) under fluctuating redox conditions. For this reason, the Fe-As biominerals formed by strain ST3 were characterised in detail using XRD and STEM-HAADF-EDS, so that the impact on long-term As immobilisation in natural environments by this process can be considered.

7.3 Materials and methods

7.3.1 Bacteria strain and culture

The facultative bacterium *Acidovorax* sp. strain ST3 was first streaked onto by this process of aerobic lysogeny broth (LB) agar plates and incubated for 48 h at 28°C in the dark. Single isolated colonies were then transferred to Erlenmeyer flasks containing 100 mL liquid LB broth and incubated in a shaker/incubator at 28°C/150 rpm for 24 h. 5 mL of this liquid culture were used to inoculate serum bottles containing 100 mL of anaerobic low-phosphate (LP) medium to switch the bacteria metabolism from aerobic to anaerobic and promote biomass production, to use as inoculum for the experiments.
The LP medium composition is (g/L): KH₂PO₄ 0.6, NH₄Cl 0.3, MgSO₄ 7H₂O 0.5, CaCl₂ 2H₂O 0.1, 10 mM sodium acetate 0.8203 g, 5 mM KNO₃ 0.505 g, 10 mM NaCO₃ 0.42, 0.5 mM sodium arsenite (As(III)), additionally 1 mL of trace elements, vitamins solution and a tungstate-selenate solution were added (Hegler et al., 2008). pH was adjusted to 6.80. Cells were incubated for 48 h at 30°C in the dark. The cells were centrifuged (2469 g/30 min) and the pellet was resuspended in 20 mL of 10 mM anaerobic piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH=7.0) and centrifuged. The cells were washed twice and the last pellet was resuspended in a small volume of PIPES buffer. These processes were performed under a N₂/CO₂ atmosphere.

7.3.2 Experimental Fe(II) and As(III)-oxidising conditions

The experiments consisted of incubating cells of strain ST3 in serum bottles containing 20 mL of LP media with the same composition as above but additionally containing 10 mM FeCl₂ with 10 mM ¹³C-labelled sodium acetate used to label metabolically active cells. FeCl₂ reacted with phosphate leading to the formation of a green-whitish precipitate, potentially vivianite and siderite (Kappler et al., 2005), which was removed by filtering the medium through a 0.2 µm pore size filter (Jet Biofil®). After filtration the final concentration of Fe(II) was 3.5-4.0 mM Fe. Two growth conditions were tested, planktonic and biofilm, depending on the imaging techniques to be used (planktonic for XRD and STEM, biofilm for SEM and NanoSIMS). For the planktonic experiments, the bottles with LP medium were inoculated with ST3 cells (final OD₆₀₀≈0.15) and three bottles were left un-inoculated and used as “no cell” controls. For the biofilm experiments, two boron-doped silicon (Si) wafers (Sigma-Aldrich®) (7.3 x 7.3 x 0.5 mm) were placed vertically in a plastic holder; these holders were fixed to the bottom of the serum bottles with silicon grease prior to addition of the medium and cells. The Si wafer supported biofilm growth and the precipitates formed. All the experiment bottles were incubated at 28°C in the dark for up to 21 days (planktonic samples). All procedures were performed under an anoxic N₂/CO₂ atmosphere.
7.3.3 Aqueous phase analytical methods

The aqueous phase was sampled and filtered using 0.2 µm pore size filters (Jet Biofil®) to remove precipitates until day 7 of incubation. These filtered samples were analysed for Fe(II), As species, Fe_{total}, A_{total}, nitrate, nitrite and acetate concentrations. Fe(II) was the only species monitored until day 21 in the planktonic growth experiments and until day 14 in the biofilm cultures. The ferrozine method was used to measure Fe(II). The HCl-extractable Fe(II) and total biologically available Fe were quantified by measuring the absorbance (Abs_{562}) of HCl-extracted samples reacted with a ferrozine solution in HEPES buffer. Total Fe(II) was measured by reducing the samples with a 6.25 M hydroxylamine hydrochloride solution (Lovley and Phillips, 1986). As species samples were diluted in d_{2}H_{2}O and quantified by ion chromatography inductively coupled plasma-mass spectrometry (IC-ICP-MS) (Gault et al., 2005) and the anions nitrate, nitrite and acetate were quantified by ion chromatography using a ionex ICS 5000 equipped with an AS11HC 0.4 mm capillary ion exchange column. Fe_{total} and A_{total} samples were extracted-diluted in 2 % HNO_{3} and analysed in ICP-AES using a Perkin–Elmer Optima 5300 DV instrument.

7.3.4 SEM-NanoSIMS sample preservation

The Si wafers in the biofilm cultures were removed from the medium in an anaerobic chamber at days 7 and 14, and preserved by chemical fixation and dehydration. The wafers were submerged in solutions containing absorbance A_{562} decreasing concentrations of glutaraldehyde in phosphate buffered saline (PBS) at pH 7.0 (2.5, 1.5 and 0.75 % v/v). The wafers were left in the 2.5 % glutaraldehyde solution for 4 h and for 30 min in the other two concentrations, afterwards, glutaraldehyde was exchanged for d_{2}H_{2}O and left for 30 min. After cell fixation, the samples were dehydrated by exchanging the d_{2}H_{2}O with ethanol solutions of increasing concentrations (25, 30, 40, 50, 60, 70, 80, 90 and 100 % v/v) and left for 30 min. The wafers were then removed from the solvent and air dried in the anaerobic cabinet overnight. Wafers collected at day 7 were also preserved by only rinsing with d_{2}H_{2}O (degassed with an anaerobic gas mix of N_{2}/CO_{2}) and air dried (in anaerobic chamber), in order to compare the cell and mineral preservation,
as this was the approach used in TEM sample preparation, as explained in the next sections. Once dry, all the Si wafers were sputter coated with 10 nm of platinum.

7.3.5 SEM and NanoSIMS analysis settings
A FEI Quanta 650 FEG SEM operating at 15 kV was used to find suitable areas prior to NanoSIMS analysis using the secondary electron detector under high vacuum. Samples were further analysed in a NanoSIMS 50L ion microprobe (CAMECA, France) using a Cs⁺ primary ion beam at 16 KeV. A beam with a current of 0.131-1.435 pA with spatial resolution from 100-300 nm (D1 aperture=5-2) was scanned over the surface of the sample and the negative secondary ions were collected and analysed using a double focusing mass spectrometer. The following masses were detected simultaneously in multicollection mode: $^{12}\text{C}$, $^{13}\text{C}$, $^{12}\text{C}^{14}\text{N}$, $^{28}\text{Si}$, $^{56}\text{Fe}^{12}\text{C}$, $^{56}\text{Fe}^{16}\text{O}^{-}$ and $^{75}\text{As}^{-}$ (subsequently referred to as $^{12}\text{C}$, $^{13}\text{C}$, $^{12}\text{C}^{14}\text{N}$, $^{28}\text{Si}$, $^{56}\text{Fe}^{12}\text{C}$, $^{56}\text{Fe}^{16}\text{O}$ and $^{75}\text{As}$ for simplicity). Additionally, an ion-induced secondary electron (SE) image was obtained. The elemental ratio $^{13}\text{C}/^{12}\text{C}$ was used to identify cells that were metabolically active; the molecular ions $^{56}\text{Fe}^{12}\text{C}$ and $^{56}\text{Fe}^{16}\text{O}$ were used to monitor $^{56}\text{Fe}$ because $^{56}\text{Fe}^{-}$ has a low ionisation yield under the Cs⁺ ion beam. Before starting the analysis the selected areas of analysis were implanted with Cs⁺ ions to a dose of $1\times10^{17}$ ions cm$^{-2}$ to remove the Pt coating and reach steady state (McPhail and Dowsett, 2009). Images were collected at a dwell time between 2000-5000 µs px$^{-1}$. CAMECA High mass resolution (HMR) spectra were acquired at masses 13, 72 and 75 using iron metal and gallium arsenide as reference materials to avoid peak overlaps of molecular interferences, primarily $^{12}\text{C}^{1}\text{H}$ at mass 13, $^{28}\text{Si}^{2}\text{16O}$ at mass 72 and $^{56}\text{Fe}^{19}\text{F}^{-}$ at mass 75; the CAMECA mass resolving power (MRP) was >7000 using ES =3 and AS=2. The image pixel sizes were 128x128 or 256x256. Acquisition raster sizes ranged from 30-60 µm to image groups of cells and minerals.

7.3.6 NanoSIMS depth profiles-Cs⁺ primary ions
After collecting images at a large field of view (30-60 µm), single cells were selected in order to collect depth profiles from a raster size of 3-5 µm. The spatial resolution was improved by using D1=4 or 5 (spot size ~120-100nm), mass resolution remained above 7000. Pixel sizes were 128x128 or 256x256, and dwell time varied from 5,000-20,000 µs
The number of planes collected were in the range of 50-170, and the scanning was stopped when the $^{12}\text{C}$ or $^{12}\text{C}^{14}\text{N}$ signal disappeared, indicating the bacterial cell had been completely sputtered away and chemical information could be reconstructed for the whole cell.

### 7.3.7 NanoSIMS depth profiles-O$^-$ primary ions

An O$^-$ beam was scanned on the sample surface during depth profiling of cells to collect positive secondary ions with a current between 1.693-4.31 pA (D1 apertures 5 or 4). The areas analysed were 3-7 µm wide and images were collected at a dwell time of 5000 µs px$^{-1}$. The masses detected were $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{28}\text{Si}^+$, $^{39}\text{K}^+$, $^{44}\text{Ca}^+$, $^{56}\text{Fe}^+$ and $^{75}\text{As}^-$. Cameca MRP was improved to ≈3000 (ES=3 AS=2) to separate the mass interferences $^{12}\text{C}^{16}\text{O}^+$ at mass 28 and $^{23}\text{Na}^{16}\text{O}^+$ at mass 39. Mass 75 had no isobaric interferences ($^{56}\text{Fe}^{19}\text{F}^+$ was not formed). In this case the disappearance of $^{56}\text{Fe}^+$ signal was monitored as an indicator of complete cell sputtering.

For both Cs$^+$ and O$^-$ analysis, depth profiled cells were not implanted to reach steady state, in order to keep the cell mineralisation coating intact, however a quick implantation of Cs$^+$/O$^-$ ions was done before the start of analysis in order to locate and focus single cells. This quick implantation consisted on changing to D1=1 on larger fields of view (≈10 µm) for 20-30 s.

### 7.3.8 NanoSIMS data analysis

L’image software was used to obtain stack NanoSIMS images (Larry Nittler, Carnegie Institution of Washington). ImageJ (https://imagej.nih.gov/ij/) with the plugin OpenMIMS (MIMS, Harvard University; www.nrims.harvard.edu) was used to create the hue saturation images (HSI) isotope ratio maps and to generate colour merge (overlay) images. Regions of interest (ROIs) were drawn around cells and minerals. ROIs were classified in active cells as “planktonic” for cells grown under those conditions or “biofilm” for that type of culture. The ion counts in these ROIs were normalised to dose, analysed and compared by creating box and whisker plots and counts comparison graphs using OriginPro® 8.5.1. A box and whisker plot was used to display the distribution of
$^{13}$C/$^{12}$C enrichment in all analysed cells, where the box represents the upper and lower quartile and whiskers represent the data range (lowest to highest $^{13}$C/$^{12}$C observed).

3D reconstructions of the depth profiles were created using the Thermo Scientific™ Avizo™ Software 9.7.0. Stack data of $^{12}$C, $^{23}$Na, $^{56}$Fe, $^{56}$Fe$^{16}$O and $^{75}$As were first extracted with ImageJ and saved in the “.raw” format file. Afterwards, these .raw files were loaded into Avizo™. The Z depth was compressed to 15-20 %, in order to reduce the volume of 3D visualisation, closer to scale. The $^{12}$C and $^{23}$Na signals were used to generate the bacteria surface when negative or positive secondary ions respectively were collected, by smoothing the signal over 2-3 pixels. The “generate surface” and “show surface” commands were used sequentially, and the “transparent” display with a transparency of 80 % was selected. $^{56}$Fe or $^{56}$Fe$^{16}$O signals were smoothed to 2 pixels and displayed as “shaded”. The $^{75}$As ion counts were smoothed to 1 pixel because of the lower ion counts and displayed as “points” to enable their visualisation.

7.3.9 Transmission electron microscopy (TEM)

Samples were obtained from the cells and precipitates grown in planktonic culture sampled at days 1, 3 and 7, and handled under anaerobic conditions for STEM analysis. 1 mL of the sample was centrifuged, the pellet was washed twice in $d$H$_2$O and diluted to an OD$_{600}$≈0.1. This dilution was suspended (1.5 µL) on TEM grids (holey carbon films on 400 mesh copper grids, Agar Scientific®) and air dried in an anaerobic cabinet. Sample transportation to the TEM laboratory was done in an airtight bottle to preserve the anaerobic conditions as long as possible. The TEM grid loading into the sample holder was done using a plastic box filled with argon at the bottom, and all the transference from the portable sample holder to the TEM instrument holder was done at the height of the argon layer inside the plastic box. This sample manipulation was very challenging but with the aim to keep the anoxic conditions for as long as possible, although we cannot rule out the presence of some ppm of O$_2$. The samples were analysed in a FEI Titan ChemiSTEM running at 200kV using a 180 pA beam current, high angle annular dark field (HAADF) images and electron dispersive spectroscopy (EDS) data were collected using the Gitan digital micrograph software. STEM-EDS data was processed using
Hyperspy, quantification of the EDS used a background correction and Cliff Lorimer factors. Moreover, the apparent nanoparticle size was manually measured with ImageJ in these TEM images.

**7.3.10 Biominerals characterisation by powder X-ray diffraction (XRD)**

The precipitates obtained from planktonic samples were centrifuged (2469 g/30 min) at days 7 and 10 of incubation, washed twice with dH_{2}O, deposited on a glass slide (1 x 0.5 cm) and dried in an anaerobic chamber. This glass holder was loaded to an anaerobic dome and analysed in a XRD Brucker D8 Advance X-ray diffractometer to identify the biominerals formed. The samples were analysed with a 40 kV beam, over the 0-70° 2θ range, with a 0.02° 2θ step size and 1 s counting time.

**7.4 Results**

**7.4.1 Aqueous geochemistry**

Fe(II) oxidation was noticeable as the formation of rust-coloured precipitates from day 1 in the experiment bottles containing active biomass, as observed previously by Zhang et al. (2017). The results of Fe(II) oxidation in the experiments were similar for planktonic and biofilm cultures; at day 7 the biofilm samples oxidised 67 % of the Fe(II) to Fe(III), while the planktonic samples oxidised 62 % of Fe(II) (Fig. S7.1A). By day 14 the biofilm samples oxidised 72 % of Fe(II), whereas the planktonic samples followed by oxidising 65 % at that time point (Fig. S7.1A). Only planktonic samples were monitored until day 21 when 81 % of Fe(II) was oxidised (Fig. S7.1A). The “no cells” control oxidised 15 % of the Fe(II) by day 7, and over the course of 21 days this abiotic Fe(II) oxidation increased to 31 % (Fig. S7.1A). It is worth noting that the “no cells” controls presented a thin red/brown layer on the surface of the medium (in the liquid-gaseous interphase), although the formation of precipitates was negligible in these control samples.

As speciation in the solution phase showed a removal of 93 % As(III) in biofilm samples at day 7 and a 65.5 % As(III) removal in planktonic samples (Fig. S7.1B). As(V) was detected in all experiments, and also dropped in concentration over the duration of the experiment (from 110 to 10 µM) (Fig. S7.1C). There was also 70 % As(III) removal in the
“no cells” controls (Fig. S7.1B). The As_{\text{total}} measurements by ICP-AES were below level of detection (10 ppb) in both biofilm and planktonic experiments at day 7, whereas the no cells controls showed 75 % removal of As_{\text{total}} (Fig. S7.1D).

The consumption of acetate and nitrate was followed until day 7 in all experiments, and it showed that 0.61 and 0.26 mM of acetate were consumed in biofilm and planktonic samples, respectively, implying a 5.5 % consumption of acetate in biofilm and 2.5 % consumption in planktonic samples (Fig. S7.2A). 1.99 mM and 0.99 mM of NO$_3$ were consumed in biofilm and planktonic samples, respectively, indicating that 17 % of nitrate was consumed in biofilm samples and 9 % in planktonic growth (Fig. S7.2B). Nitrite was not detectable in the supernatants over the duration of the experiment. The “no cells” controls showed no changes in the acetate and NO$_3$ concentrations, and NO$_2$ was also not detected.

7.4.2 Sample preservation effect imaged in SEM

Sample preservation is often overlooked but is a key determining factor for accurate image analyses, including electron microscopy and SIMS techniques. SEM images were collected to compare the effect of the following methodologies on cells and biominerals; rinsing-air drying (anaerobic), the approach used to prepare TEM samples, and chemical fixation-dehydration, chosen for NanoSIMS imaging. Abundant biomass colonising the Si wafer was observed in the biofilm samples in SEM at day 7, regardless of the preparation method used (Fig. 7.1), although substantial surface colonisation was already present by day 4 (images not shown). The chemical fixation-dehydration approach preserved the structure of the cells (Fig. 7.1B), in contrast to the samples that were rinsed with $d$H$_2$O-air dried, which showed many disrupted cells (images shown in section 3.5.3.6 in Chapter 3). Cells were heavily mineralised after 7 days of incubation and processing by both treatments, although the cells in the chemically fixed samples showed biominerals in the shape of sheaths (yellow arrow in Fig. 7.1B), while the samples rinsed with $d$H$_2$O-air dried showed nanoparticles of a few tens of nm on the cell surfaces (magenta arrows in Fig. 7.1D). The Si wafer surfaces were coated with small particles, and these solids were similar in morphology to the biominerals on the surface of the cells with each type of
sample preparation (Fig. 7.1B, D). Moreover, the samples rinsed with \( \Delta H_2O \)-air dried showed a layer covering the cells (orange arrows in Fig. 7.1C), potentially extracellular polysaccharides substances (EPS) or other organic material, which was not observed in the chemically fixed-dehydrated samples.

**Figure 7.1.** SEM images of *Acidovorax* sp. ST3 growing in biofilm conditions after 7 days of incubation and preserved by chemical fixation-dehydration (A & B) or rinsed–air dried (C & D). Abundant biomass is observed with both preservation methods, but differences are noticeable at the cell level and the morphology of the biominerals on the cell surface. The white arrow in (B) indicates a cell with low mineralisation, the yellow arrow in (B) is a heavily mineralised cell showing “flakes” of biominerals, and the cell in (D) shows spherical particles of tens of nm on the cell surface (magenta arrows). The blue arrows in (C) indicate groups of cells covered by a layer (potentially EPS or other organic material).

**7.4.3 NanoSIMS imaging**

By day 7, many cells showed high counts of Fe (as \(^{56}\text{Fe}^{16}\text{O}\)) when imaged with NanoSIMS, suggesting a mineral coating on the surface of cells (encrusted or mineralised cells) (Fig. S7.3A-C). The \(^{75}\text{As}\) signal was co-located with \(^{56}\text{Fe}^{16}\text{O}\) on the surface of cells, although \(^{75}\text{As}\) was not detected in all mineralised cells or extracellular biominerals. The poles of the cells showed high \(^{56}\text{Fe}^{16}\text{O}\) counts, suggesting mineral encrustation at the cell pole.
(Fig. S7.3C), as observed in strain BoFeN1 (Miot et al., 2009b). NanoSIMS analyses at day 7 showed negligible $^{13}$C enrichment of the cells (Fig. S7.3D-F). Some regions in the cells showed up to 2 % $^{13}$C enrichment, and interestingly the parts that were not $^{13}$C enriched showed $^{56}$Fe$^{16}$O counts (Fig. S7.3C & F). Many cells were completely covered in mineral coating showing no $^{12}$C or $^{13}$C signal (Fig. S7.3B & E).

By day 14 NanoSIMS images of biofilm samples showed heterogeneous $^{13}$C accumulation of up to 4 %, or almost four times the natural $^{13}$C/$^{12}$C ratio (Fig. 7.2D-F). In fact, a high proportion of cells were not active and had a $^{13}$C/$^{12}$C ratio close to the natural abundance of 1.11 %. Some cells showed $^{56}$Fe$^{16}$O counts on their surface (white arrows in Fig. 7.2A-C) with a similar intensity to the $^{56}$Fe$^{16}$O counts on the biominerals at a distance from the cells (yellow arrows in Fig. 7.2A-C), indicating heavy Fe mineral encrustation of the cells. The cells that were mineralised were not active and showed low $^{12}$C and $^{13}$C counts, whereas the active cells didn’t show Fe or As on their surfaces. The surface of the cells presented a higher intensity signal for $^{56}$Fe$^{16}$O than for $^{56}$Fe$^{12}$C, despite the natural high C abundance of the cells. This suggests that the thick mineral coating on the cell surface was probed by the Cs$^+$ beam and not the actual cell surface, as the data presented would be consistent with the presence of an Fe(III) oxide or Fe(III)-oxyhydroxide coating. In these conditions $^{56}$Fe$^{16}$O is a molecule more likely to be formed than $^{56}$Fe$^{12}$C. In some cases, cells showed Fe encrustation, $^{75}$As on the surface and also $^{13}$C accumulation. The surface of most inactive cells also generated $^{75}$As counts, which were higher on mineralised cells (purple coloured cells in Fig. 7.2B & C). The $^{75}$As counts on the surface of biominerals at a distance from the cells were lower than expected, seen as bright magenta surfaces (indicating mostly $^{56}$Fe$^{16}$O in Figures 7.2A-C), as opposed to purple surfaces indicating a co-location of $^{75}$As and $^{56}$Fe$^{16}$O.
7.4.4 Biofilm vs planktonic growth in NanoSIMS

By day 14, cells in the biofilm cultures accumulated on average 2.59 ± 1.33 % of \(^{13}\text{C}/^{12}\text{C}\). Thus cells accumulated on average 2.3 times more \(^{13}\text{C}\) than natural abundance, whereas planktonic cells accumulated 1.51 ± 0.36 % of \(^{13}\text{C}/^{12}\text{C}\), implying that cells only accumulated 1.3 times more \(^{13}\text{C}\) than the natural \(^{13}\text{C}/^{12}\text{C}\) abundance (Fig. 7.3A). \(^{56}\text{Fe}^{16}\text{O}\) counts on the cell surface (normalised to dose) were used to estimate the level of cell Fe mineralisation. When plotting these counts against the \(^{13}\text{C}/^{12}\text{C}\) ratio (%, as indicator of metabolic activity) a curve was obtained, which was more apparent for biofilm cells (Fig. 7.3B). The fit for this curve was an exponential decay 3-parameter model. This curve describes an inversely proportional correlation between metabolic activity and Fe.
mineralisation on the cell surface, where it appears that decreasing metabolic activity can halt encrustation.

7.4.5 NanoSIMS depth profiles

The selected areas for depth profiles were not implanted with Cs⁺ or O⁻ ions prior to starting the analysis, in order to preserve and probe the intact biomineral coating on the cells. Both Cs⁺ (negative secondary ion) and O⁻ (positive secondary ion) were used as primary ions for depth profiling. Under O⁻ bombardment ⁵⁶Fe⁺ has a higher ionisation efficiency, therefore this secondary ion was detected with a higher intensity (Fig. 7.4H), typically one order of magnitude higher than ⁵⁶Fe¹⁶O⁻ when imaged with Cs⁺ (Fig. 7.4C). However, ⁷⁵As has a lower ionisation efficiency under the O⁻ beam than under Cs⁺ bombardment. Regardless of the secondary ion collection mode, the ⁵⁶Fe⁻ signal was more intense than ⁷⁵As in depth profiles. Cells with asymmetric Fe encrustation were observed and the cell poles were particularly encrusted (see arrow number 2 in Fig. S7.4B). ⁷⁵As was observed associated with all cell surfaces, regardless of the Fe encrustation level of the cell, and was observed as intense counts at the end of the depth profiles (see arrow 1 in Fig. S7.4A). However, cells that were metabolically active (higher
only showed $^{75}\text{As}$ but no $^{56}\text{Fe}^{16}\text{O}^{-}$ on the surface, confirming that metabolic activity deterred mineralisation (see bottom cell in Fig. 7.4A-E and arrow #1 in Fig. S7.4A), rather than cellular interactions with As. In all these depth profiles the estimated spatial resolution was 100-150 nm. Surprisingly, $^{75}\text{As}$ did not co-locate completely with precipitated Fe (when present), presumably in the periplasm (Fig. 7.4A-B & S7.4A-B), where the encrusted Fe was expected to sorb As. The 3D reconstructions presented show this asymmetrical As and Fe distribution at the cell surface, in contrast to NanoSIMS stack images (Fig. 7.4C, D, H & I and supporting Fig. S7.4C, D, H & I) which only show the total sum of each secondary ion in 2D. Mineralised *Acidovorax* sp. strain BoFeN1 cells had been analysed in 3D via a TEM, scanning transmission X-ray microscopy (STXM) and focused ion beam (FIB/SEM) approach, where different Fe biomineralisation patterns were determined (Schmid et al., 2014). To our knowledge though, the work presented here is the first to use NanoSIMS imaging to generate single-cell 3D models of microbial biomineralisation, in this case linked to nitrate-dependent Fe(II)-oxidising cells in simultaneous incubations with As(III).

**7.4.6 TEM analysis of Fe-As minerals**

TEM images were collected of planktonic cells collected on days 1, 3 and 7, to observe the aging of cell associated biogenic minerals. Cells showed encrustation from day 1, where extracellular biominerals where present as amorphous nanoparticles (at the cell surface or at a distance from the cell). At this point the cells showed heavier mineralisation at the poles than at the centre of the cells (red squares in Fig. 7.5A, B & C). These markedly encrusted cell poles are consistent with the observations of NanoSIMS depth profiles (Fig. S7.4). However, the TEM images are 2D representations of a 3D object, and understandably, this higher electron density interpreted as heavier mineralisation could be an artifact of the technique, where thinner areas of the cells (the poles) are allowing a higher transmission of electrons. At day 1 the average biomineral nanoparticle size was $48 \pm 25$ nm. By day 3, cells were coated with amorphous nanoparticles of similar morphology, although the cell pole coating appeared thicker (see red square in Fig. 7.5C). By day 3 the average particle size increased to $62 \pm 26$ nm.
Figure 7.4. 3D reconstructions of NanoSIMS depth profiles of *Acidovorax* strain ST3. (A), (B), (F) and (G) are 3D models, where (A) are cells sputtered with Cs⁺ and (B) is the side view of the same cells; (F) is a cell sputtered with O⁻ and (G) is the side view of the same cell. In these 3D reconstructions Fe is shown in red (either $^{56}\text{Fe}^{16}\text{O}$ or $^{56}\text{Fe}^+$), $^{75}\text{As}$ in yellow and $^{12}\text{C}$ (A & B) or $^{23}\text{Na}^+$ (F & G) in blue. (C) and (D) are stack images of $^{56}\text{Fe}^{16}\text{O}$ and $^{75}\text{As}$, respectively, of the cells in panel (A), (E) is the $^{13}\text{C}/^{12}\text{C}$ ratio of the same area, sum of 74 planes. (H) and (I) are stack images of $^{56}\text{Fe}$ and $^{75}\text{As}$, respectively, of the cell in panel (F), sum of 71 planes. The vertical colour bars in panels (C), (D), (H) & (I) are the SI intensities.
suggesting growth of these minerals (Fig. 7.5D). At day 7 similar mineralisation and morphologies were observed, and at this point another biomineral phase was noted on the surface of the cells. This new biomineral appeared to have a more crystalline structure, resembling flakes (Fig. 7.5E & F). By day 7 the average particle size increased to 104 ± 43 nm, where the particles at a distance from the cells were twice as big as on the cell surface (Fig. 7.5E). Moreover, regardless of the time point, the amorphous nanoparticles formed aggregates at a distance from the cells (Fig. 7.5D) or aggregated as filaments associated with the cell surface (red arrows in Fig. 7.5A, C & E).

Figure 7.5. TEM images of planktonic ST3 samples at days 1 (A & B), 3 (C & D) and 7 (E & F). Mineralised cells can be observed from day 1, showing a thicker mineralisation by day 3 and the appearance of a more crystalline structure by day 7. The red squares in (A), (B) and (C) highlight the cell poles with heavier mineralisation than the rest of the cell. The yellow square in (E) is a selected region with “flake-like” biominerals and magnified in (F). Red arrows in A, C & E are indicating the nanoparticles aggregated as filaments associated with the cell surface. Scale bars are 500 nm in (A), (B), (C) and (E), 300 nm in (D) and 20 nm in (F).

7.4.7 Quantitative high-resolution imaging with STEM-HAADF-EDS

At day 7, high resolution STEM-HAADF-EDS data of the biominerals were collected primarily at the outer regions of the particles, given that the areas need to be thin for the analysis. The samples were kept under anaerobic conditions, with exposure to
oxygen kept to a minimum, given that oxygen was one of the elements of interest in EDS mapping. As mentioned previously, two main mineral morphologies were observed and selected for analysis: crystalline flakes (not in direct contact with the cell surface) and amorphous nanoparticles (attached to the cell surface). All the biominerals selected for STEM-HAADF-EDS analysis were composed mainly of iron, oxygen and arsenic and their abundance was quantified. The As to Fe ratio (normalised to Fe) varied from 2.3 to 25 % in all the regions analysed (Fig. 7.6E & J). The crystalline flakes showed lower As/Fe ratios (2.3-6 %) (Figs. 7.6E and S7.6J), whereas the cell surface and the attached amorphous nanoparticles had higher As/Fe ratios (15-25 %) (Fig. 7.6J). The amorphous nanoparticles at a distance from the cells also presented 23-25 % As/Fe (Fig. S7.5E). Oxygen was the most abundant element in these amorphous nanoparticles where the ratio normalised to O (Fe:O) was 0.7-0.8:1. However, the crystalline flakes were deficient in oxygen, where the Fe:O increased to approximately 1:0.5.

Planktonic precipitates were also analysed by powder XRD. At day 7 an amorphous mineral was identified, and by day 10 the bulk biomineral identified was the Fe(III)-oxyhydroxide lepidocrocite (γ-Fe³⁺OOH) (Fig. S7.6).

7.4.8 Periplasm encrustation

STEM-HAADF-EDS is based in the transmission of electrons and is therefore limited by sample thickness. Although this technique cannot directly probe the periplasm in whole cells without sectioning, HAADF forms images by Z-contrast, and the bright region highlighted in the green square in Figure 7.6F is rich in high atomic number elements, such as Fe and As, seen in Fig. 7.6 panels G and H, respectively, and matches the reported size of the periplasm (Miot et al., 2011). Therefore, the HAADF images are consistent with the periplasm being encrusted (orange arrows in Fig. 7.6F).
7.5 Discussion

7.5.1 Aqueous As removal and the effect of growth conditions

The comparison between growth conditions was not an objective in this work, however, biofilm growth was more favourable for As(III) removal, Fe(II) oxidation and denitrification. Bacteria are commonly found growing in biofilms in natural systems. Even though biofilms are heterogeneous, they provide resistance to environmental stress conditions, which could explain these results. Additionally, biofilms contain metal-reactive ligands, for example in the EPS fraction, which could have complexed Fe(II) and promoted its precipitation (French et al., 2013) and subsequent sequestration of As(III/V).
The Si wafer surface in the biofilm samples could have played a role as a biomineral precipitation site, which itself worked as a nucleation site for the minerals, retaining Fe and As, although not necessarily as the oxidised species. The consumption of acetate was only 0.3-0.6 mM, however, Fe(II)-oxidisers have been found to only require organic co-substrates at low concentrations, typically between 0.5 to 1.0 mM (Chakraborty et al., 2011). In contrast to other NDFO organisms, nitrite was not detected in strain ST3 cultures, although, considering the low nitrate consumption it is possible that it was produced at much lower concentrations, and given that the samples were diluted for ion chromatography analysis, the concentration could have been below the technique’s detection limit (1 µM). Moreover, the head space of the bottle was not analysed for all gaseous denitrification products (N₂O, NO and N₂), therefore a N mass balance is not possible in this experiment. No As(V) generation was quantified in the supernatants, although it is possible that the As(V) was sorbed to the solid biominerals, which were removed from the aqueous phase by filtration prior to As species analysis. In addition, spontaneous oxidation of As(III) by exposure to oxygen would not explain the As(V) quantified during all time points, as this process is slow, in the range of months to years (Gorny et al., 2015).

7.5.2 Metabolic heterogeneity revealed at the nanoscale

In this study ST3 cells accumulated heterogeneous ¹³C levels. By day 7 of incubation, cells didn’t appear to accumulate ¹³C/¹²C beyond the natural abundance, but by day 14 higher ¹³C/¹²C concentrations (up to 4 %) were detected, although most cells were not ¹³C-labelled and were therefore inactive. The active cells with ¹³C/¹²C > 1.11 % showed low levels of Fe (as ⁵⁶Fe¹⁶O or ⁵⁶Fe¹²C), whereas the mineralised cells (with high ⁵⁶Fe¹⁶O counts) showed low ¹³C accumulation. This suggests that cell encrustation affects the metabolic activity, as observed in strain BoFeN1 (Miot et al., 2015). The equation showed in Fig. 7.3B proposed a threshold where only low levels of cell mineralisation and metabolic activity were compatible, implying that highly active cells can deter cell encrustation, and on the contrary, high cell mineralisation appears to be detrimental for the metabolism of ST3 cells.
This phenotypic diversity observed as heterogeneous Fe mineralisation and $^{13}$C accumulation is intriguing in genetically identical cells cultured under the same environmental conditions. Metabolic heterogeneity is an ongoing research field, where not only the genotype and environment regulate these variations but also stochastic gene expression (Takhaveev and Heinemann, 2018). In microbial populations ecological factors such as surfaces or cell to cell communication, as well as the cell cycle and cell ageing can affect gene expression, leading to metabolic variations. The cell cycle and cell ageing might explain the different biomineralisation patterns, particularly the marked mineralisation at the (older) cell poles, which could be explained by protein aggregation in this part of the cells, as proposed earlier by Miot et al. (2015).

7.5.3 Cell encrustation is harmful for the cells

The biogenic minerals studied through different nanoscale approaches in this work, showed a removal of both As(III) and Fe(II) in the experiments with cells, confirming that cells are directly involved in this removal. Acidovorax sp. strain ST3 had been previously tested and isolated for its As(III) oxidation capabilities, although this capability was not tested for simultaneous As(III) and Fe(II) oxidation under denitrifying conditions. From the NanoSIMS and STEM biomineralisation studies it can be inferred that cell encrustation was detrimental for the cells, although it is not clear if Fe was encrusted as a protection mechanism against Fe(II). This Fe encrustation could also have an effect on the enzymatic oxidation of As(III) and reduction of NO$_3^-$, which take place in the periplasm. Additionally, this encrustation could stop the flow of substances inside and outside of the cell. The relatively high concentrations of As(III) and Fe(II) could have been toxic for ST3 cells, despite this strain’s high tolerance to arsenite [up to 5 mM As(III)] (Zhang et al., 2017). Strain ST3 was isolated from a soil that contained high Fe and high As concentrations (4.7 g kg$^{-1}$ of total Fe and 340.6 mg kg$^{-1}$ of total As), where it can be assumed that a high percentage of these two elements was in their reduced form, due to the flooded soil conditions deficient in oxygen (Zhang et al., 2017). Therefore, cell encrustation with Fe(III) minerals appears to be a disadvantageous evolutionary step for these bacteria.
7.5.4 Spatial distribution of As and Fe in single cells

Single-cell depth profiles were performed to compare the $^{56}\text{Fe}^{56}\text{Fe}^{16}\text{O}$ and $^{75}\text{As}$ distribution in whole cells at high resolution and the 3D reconstructions helped to observe the relative distribution of As and Fe. In this study many ST3 cells appeared encrusted in Fe minerals at day 7, and this mineralisation, detected with NanoSIMS, indicated that $^{75}\text{As}$ co-located with $^{56}\text{Fe}^{16}\text{O}$ in many cases, suggesting that these Fe precipitates sorbed or co-precipitated As. However, other cells did not show $^{75}\text{As}$ and $^{56}\text{Fe}^{16}\text{O}$ spatially co-located, which could indicate that As was bound to the cell possibly as As(III), binding proteins in the cell membranes (Shen et al., 2013), whereas the $^{75}\text{As}$ observed co-locating to the Fe encrustation could be mixed valence As, given that Fe(III) minerals can efficiently sorb both As(III) and As(V) (Dixit and Hering, 2003b).

7.5.5 Site of Fe mineralisation

The techniques used in this study did not allow the direct probing of the periplasm. In order to do this, the cell would need to be sectioned and then analysed using, for instance, TEM. However, by studying TEM-HAADF images (Fig. 7.6F) the encrusted region has an average size of 25.8 ± 6.2 nm, which is within the periplasm size range of what has been determined using traditional TEM (Miot et al., 2011). Thus, the size of the encrusted region suggests that the periplasm is a site of Fe encrustation in *Acidovorax* sp. strain ST3, additional to the cell surface, and no cytoplasm encrustation was observed. The location of the site of mineralisation can be used to infer the presumable mechanism of Fe(II) oxidation in strain ST3, where enzymatic Fe(II) oxidation as well as oxidation through nitrogen species in the periplasm are plausible pathways. The export of oxidative nitrogen species from the periplasm could explain the extracellular Fe mineralisation observed, whereas the precipitation of Fe minerals at the cell vicinity could also be an effect of the negatively charged cell surface acting as nucleation site for positively charged Fe minerals (Miot et al., 2011).

The biomineralisation patterns appear to be characteristic of the specific Fe(II) oxidation mechanism used by bacteria. Periplasmic biomineralisation could be a common trait in the *Acidovorax* genus and perhaps in other NDFO organisms. In contrast,
photoautotrophic Fe(II)-oxidisers possess outer cell membrane c-type cytochromes that catalyse Fe(II) oxidation (Bryce et al., 2018) producing extracellular Fe(III) minerals (or rapidly exporting them from the cell), which keeps cells free from mineral encrustation (Kappler and Newman, 2004).

**7.5.6 Biominerals produced by strain ST3 and their implications in As sequestration**

The bulk minerals identified are a product of the particular culture conditions; the amorphous Fe(III)-oxyhydroxide lepidocrocite was identified in the present study, and other Fe(II)-oxidising bacteria have also produced lepidocrocite under similar conditions (Kappler and Newman, 2004; Larese-Casanova et al., 2010; Xiu et al., 2016; Li et al., 2017). Fe(III) minerals can incorporate both As(III) and As(V), where sorption for both As species is similar at neutral pH, although greatly influenced by the presence of competing sorbates such as phosphate (Dixit and Hering, 2003a). Unfortunately, lepidocrocite may not be the ideal host for long term As immobilisation, as this poorly ordered Fe(III) mineral is not thermodynamically stable over a wide range of pH and redox conditions. Longer incubation periods (>30 days) could potentially allow mineral evolution, in order to produce more kinetically stable Fe minerals, such as magnetite, which could adsorb As(III) and even incorporate As(V) to its structure during NO₃-dependent Fe(II) oxidation (Coker et al., 2006; Sun et al., 2016). Interestingly, the amorphous nanoparticles coating the cell surface retained more As (III/V), potentially due to their higher surface area, in comparison to the crystalline flakes (as shown in EDS maps), when these flakes were developed after a longer incubation period. Further STEM analysis using selected area electron diffraction (SAED) and electron energy loss spectroscopy (EELS) could reveal the oxidation state of Fe along with the biomineral identification at the nanoscale, allowing to delve into the mechanism of Fe mineralisation and concomitant As sequestration. This information would complement the assessment of long term As removal by these biominerals, and this is being investigated in our laboratory.

**7.6 Conclusion**

The aim of the present study was to understand the structure and composition of biominerals produced by *Acidovorax* sp. strain ST3 and their effect on cellular
metabolism. In this system the application of complementary nanoscale techniques was vital for a comprehensive analysis. NanoSIMS allowed to simultaneously depth profile active/inactive and encrusted/non-encrusted cells with varying $^{13}$C-accumulation, and therefore level of metabolic activity, which can only be achieved with NanoSIMS. STEM allowed to quantitatively analyse the composition of biominerals at high spatial resolution and to identify the intracellular compartment where cell encrustation occurs, moreover, the bulk biomineral was identified with XRD. Furthermore, Fe(III) minerals were expected to sorb and co-localise with As in these conditions, however, the NanoSIMS depth-profiles produced an insightful 3D analysis of the intracellular As and Fe distribution, revealing the unexpected spatial delocalisation of As and Fe at high resolution.

In our experimental conditions nitrite accumulation was not noted, and would not explain the Fe(II) oxidation observed, hence, other more direct Fe(II) oxidation mechanism might be at play. Further studies with this and other nitrate-dependent Fe(II)-oxidisers could focus on the role of organics, where EPS are key components that could complete the principles of biotic Fe(II) oxidation by bacteria, beyond chemodenitrification promoted by NO$_2$ and NO.

It is unclear if the cell encrustation observed was an artefact of the relatively high Fe(II) concentration in the experimental conditions, as suggested previously by Chakraborty et al. (2011), despite ST3 cells exposure to high Fe(II) concentrations in their natural environment. Nonetheless, Fe(II) oxidation and biomineralisation by strain ST3 could be stimulated by adding nitrate and exploited for *in situ* remediation of high concentrations of arsenite, as demonstrated in the present work. Further studies should aim to investigate microcosms and mixed communities from representative environmental conditions and be further imaged by the combination of stable-isotope labelling and nanoscale approaches used in this work.
7.7 Acknowledgments

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7.8 References


7.9 Supporting information

Research article: Elucidating Iron Biomineralisation Patterns in Denitrifying As(III)-Oxidising Bacteria: Implications for Arsenic Immobilisation

Figure S7.1. Fe(II) and As species measurements in the aqueous phase in planktonic and biofilm cultures of strain ST3. (A) Fe(II) by ferrozine until 21 days of incubation, except for biofilm growth; As species were measured until 7 days of incubation: (B) As(III), (C) As(V) and (D) total As by ICP-AES. Notice the abiotic removal of As from solution, probably through sorption to abiotically formed precipitates or minerals. Bars are the standard deviation, n=3.
Figure S7.2. Acetate and nitrate measurements in the aqueous phase of strain ST3 in biofilm and planktonic growth. Bars are the standard deviation, n=3.
Fig. S7.3. NanoSIMS images of strain ST3 chemically fixed and dehydrated at day 7. Top row (A-C) are overlay images of cells displaying $^{13}$C (cyan), $^{56}$Fe$^{16}$O (magenta) and $^{75}$As (blue). Bottom row (D, E & F) are the $^{13}$C/$^{12}$C HSI images of (A), (B) and (C), respectively. Note that only parts of cells appear $^{13}$C-enriched at 1.5-2 % (yellow arrows in F), which doesn’t overlap with the Fe mineralisation in cells (yellow arrows in C). The white arrow in (B) is indicating a cell fully coated with Fe minerals, showing no $^{13}$C signal (white arrow in (E)).
Figure S7.4. NanoSIMS depth profile 3D reconstructions of Acidovorax sp. strain ST3 cells at day 7 of incubation. (A), (B), (F) and (G) are 3D reconstructions, where (A) & (B) were sputtered with Cs\(^+\) (negative SI mode), (A) is the whole area analysed and (B) are the cells selected in the red dashed oval in (A) seen in another angle; (F) and (G) are cells sputtered with O\(^-\) (positive SI mode), where (G) is a side view of cells from (F). In these 3D reconstructions Fe is shown in red (either \(^{56}\text{Fe}^{16}\text{O}\) or \(^{56}\text{Fe}^+\)), \(^{75}\text{As}\) in yellow and \(^{12}\text{C}\) (A & B) or \(^{23}\text{Na}^+\) (F & G) in blue. (C) and (D) are stack images of \(^{56}\text{Fe}^{16}\text{O}\) and \(^{75}\text{As}\), respectively, of the cells in panel (A); (E) is the \(^{13}\text{C}/^{12}\text{C}\) ratio in this area, sum of 170 planes. (H) and (I) are stack images of \(^{56}\text{Fe}\) and \(^{75}\text{As}\), respectively, of the cells in (F), sum of 101 planes. The arrow #1 in (A) indicates a cell with no Fe encrustation but As on the surface, whereas arrows #2 in (B) indicate the encrusted cell poles.
Figure S7.5. Additional STEM/HAADF/EDS images of mineralised ST3 cells. A & F are HAADF images, B-D and G-I are the EDS maps of the selected regions (green squares) in A and F, respectively. B & G are EDS maps of $^{56}$Fe, C & H are $^{75}$As and D & I are $^{16}$O. E and J are the As/Fe ratio maps (colour scale on the right, atomic %), where the average As/Fe ratio was 23.4 % in E and 6.7 % in J. Notice that Fe and O co-locate in both mapped regions. The scale bars are 100 nm for the images in the red squares in A and F.
Figure S7.6. Diffractogram of the bulk precipitates of *Acidovorax* sp. strain ST3. The black diffractogram is at day 7 (anaerobic analysis); the blue and red diffractograms are the sample at day 10, aerobic and anaerobic analysis, respectively. At day 10 lepidocrocite (L) was the main mineral phase detected.
Chapter 8: Conclusions and Future Work

The transformation of As and Fe by bacteria were at the centre of the research described in this thesis, and these were studied through novel techniques that provided spatially resolved chemical information from these systems. One of the main reasons to opt for SIMS techniques, and principally NanoSIMS, was the capability to image metabolically active cells. Genes associated with arsenic oxidation and reduction are ubiquitous in many microorganisms from As-impacted environments (Gnanaprakasam et al., 2017). However, carrying these genes does not confirm the expression of these enzymes and thus a direct responsibility in As oxido-reduction. NanoSIMS allowed us to fill this knowledge gap, where microbial activity (\(^{13}\)C uptake) was successfully linked to the observed metabolic function (via changes in As-Fe chemistry). In such conditions, Fe minerals are essential agents driving the mobilisation and immobilisation of As, where Fe minerals are also susceptible to oxidation or reduction by bacteria. Therefore, the mineralogy of both As and Fe are interconnected.

The applicability of NanoSIMS to spatially correlate Fe(III)-reducing bacteria and the mineral they were respiring was illustrated in Chapter 4. The results presented in this Chapter provided evidence for the contrasting extracellular electron transfer mechanisms used by \textit{Geobacter sulfurreducens} and \textit{Shewanella} sp. strain ANA-3. In this system As was analysed at the single-cell level by studying the bacterial mobilisation of sorbed As(V) on an Fe(III)-oxyhydroxide mineral. It was determined that the non As(V)-respirer \textit{G. sulfurreducens} mobilised As(V), and preferentially deposited this As(V) on the Fe(III) mineral where the active cells were located, in contrast to the As(V)-respirer \textit{S. ANA}-3, which did not require direct contact with the Fe(III) mineral, and mobilised As(III). This study set the groundwork to further investigate the As(V) reduction mechanism in \textit{S. ANA}-3, and it was found that As(V) is respired once it has been solubilised and not via direct reduction of sorbed arsenate, as illustrated in Chapter 5. Moreover, in this experiment As(III) remained in the aqueous phase, suggesting that the impact of As(V)
respiration is potentially more significant for As mobilisation than As(V) reduction for detoxification, as shown through the comparative results with the non As(V)-respirer, S. ANA-3 strain ARRA3. Future work in these systems should involve the study of environmental samples including mixed microbial communities with As(V) and/or Fe(III)-respiring bacteria. However, environmental samples can be challenging for the location of minerals and cells, additional to their complex and heterogeneous composition, which could complicate and require prolonged analysis in surface sensitive techniques, such as SIMS. Hybridisation approaches (FISH, CARD-FISH) have been used complementary to NanoSIMS to efficiently locate active microorganisms from environmental samples (Eickhorst and Tippkötter, 2008; Braun et al., 2018) and to infer metabolic traits, however these tools are limited for the study of the associated mineralogy. Alternative devices to collect environmental microbes were recently developed by one member of the Manchester Geomicrobiology group (Milton, 2019). This method involves the incubation of a porous material coated in Fe and As (“microbial bait”) in As-impacted sites that work as a matrix for the collection of As and Fe-respiring microbes from their native environments. This tool could improve the intrinsic drawbacks involved in the analysis of environmental samples, while maintaining representative environmental conditions. Samples obtained from these devices could be further tested and analysed with the multi-nanoscale approaches presented in this work for the assessment of the pertinent microbial metabolisms and mineralogy.

Furthermore, the capability of denitrifying bacteria to catalyse As(III) oxidation was assessed in Chapter 7, where Acidovorax sp. strain ST3 was incubated under nitrate-reducing conditions in the presence of Fe(II), and cells and biogenic minerals were analysed. This system promoted a removal of more than 0.5 mM As(III) in 7 days of incubation, an arsenite concentration much higher than found naturally in heavily As-impacted aquifers (Gnanaprakasam et al., 2017), although representative of the soil from where this bacterium was isolated. The complementary nanoscale techniques used (SEM, STEM and NanoSIMS) revealed encrustation of the majority of cells, while others remained metabolically active and free from mineralisation, indicating metabolic
heterogeneity. NanoSIMS helped to identify the active cells, leading to the inference that moderate cell growth was promoted by As(III) and Fe(II). However, some questions still remain regarding nitrate-dependent Fe(II) oxidation in strain ST3. For instance, what role does the cell growth stage play in the formation of harmful periplasmic encrustation in some cells and its absence in others? One way to address this would be by culturing strain ST3 in a similar medium composition but in a continuous-flow system, where cells can be kept at the same growth phase (Maier and Pepper, 2015). This setting could reduce metabolic heterogeneity and stochastic effects. Moreover, it is still not clear if Fe(II) oxidation to insoluble Fe(III) and the resulting cell encrustation is an Fe(II) detoxification mechanism, but proteomics could help identify the expression of stress-related proteins under these conditions and in the search of a dedicated ferroxidase in the bacteria studied in Chapter 7 and Appendix A. In addition, cell encrustation could be dependent on Fe(II) concentration, as shown in Acidovorax sp. strain 2AN, where no encrustation was observed when cells were incubated in a range of much lower Fe(II) concentrations (50-250 μM) (Chakraborty et al., 2011) than the concentration tested in the experiments in Chapter 7 and Appendix A (3.5-4 mM). In order to investigate this, Acidovorax sp. strain ST3 and Paracoccus sp. strain QY30 could be cultured in a range of Fe(II) concentrations below millimolar levels, and single cells could be further imaged at high resolution. Similarly, simultaneous incubations with varying concentrations of arsenite would be advisable in order to investigate a toxicity effect boosted in synergy with arsenite, as suggested by the Paracoccus sp. strain QY30 results, presented in Appendix A.

8.1.1 Subcellular spatial distribution of As and Fe

The notable capabilities of NanoSIMS to study single bacterial cells were exploited in this project as well, in order to analyse intracellular distribution of As and Fe. NanoSIMS had not been used to study bacteria respiring As and Fe, and it allowed the subcellular localisation of both elements in depth-profiled cells, providing information about the relative 3D distribution of As and Fe. In reducing conditions, As and Fe, presumably as As(III) and Fe(II), showed preferential localisation for the cell surface of S. ANA-3 WT,
whereas S. ANA-3 strain ARRA3 only showed interactions with soluble Fe, as this strain didn’t respire As(V), suggesting that S. ANA-3 strains only sorbed the soluble species, as shown in Chapter 5. The results were different under oxidising conditions. Strain ST3 showed encrustation of Fe(III) mineral at the periplasm and cell surface, and this Fe(III) mineral was predicted to sorb or even co-precipitate As. However, As co-localised with Fe in some cases, but in others these two elements were delocalised, as shown in the single-cell depth profiles in Chapter 7. This startling delocalisation exhibits the sensitivity and high spatial resolution of NanoSIMS, and also shows the close cell membrane-metal interaction, which is assumed for As, an element that easily binds to proteins, and Fe, an element that is present in metalloproteins (e.g. membrane-associated c-type cytochromes), in addition to electrostatic interactions, which had not been imaged at this level of detail before.

8.1.2 Arsenite remediation and global warming
Arsenite is the most threatening and persistent arsenic oxyanion polluting soils and aquifers worldwide. The oxidation of As(III) can be stimulated via addition of NO\textsuperscript{3-} in aquifers and soils, as seen in Chapter 7. NO\textsuperscript{3-} can easily be added to soils through fertilisers, which usually contain high amounts of urea or ammonium that can be transformed to nitrate by nitrifying bacteria. Nevertheless, nitric oxide (NO) and nitrous oxide (N\textsubscript{2}O) are intermediate gases produced during denitrification, where NO is a precursor of tropospheric ozone (a greenhouse gas) and N\textsubscript{2}O is a greenhouse gas with a global warming potential 298 times higher than CO\textsubscript{2} (Smith et al., 2018). N\textsubscript{2}O contributes an estimated 5.6 % of all greenhouse gas emissions from the U.S.A, and agriculture is the largest source of N\textsubscript{2}O emissions, contributing with 73.9 % of all anthropogenic N\textsubscript{2}O (EPA, 2019). Natural microbial soil processes contribute to the emission of NO and N\textsubscript{2}O, but it is clear that the stimulation of As(III) oxidation via addition of NO\textsuperscript{3-} could increase the production of these intermediate nitrogen species, thus, this remediation approach must be planned carefully in order to not aggravate another highly important matter (global warming and the climate crisis), while aiming to correct the initial problem. Moreover, the identification of the full denitrification enzymatic pathway in the bacteria studied in
Chapter 7 and Appendix A could complete the detection and quantification of the nitrogen species generated, allowing an appropriate nitrogen mass balance, including the intermediate greenhouse gases (where naturally, the anoxic experimental conditions will need to be kept using other gas than routine N\textsubscript{2}). Isotopically $^{15}$N-labelled NO\textsubscript{3} would facilitate the assessment of this mass balance in both the gas and liquid states.

Furthermore, heterotrophic bacteria, including the As and Fe reducers/oxidisers, contribute to the global CO\textsubscript{2} production, which so far has only been estimated in closed systems, where 8-14 % of the originally fixed CO\textsubscript{2} is oxidised back to CO\textsubscript{2} (Oremland et al., 2004), but this potential CO\textsubscript{2} input should also be accounted in the design of As bioremediation approaches.

### 8.1.3 Biogeochemical cycling of As and other pollutants

It is clear that the behaviour of As and Fe are interconnected through numerous biotic and abiotic processes in the geosphere, and these intersect with the behaviour of other elements and the minerals they form. Consequently, their biogeochemical cycles are mutually influenced along with that of organic matter cycling in the environment. Much attention has been given to As and its mobilisation in the environment due to the numerous sites worldwide with unsafe As levels. However, iron minerals are not only good sinks for As, but also for other pollutants or trace elements such as cobalt, copper, chromium, mercury, antimony, technetium, and the actinides uranium and plutonium, in addition to organic contaminants such as nitrobenzene and chlorinated solvents (Borch et al., 2010). Therefore, the biogeochemical cycling of Fe might well affect the solubility and redox transformations of these elements and pollutants, posing another set of plausible systems that could be carefully analysed at the nanoscale.

### 8.1.4 The future of Geomicrobiological SIMS

NanoSIMS was successful in analysing the distribution of species such as Fe and As within single bacterial cells. ToF-SIMS offers the possibility of detecting most elements and a wide range of molecules, although the instruments used here proved to have insufficient sensitivity to detect flavins at the level of single bacterial cells, as detailed in Chapter 6.
However, these and other SIMS instruments are under constant development, and this next decade will likely see many advances in both spatial resolution and sensitivity.

NanoSIMS is quickly becoming a key imaging tool in Environmental Microbiology and Ecology, and this technique could be applied for the study of pure cultures, mixed communities and even environmental samples in a wide range of Geomicrobiological conditions and systems. Even though every year more NanoSIMS instruments become available in many research laboratories worldwide, NanoSIMS analysis is still a costly technique where the time allocated in the instrument has to be optimised, and an adequate sample preparation is essential in that regard. NanoSIMS and other SIMS techniques could aid in the advancement of interdisciplinary fields such as Geomicrobiology, however this field would greatly benefit from alternative environmental sampling collection tools, as discussed previously, and from a combination of multiple techniques in order to assure an insightful analysis of these complex systems.

8.2 References


Appendix A - Imaging Fe Mineralisation Patterns in the Chemolithoautotroph As(III)-Oxidising Bacterium Paracoccus sp. strain QY30

A.1 Introduction

This appendix is a compilation of the explorative analysis performed on the nitrate-dependent Fe(II)-oxidising and As(III)-oxidising bacterium, Paracoccus sp. strain QY30, isolated from an As-polluted paddy soil (Zhang et al., 2015). Members of the Paracoccus genus are gram-negative facultative chemolithoautotrophic bacteria. Paracoccus species show polymorphism, where under anaerobic conditions cells usually grow as coccoids whereas in aerobic rich media cells grow as rods, in some cases forming chains (Kumaraswamy et al., 2006). The type species, Paracoccus denitrificans, is a well-known model organism studied for its cytochrome system, denitrification and electron transfer mechanisms (Kelly et al., 2006). Paracoccus strains are usually among the most abundant species identified in soils under denitrifying conditions and even after stimulating Fe(III) reduction, suggesting a close interaction between Fe and N cycling, promoted by these bacteria (Zhang et al., 2019).

Strain QY30 showed to be a nitrate-dependent Fe(II)-oxidising organism in incubations with NO$₃^-$ and an organic co-substrate (e.g. acetate or lactate). This bacterium putatively possesses the periplasmic arsenite oxidase AioA, along with the nitrate reductases enzymatic pathway (NAR, NIR, NOR and NOS), although its full enzymatic pathway has not been confirmed (Zhang et al., 2015).

Strain QY30 was isolated from a flooded soil containing 80 mg kg$^{-1}$ (80 ppm) of total As and 24.6 g kg$^{-1}$ of total Fe; in these conditions most of the Fe and As are expected to be in the reduced species, As(III) and Fe(II). Consequently, this bacterium tolerates high concentrations of arsenite (up to 5 mM) (Zhang et al., 2017), although the concomitant arsenite and ferrous iron in the medium could be detrimental for the cells in anoxic medium. The toxic effect of Fe(II) in synergy with Cu(II) has been observed in other bacteria (Bird et al., 2013).
Few *Paracoccus* strains have been imaged after growth in denitrifying conditions with Fe(II), including strains of the type species *P. denitrificans* (Kumaraswamy et al., 2006; Klueglein et al., 2014). These previous works showed a consistent mineralisation pattern on the periplasm and at the cell surface consisting on fibrils surrounding the cell. Additional to the original work that reported the isolation of strain QY30, there are no further studies with this bacterium, including the use of imaging tools, except for a few SEM images of the cells identifying its coccoid shape (Zhang et al., 2015). The aim of the experiment presented in this appendix was to study the simultaneous Fe(II) and As(III) oxidation by strain QY30 under denitrifying conditions, in order to understand its mineralisation patterns, the mineralisation effect on metabolism (if any) and ultimately assess the biogenic minerals produced as hosts for As removal. *Paracoccus* members have diverse metabolisms, they can grow as autotrophs or chemolithotrophs using a variety of terminal electron acceptors (Kelly et al., 2006), and for this reason the mineralisation patterns and products are expected to be different from other model nitrate-dependent Fe(II) oxidisers. In these systems it is recognised that the medium composition (carbon source, pH, CO₃, etc.) will affect the denitrification end products (Blaszczyk, 1993) and the minerals produced (Larese-Casanova et al., 2010). In order to test this, *Paracoccus* sp. strain QY30 was grown under mixotrophic culture conditions, with NO₃⁻, As(III), Fe(II) and ¹³C-labelled acetate. Exploratory NanoSIMS, TEM and XRD data were collected in these samples as detailed below.

**A.2 Methodology**

**A.2.1 Microbial culture**

*Paracoccus* sp. strain QY30 was kindly donated by Prof Fanjie Zhao (Nanjing Agricultural University, China). The bacteria was re-inoculated on aerobic plates of R2A medium to reactivate the cells, and incubated at 30°C. Single colonies were suspended in flasks containing the same liquid medium R2A, in order to obtain a liquid inoculum for the experiments. The flasks were incubated in a shaker incubator. 5 mL of this culture were transferred to 100 mL serum bottles containing minimal salts medium (MSM) with nitrate 10 mM and arsenite 0.5 mM (composition available in table 3.5 in Chapter 3).
These bottles were incubated for 48 h until a light pink colour appeared. After this incubation period the cells were harvested by washing with PIPES buffer, as detailed in Chapter 3.

**A.2.2 As(III)-Fe(II) oxidation experiments**

For the simultaneous As(III) and Fe(II)-oxidising experiments QY30 cells were grown in planktonic conditions in serum bottles containing MSM amended with 5 mM NO₃, 0.5 mM As(III), 3.5 mM Fe(II) and 10 mM ¹³C-labelled acetate. Samples were withdrawn after 7, 14 and 21 days of incubation. The precipitates were only washed with dH₂O and pipetted on Si wafers to be analysed in NanoSIMS, in TEM grids for TEM analysis and on glass slides for XRD.

**A.2.3 Aqueous phase analysis**

The aqueous phase was monitored for the analysis of Fe(II), As(III) and cell growth (by spectrophotometry) as detailed in Chapter 3.

**A.2.4 Solid phase analysis**

The solid phase analysis consisted in imaging the planktonic cells in NanoSIMS and Transmission electron microscopy (TEM), where energy-dispersive X-ray spectroscopy (EDS) maps were also collected. Additionally, bulk sample XRD spectra were obtained to identify the biominerals after 7, 14 and 21 days of incubation.

**A.2.4.1 NanoSIMS**

The samples were analysed in a NanoSIMS 50L ion microprobe (CAMECA, France) using Cs⁺ as the primary beam at 16 KeV to collect the following negative secondary ions: ¹²C⁻, ¹³C⁻, ¹²C¹⁴N⁻, ²⁸Si, ⁵⁶Fe¹²C⁻, ⁵⁶Fe¹⁶O⁻ and ⁷⁵As⁻. The D1 apertures used for imaging ranged from D1=2-4, for all fields of view (10-40 µm). No depth profiles were performed on these cells. The CAMECA mass resolving power (MRP) was 5500 for detector 2 (mass 13) and 7000 for detector 7 (mass 75), in order to resolve the isobaric interferences, as detailed in Chapter 3. The slits ES and AS were used at ES=3 and AS=2 to improve mass resolution. Images were collected at a dwell time of 5000 µs px⁻¹ and pixel size was 256 x 256. The ¹³C/¹²C ratio was used to identify active cells by their ¹³C accumulation, above
the 1.11 % natural abundance, as discussed in previous chapters. The images were processed using L’IMAGE software (Larry Nittler, Carnegie Institution of Washington) and the plugin OpenMIMS for ImageJ (MIMS, Harvard University; www.nrims.harvard.edu).

A.2.4.2 Transmission electron microscopy (TEM)

TEM analysis was performed using a FEI Tecnai F30 at an acceleration voltage of 200 kV. Bright-field (BF) images and EDS maps were collected in cells and biominerals. The results were processed in INCA Energy software (Oxford instruments®). The anoxic conditions in these samples were only kept until the sample preparation step, and the transportation and loading to the TEM instrument exposed the samples to oxygen.

A.2.4.3 Powder X-ray diffraction (XRD)

Powder X-ray diffraction was performed in bulk samples using a Bruker D8 advance X-ray diffractometer with a 40 kV beam. Analyses were run at a step size of 0.02 theta and dwell time of 1 second, due to the low crystallinity of the samples.

A.3 Results and discussion

A.3.1 Growth of strain QY30

The biomass generation by this strain is low, and after 48 h of culture, the biomass showed an $A_{600}=0.2$, when other bacterial strains typically show much higher absorbances ($A_{600}>1.0$), even in anaerobic culture. This suggests that, even though thermodynamically favourable, denitrification coupled to As(III) oxidation is not a high energy yielding process for this microorganism. Strain QY30 also formed small colonies after 48 h of culture in aerobic R2A agar plates, implying that overall this strain has slow growth rates.

A.3.2 Aqueous Fe and As in oxidation experiments

The aqueous phase results of the experiments of QY30 cells in planktonic growth to obtain NanoSIMS and TEM samples showed an Fe(II) oxidation of 40 % at day 21 (Fig. A1) and an abiotic Fe(II) oxidation of 10 % at that same time point. The samples with QY30 cells oxidised 56 % of As(III) by day 21 of incubation, whereas the abiotic samples showed
10% of As(III) removal by day 21 (Fig. A2). The first 7 days of the experiment showed the highest Fe(II) and As(III) oxidation, and after this period the oxidation rate remained semi-stable in both cases (see the experiment bottles with the precipitates in Fig. A3). This implies that shorter incubation periods are sufficient to remove up to 0.28 mM As(III) in the conditions assessed. The concentration tested [equal to 21,000 ppb As(III)], is much higher than is usually found in aquifers polluted with As, for instance in West Bengal (up to 2500 ppb) (Gnanapprakasam et al., 2017), and for this reason this high removal/oxidation of As(III) is encouraging.

Figure A1. Fe(II) oxidation by planktonic strain QY30 cells with 0.5 mM As(III). At 21 days of culture 40% of Fe(II) was oxidised by cells and 10% of Fe(II) was oxidised abiotically.

Figure A2. As(II) oxidation by planktonic Paracoccus sp. QY30 cells with 0.5 mM As(III). At 21 days of culture 56% of As(III) was oxidised by cells.
A.3.3 NanoSIMS

The simultaneous Fe(II) and As(III) oxidation experiments with strain QY30 were initially tested under a high concentration of As(III) (0.5 mM). The cells were imaged after 7, 14 and 21 days of incubation and in these conditions the cells did not accumulate $^{13}$C above 1.11 %, implying that the cells were not metabolically active (Fig. A4). Moreover, the cells presented a mixture of rod and coccoid shapes, and budding and deformities were also observed, indicating cell stress (see orange arrows in Fig. A4). For these reasons, new samples were prepared with a lower As(III) concentration (0.02 mM). The results were

Figure A3. Experiment bottles with MSM medium ($\text{NO}_3$, As(III), Fe(II) & $^{13}$C-acetate) and QY30 cells at 7 and 21 days of incubation.

Figure A4. NanoSIMS images of strain QY30 at day 7 of incubation grown with 0.5 mM As(III). The first panel is the secondary electron image to observe the sample surface, the second panel are overlays of $^{13}$C (green), $^{56}$Fe$^{16}$O (red) and $^{75}$As (blue), whereas the third panel are the $^{13}$C/$^{12}$C ratio images. Notice that cells did not accumulate $^{13}$C, implying they didn’t metabolise the $^{13}$C-labelled acetate. These cells presented rod and irregular cell shapes with sizes between 1.0-2.0 µm (mustard arrows).
strikingly different, as these cells were metabolically active, accumulating up to 20 times the natural $^{13}\text{C}/^{12}\text{C}$ ratio (Fig. A5). In the lower arsenite conditions the cell shapes were coccoids of $\approx 0.5 \mu\text{m}$, suggesting that the higher arsenite concentration was toxic for the cells. Even though strain QY30 proved to tolerate high arsenite concentrations, up to 5 mM, these initial growth tests did not contain ferrous iron, which could contribute to the overall system toxicity, as noted in other bacterial strains (Bird et al., 2013).

Figure A5. NanoSIMS images of strain QY30 at day 7 of incubation grown with 0.02 mM As(III). The first panel is the secondary electron image to observe the sample surface, the second panel are overlays of $^{13}\text{C}$ (green), $^{56}\text{Fe}^{16}\text{O}$ (red) and $^{75}\text{As}$ (blue), whereas the third panel are the $^{13}\text{C}/^{12}\text{C}$ ratio images. Notice that these cells accumulated up to 20 times the $^{13}\text{C}$ natural abundance and the cells kept their typical coccoid shape and size of 0.5-1.0 µm.
Regarding the biominerals, a close spatial association between minerals and bacteria was observed in both kinds of samples (regardless of the As(III) concentration). The majority of the cells were found in clusters of mineral and cells, in rare cases the cells were found isolated on the Si surface. The biominerals showed a good correlation of $^{75}$As$^{-}$ and $^{56}$Fe$^{16}$O$^{−}$. Moreover, the cells showed low $^{75}$As and/or $^{56}$Fe$^{16}$O counts on the cell surface, suggesting that the cells were not encrusted or mineralised, and the biomineralisation occurred extracellularly.

A.3.4 TEM-EDS

The cells and associated biominerals were imaged in TEM and EDS maps were also collected for the samples grown at 0.5 mM As(III) after 14 and 21 days of incubation. These cells grew as polymorphs with coccoid and rod shapes, with budding and deformities, also observed in NanoSIMS. These cells were heavily coated with nanoparticles on the cell surface, but these biominerals were also imaged at a distance from the cells (Fig. A6). The nanoparticles maintained their nanoparticle size (<100 nm) until day 21 of incubation at the cell surface (blue arrows in Fig. A6-E). Interestingly, the biominerals seemed to aggregate when they were at a distance from the cells (Fig. A6-C).

These TEM images showed the encrusted periplasm, similar to the observations in Acidovorax sp. strain ST3 (Chapter 7), although this contrasts with the observations in NanoSIMS of the QY30 samples, which showed very low $^{75}$As and $^{56}$Fe$^{16}$O counts on the surface of the cells. However, no TEM images were collected at day 7 of incubation, when cells could have presented lower levels of mineralisation, explaining the differences observed in NanoSIMS. Thus, it is unlikely that these cultures presented metabolic heterogeneity, in contrast to strain ST3, where the encrusted cells were not metabolically active. The NanoSIMS results in strain QY30 show non-mineralised metabolically active cells (consistently accumulating 20 % of $^{13}$C/$^{12}$C) and non-mineralised inactive cells. It appears that regardless of the mineralisation in cells, the limiting growth factor was arsenite and not ferrous iron. In this mixotrophic system arsenite could contribute as an electron donor coupled to NO$_3^−$ reduction, and promote
indirect Fe(II) oxidation (for instance via NO$_2^-$). However, high concentrations of arsenite affected growth and possibly stressed the cells as shown by the budding and deformed
cells, best observed in TEM (Fig. A6).

Unfortunately, during the EDS mapping Pt was mistakenly identified as the most abundant element in the samples (Fig. A6.G-J). This Pt likely originated from a slit in the TEM instrument, as no Pt was present in the samples. Fe and As were also identified, but these results are not reliable due to this technical issue.

**A.3.5 XRD**

The bulk biominerals were analysed by X-ray diffraction. At day 7 of incubation an amorphous and unidentified phase was obtained, but at days 14 and 21 of incubation the main mineral phases identified were lepidocrocite ($\gamma$-Fe$^{3+}$OOH), goethite ($\alpha$-...
Fe$_{3+}$OOH) and possibly parasymplesite (Fe$_{2+3}$As$_4$O$_{12}$·8H$_2$O) (Fig. A7). Moreover, this mineral remained practically unaltered from days 14 to 21 of incubation. Contrary to what was expected, no hematite or magnetite were detected, as these crystalline Fe(III) minerals could develop under longer incubation periods (Sun et al., 2016), but this only shows that the conditions and length of the experiment were not suitable for the formation of those mineral phases.

**A.4 Conclusions and future work**

These preliminary study of *Paracoccus* sp. strain QY30 showed that an anaerobic mixotrophic culture containing Fe(II), NO$_3^-$, As(III) and acetate as co-substrate was toxic at high concentrations of As(III), contrary to the expectations, given that ferrous iron was thought to affect the cells’ metabolism through cell mineralisation/encrustation. However, NanoSIMS and TEM was a complementary approach to initially image the mechanism of mineralisation in this bacterial strain, which presented mineral encrustation in the periplasm and extracellularly at the vicinity of cells, when grown under high arsenite concentrations. The experiment comprising 21 days of incubation was not adequate to produce a thermodynamically stable mineral phase, which could sequester As in the long term. Nevertheless, the first 7 days of incubation were the most active days for the cells, and during this period the highest oxidation rates of As(III) and Fe(II) took place. At this time point, an amorphous mineral was produced, which could potentially sorb high concentrations of both As(III) and As(V). More studies are needed to quantitatively assess the As sequestration and overall composition of these biogenic minerals at all the time points in order to produce an adequate appraisal of this system. High resolution approaches such as S/TEM and NanoSIMS, particularly single-cell depth profiles, are recommended, which could reveal a detailed distribution of As and Fe in QY30 cells. Moreover, experiments using the strain QY30 could benefit by testing cell growth with other favourable organic co-substrates such as succinic acid and ethanol, although these organic compounds are not as accessible as acetate or lactate for $^{13}$C-labelling.
Furthermore, this strain offers a wide possibility of future research pathways, due to its potential metabolic versatility. QY30 could be used to study denitrification in depth and assess its contribution to N cycling in soils, although the end products of denitrification need to be evaluated in this Paracoccus strain along with the identification of the full enzymatic denitrification pathway.

A.5 References


