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INTERACTION BETWEEN MICROBES, IRON AND CHLORINE FOR THE DEVELOPMENT OF BIOTECHNOLOGICAL APPROACHES TO STABILIZE CORRODED IRON

Lucrezia Comensoli

A dissertation submitted to the University of Neuchâtel

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UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

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Madame Lucrezia COMENSOLI

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sur le rapport des membres du jury composé comme suit:

- Prof. Pilar Junier, co-directrice de thèse, UniNE
- Prof. ass. Edith Joseph, co-directrice de thèse, UniNE
- Prof. Geoff M. Gadd, University of Dundee, UK
- Dr Julien Maillard, EPF Lausanne, Suisse
- Dr Mauro Tonolla, SUPSI, Bellinzone, Suisse

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Le Doyen, Prof. R. Bshary

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Presented by Lucrezia Comensoli, MSc Biogeosciences

Manuscript for the Jury:

Prof. Dr. Pilar Junier, thesis co-director, University of Neuchâtel

Prof. Dr. Edith Joseph, thesis co-director, University of Neuchâtel

Prof. Dr. Geoffrey M. Gadd, University of Dundee, United Kingdom

Dr. Julien Maillard, EPFL, Lausanne

Dr. Mauro Tonolla, SUPSI, Manno

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“You can’t make everyone happy. You’re not a Nutella Jar”

Anonymous

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Summary

Iron objects suffer inexorable oxidation and without any human intervention they would be completely damaged. This phenomenon occurs for iron surfaces, outdoor exposed structures as well as for archaeological iron objects. Several methods are currently available for the stabilisation of this type of metallic substrate, however, none of them is completely efficient, and several relay on the use of hazardous compounds. In addition, especially for outdoor iron and pipelines structure a permanent protective treatment does not exist. After few years these corroded surfaces have to be re-treated and in some cases replaced. This causes substantial maintenance costs having an important economic impact on our society. Regarding archaeological iron objects, an additional issue has to be considered. In fact, each object consists of a unique testimony of our past that should be preserved and studied. An archaeological object is usually unique and if the conservation interventions fail, all the information that the object could have revealed will be lost. Scientists agree with the fact that until now an efficient and durable stabilisation treatment for corroded iron does not exist. As a consequence, there is a pressing need to investigate new approaches. To this purpose, the present thesis investigated the potential of microorganisms (bacteria and fungi) for the development of stabilisation methods for corroded iron. Since one of the main issues for this metal is chlorine, this study examined two different strategies of chlorine removal and conversion of the unstable iron compounds into more stable biogenic minerals.

The first approach was an indirect chlorine extraction, consisting on the microbial removal of iron ions present in chlorinated corrosion compounds. For this purpose, microbial biogenic minerals production and fungal iron adsorption were investigated. In particular, exploiting biogenic minerals production of the strains TCE1 and LBE of the anaerobic bacterium *Desulfitobacterium hafniense*, it was possible to convert a part of the corrosion layer of corroded iron coupons, as well as of archaeological iron nails, into biogenic vivianite and magnetite. In addition, this study allowed definitely to assess that fungi are not the best candidates to develop stabilisation methods for corroded iron based on biogenic minerals production. In fact, even though *Beauveria bassiana* produced some biogenic crystals their amount was not sufficient for a precise characterisation, and none of the factors tested stimulated a higher production. Nevertheless, interesting results were obtained for fungal iron uptake. Indeed, iron uptake of the fungus *Alternaria* sp. was successfully used for a biocleaning of corroded iron coupons. In addition, another biotechnological application

exploiting fungal iron uptake was investigated. In this study the ability of bacteria to use iron chelated in fungal dead biomass as a bioavailable source of iron was proved for *Pseudomonas fluorescens*. This could then be exploited to improve iron bioavailability, as well as availability of organic carbon in soil for other microbes and maybe also plants.

A second approach regarding a direct method for the removal of chlorine was also studied. Uptake of potassium and chlorine was proved for *B. bassiana* that produced aggregates containing these elements onto its biomass when exposed to $FeCl_2$. However this ability could not be further exploited, as chlorine uptake was not the main resistance mechanism used by this fungus against chlorine, and an efficient uptake of this ion was not measured. Finally, aiming to remove chlorine from corroded iron, volatiles organic compounds production was studied. Preliminary results showed that NaCl stimulates the production of particular compounds not present in absence of this substance. Overall it can be affirmed that this study allow to assess that microorganisms are a valuable alternative for the stabilisation of corroded iron. Bacteria could be employed to stabilize the corrosion layer by producing stable biogenic minerals, while fungi could be used for biocleaning of corroded iron.

Keywords: Iron, Corrosion, Stabilisation, Biotechnology, Microorganisms, Biogenic minerals, adsorption.

Résumé

Les objets en fer subissent une inexorable oxydation, qui sans intervention de conservation-restauration cause une complète perte de l'objet en question. Ce phénomène concerne les superficies en fer, plusieurs infrastructures ainsi que les objets archéologiques. Nombreux méthodes sont disponibles pour la stabilisation de ces types de surfaces métalliques, toutefois, aucun n'est complétement efficace, et plusieurs ils emploient des substances toxiques. De plus, pour les surfaces exposées et les plomberies il n'existe pas un traitement définitif. Après seulement quelques années, ce genre d'infrastructures doit être retraité ou carrément remplacé. Cela cause des coûts de maintenance très élevés ainsi qu'un grand impact économique dans notre société. Concernant les objets archéologiques, il existe un problème supplémentaire. En effet, chaque objet constitue un témoignage unique du notre passé qui doit être protégé et étudié. Un objet archéologique est souvent unique et, si la restauration devait échouer, toutes les informations que l'objet pourrait révéler seraient perdues. Les scientifiques sont de l'avis qu'actuellement il n'existe pas un traitement efficace pour stabiliser le fer corrodé. Par conséquent, il y a une forte nécessité d'étudier et développer des nouvelles méthodes. Dans ce but, dans cette thèse, le potentiel des microorganismes (bactéries et champignons) pour le développement de nouvelles méthodes qui stabilisent le fer corrodé a été étudié. Étant donné qu'un des problèmes majeurs pour ce métal est le chlore, dans cette étude, l'efficacité de deux stratégies pour enlever cet élément depuis les objets et convertir les composées de corrosion instable en minéraux biogénique protecteurs, ont été évaluées.

La première approche, a été l'extraction indirecte du chlore en exploitant la capacité des microbes d'enlever les ions de fer présents dans les composées de corrosion contentant du chlore. Dans ce but, deux phénomènes ont été étudiés : la production microbiologique de minéraux biogénique et la capacité des champignons d'adsorber du fer dans leur biomasse. Plus précisément, en exploitant la production de minéraux biogéniques des souches TCE1 and LBE de la bactérie anaérobique *Desulfitobacterium hafniense*, il a été possible de convertir une partie de la couche de corrosion de plaques de fer et de clous archéologiques, en vivianite et magnétite. De plus, cette étude a aussi permis de conclure que les champignons ne sont pas les candidats les plus adéquats, pour le développement de méthodes de stabilisation du fer corrodé basés sur la production de cristaux biogéniques. En effet, en dépit du fait que *Beauveria bassiana* ait produit quelques cristaux, leurs quantité n'a pas été suffisante pour une précise caractérisation, et aucuns des facteurs testés a stimulé une production majeure.

Toutefois, pour ce qui concerne l'adsorption de fer par les champignons, des résultats intéressants ont été obtenus. En effet, la capacité du champignon *Alternaria* sp. d'adsorber le fer a été utilisé avec succès pour le nettoyage de plaques corrodées. De plus, une application biotechnologique supplémentaire, qui exploite l'adsorption de fer par les champignons, a été étudiée. Dans cette étude, la capacité des bactéries d'utiliser le fer chélaté dans la biomasse fongique comme source de fer biodisponible a été prouvée avec la bactérie *Pseudmonas fluorescens*. Ce mécanisme, pourrait être utilisé pour améliorer la biodisponibilité de fer et la présence de carbone organique dans le sol pour les autres microorganismes, et peut-être aussi pour les plantes.

Ensuite, une deuxième approche concernant une méthode directe pour enlever le chlore a été étudiée. L'absorption de potassium et chlore a été prouvée pour *B. bassiana*, qui a produit des agrégats composés de ces deux éléments dans sa biomasse, quand exposé au $FeCl_2$. Toutefois, cette capacité n'a pas pu être exploitée, car l'absorption de chlore n'était pas le mécanisme principal utilisé par ce champignon pour contraster la toxicité du chlore. En effet, il n'a pas été possible de mesurer une absorption suffisante de cet ion. Pour finir, la production de composés organiques volatiles a été explorée dans le but d'enlever le chlore du fer corrodé. Résultats préliminaires ont démontrés que le chlorure de sodium stimule la production de composés spécifiques, pas détectés en absence de cette substance. En général, on peut affirmer que cette étude a permis de montrer que les microorganismes sont une valable alternative pour la stabilisation du fer corrodé. Les bactéries peuvent être utilisées pour stabiliser la couche de corrosion en produisant des minéraux biogéniques stables, tandis que les champignons peuvent être employés pour le nettoyage du fer corrodé.

Mots clés : Fer, Corrosion, Stabilisation, Biotechnologie, Microorganismes, minéraux biogéniques, adsorption.

Riassunto

Gli oggetti in ferro sono soggetti ad un'inesorabile ossidazione e senza alcun intervento di restauro sarebbero completamente danneggiati. Questo fenomeno concerne superfici in ferro, strutture esposte all'esterno e anche oggetti archeologici. Svariati metodi sono disponibili per stabilizzare questo tipo di superfici metalliche, tuttavia, nessuna di questi è completamente efficiente, e numerosi necessitano l'uso di sostanze dannose per la salute. Inoltre, specialmente per le superfici esposte e le tubature in ferro, non esiste un trattamento protettivo definitivo. Dopo solamente qualche anno, questo tipo d'infrastrutture devono essere ritrattate o addirittura sostituite. Questo causa elevati costi di manutenzione e un grande impatto economico nella nostra società. Riguardo gli oggetti archeologici, esiste un problema aggiuntivo. Infatti, ogni oggetto rappresenta una testimonianza unica del nostro passato che dovrebbe venir protetta e studiata. Un oggetto archeologico è solitamente unico e, nel caso in cui il suo restauro dovesse fallire, tutte le informazioni racchiuse nel oggetto sarebbero perse. Gli scienziati concordano col fatto che attualmente non esiste un trattamento efficiente per stabilizzare il ferro corroso. Di conseguenza, esiste un crescente bisogno di studiare e sviluppare nouvi metodi. A questo scopo, nella presente tesi è stato studiato il potenziale dei microorganismi (batteri e funghi) per lo sviluppo di nuovi metodi che stabilizzino il ferro corroso. Siccome uno dei maggiori problemi per questo metallo è il cloro, nel presente studio sono state esaminate due strategie differenti per rimuovere questo elemento dagli oggetti e convertire i composti di corrosione instabili in minerali biogenici protettivi.

Il primo approccio è stato l'estrazione indiretta del cloro sfruttando la capacità dei microbi di rimuovere gli ioni di ferro presenti nei composti di corrosione contenenti cloro. A questo scopo, sono state studiate la produzione microbica di minerali biogenici e l'abilità dei fungi di adsorbire il ferro nella loro biomassa. In particolare, sfruttando la produzione di minerali biogenici dei ceppi TCE1 and LBE del battere anaerobico *Desulfotobacterium hafniense,* è stato possibile convertire una parte dello strato di corrosione sviluppatasi su placche di ferro e chiodi archeologici, in vivinite e magnetite. Questo studio ha inoltre permesso di concludere che i funghi non sono i migliori candidati per lo sviluppo di metodi di stabilizzazione del ferro corroso basati sulla produzione di cristalli biogenici. Infatti, nonostante *Beauveria bassiana* abbia prodotto qualche cristallo, la loro quantità non è stata sufficiente per una precisa identificazione, e nessuno dei fattori testati ne ha stimulato una maggiore produzione. Tuttavia, dei risultati interessanti, sono stati ottenuti con l'adsorbimento di ferro nei fungi.

Infatti, l'abilità del fungo *Alternaria* sp. di adsorbire il ferro è stata usata con successo per la pulitura di placche corrose. Inoltre, è stata studiata un'ulteriore applicazione biotecnologica che sfrutta l'adsorbimento fungico di ferro. In questo studio, è stata verificata la capacità dei batteri di usare il ferro chelato nella biomassa morta dei fungi come fonte di ferro biodisponibile con il battere *Peudomonas fluorescens.* Questo meccanismo potrebbe venir sfruttato in futuro per migliorare la biodisponibilità del ferro e la presenza di carbonio organico nel suolo per altri microorganismi e magari anche per le piante.

Inseguito, un secondo approccio concernente un metodo diretto per la rimozione del cloro è stato studiato. L'assorbimento di potassio e cloro è stato dimostrato per *B. Bassiana*, che ha prodotto aggregati composti di questi elementi nella sua biomassa quando esposto al $FeCl_2$. Tuttavia quest'abilità non ha potuto essere sfruttata inquanto l'assorbimento di cloro non è stato il principale meccanismo di resistenza usato da questo fungo per contrastare la tossicità del cloro. Infatti, non è stato possibile misurare un assorbimento considerevole di questo ione. In fine, la produzione di composti organici volatili è stata studiata allo scopo di rimuovere il cloro dal ferro corroso. Risultati preliminari hanno mostrato che il cloruro di sodio stimola la produzione di specifici composti, non presenti in assenza di questa sostanza. Complessivamente, si può affermare che questo studio ha permesso di dimostrare che i microorganismi costituiscono una valida alternativa per la stabilizzazione del ferro corroso. I batteri possono venir utilizzati per stabilizzare lo strato di corrosione producendo stabili minerali biogenici, mentre i funghi possono venir impiegati per la pulitura del ferro corroso.

Parole chiave: Ferro, Corrosione, Stabilizzazione, Biotecnologia, Microorganismi, Minerali biogenici, adsorbimento.

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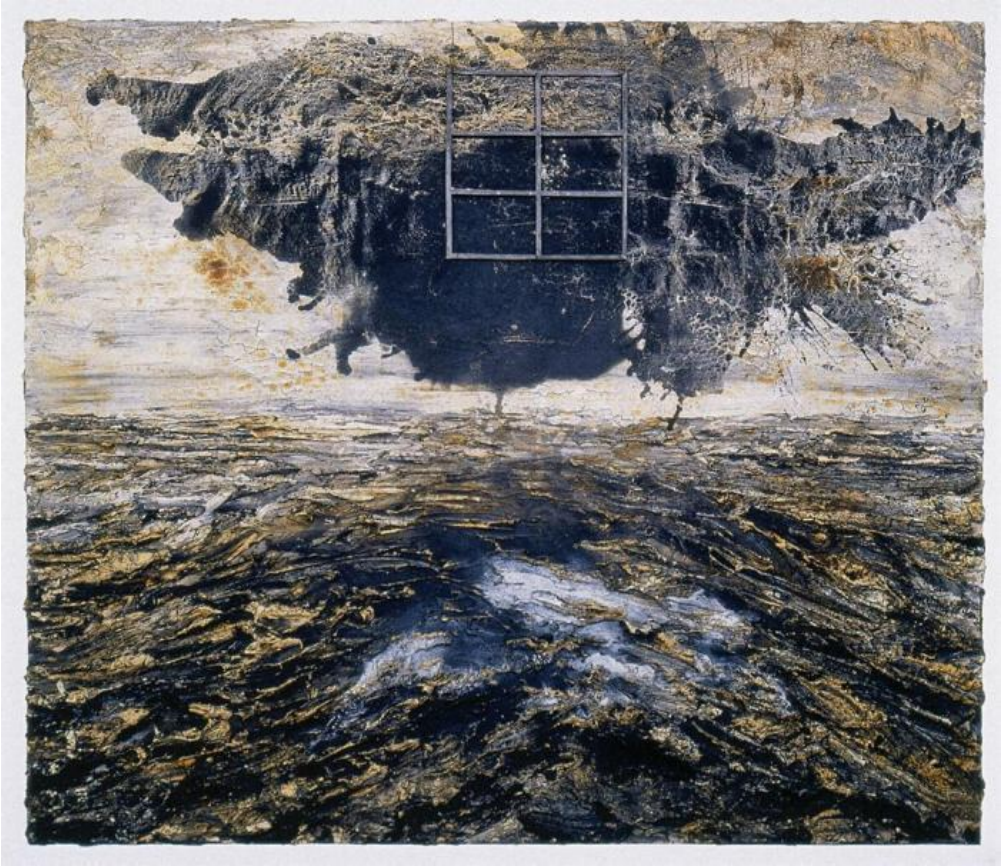
CHAPTER 1

General introduction



Anselm Kiefer – Anschenblume – 2004

1.1. Microbes, art, and the art of survival

Science and art are more closely related than it can be imagined at first glance. It appears clear when we think of Leonardo da Vinci, or of all these wonderful hand illustrations of plants, insects and animals made by scientists in the past. In addition, some researchers suggest also that science and art have the same goal: "investigate and understand nature and (as a part of it) ourselves" (Nai and Meyer 2016). Several definitions of art have been formulated from researchers coming from different fields. The philosopher Friedrich Engel, described art as a result of the attempt to conciliate nature and the infinite freedom of thinking (Knox 1976). The artist Vincent Van Gogh defined art as the result of the interpretation of the meaning of the human presence on Earth (Jansen, Luijten et al. 2009). While the psychoanalyst Mélanie Klein suggests that art is the result of an impulse of artists to rescue their own internal world (Klein 1929). I suppose that everyone has an opinion about that, but personally I share the last definition.

In fact, anger, sadness, happiness, passion, and peace are some of the emotions that the observation of a painting or a statue can elicit. Irrepressible emotions that the artist discloses in order to survive, creating what we commonly call an artwork. We could then assume that artworks are the results of a resistance mechanism that allow the purification of the artist soul. But, what about microbial resistance mechanism and the different metabolic strategies developed by microbes in order to survive? Following the previous definition, the conclusion would be that the results of these microbial activities could also be considered as an artwork. Personally, I believe this to be true. When I look to the biogenic crystals of fungi produced in order to purify their growth environment from toxic heavy metals (Figure 1 a); or when I observe an intensely colored microbial mat in the middle of a volcano (Figure 1 b); I can only agree with the fact that, yes, microbes definitely ought to be considered as artists, able to produce magnificent artworks. For this reason, I like to call the microbial ability to deal with harsh conditions the art of survival, and in this study we will use the microbial art of survival to make iron artworks survive.

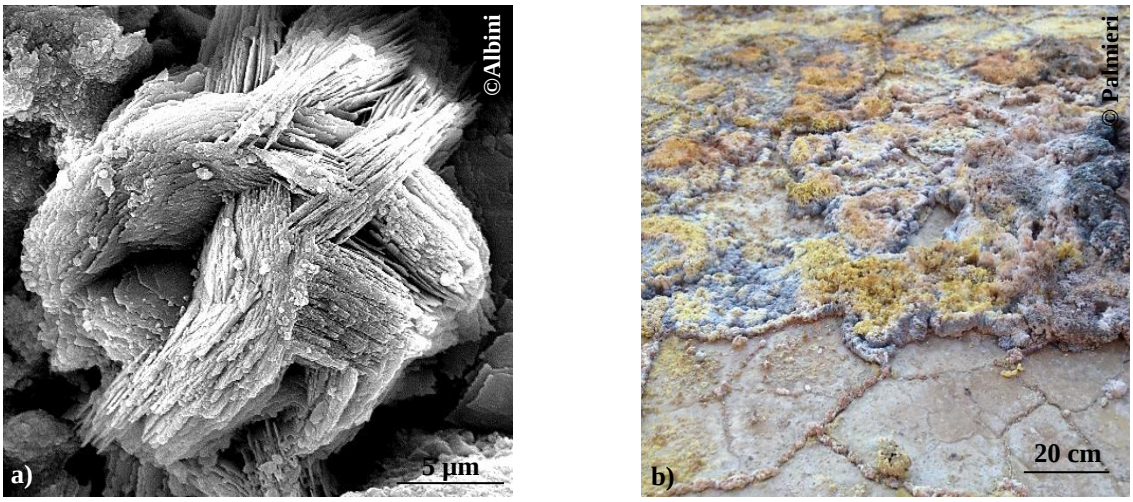


Figure 1: a) SEM image of a biogenic crystal of copper oxalate produced by *Beauveria bassiana* on copper amended medium, b) image of a microbial mat discovered in the caldera of the volcano Alexandros on the island of Nisyros (Greece).

1.2. Iron corrosion

The interaction between iron objects and their surrounding environment leads to a change in the oxidation state of the metal substrate. This change is due to the reaction of the metallic surface with oxygen and moisture causing its oxidation (anodic reaction) [eq. 1]. Free electrons resulting from this oxidation will reduce atmospheric oxygen producing OH^- (cathodic reaction) [eq. 2].

$$Fe(s) \rightarrow Fe^{2+} + 2e^- \qquad [1]$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \qquad [2]$$

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad [3]$$

Reduced iron (Fe^{2+}) and OH^- react to produce $Fe(OH)_2$. These molecules are not stable and are easily oxidized to for example hematite (Fe_2O_3), magnetite (Fe_3O_4), wüstite (FeO), lepidocrocite (γ-FeO(OH)), and goethite (α-FeO(OH)), which are the main components of a corrosion layer. In addition, free H^+ and electrons reacting with O_2 produce water droplets, which will instigate further corrosion process. This reaction can transform completely an iron object in rust, in fact some archaeological iron objects are

entirely mineralised and composed by corrosion products, and they do not contain any metal core (Scott and Eggert 2009).

The stability of a corrosion layer depends on its composition, and its composition is highly influenced by the surrounding environment. For this reason three types of corrosion regarding iron are defined depending on the exposure environment: buried archaeological iron (soil), submerged objects (water), and iron surface exposed to atmosphere (Table 1).

Table 1: Summary of the different environments causing corrosion, as well as, the principal objects found, the main issues concerning objects found in these environments, and the possible stabilisation treatments.

	Soil	**Water**		**Atmosphere**			
Sous-environment		**Fresh**	**Marine**	**Rural**	**Urban**	**Industrial**	**Costal**
	Archaeological buried objects	Archaeological submerged objects		Outdoor sculptures and infrastructures			
Objects							
Main issues	- Chlorine contamination - Instability due to the change in the external conditions after excavation	- Chlorine contamination - Incrustation of calcium carbonates due to massive colonisation of marine organisms		- Chlorine contamination - Sulphate contamination			
Stabilisation treatments	- Mechanical and chemical cleaning - Electrolytic reduction - Gas plasma reduction - Aqueous reduction	- Mechanical and chemical cleaning - Electrolytic reduction - Gas plasma reduction - Aqueous reduction		- Inorganic coating - Organic coating - Corrosion inhibitors			

Regarding buried archaeological iron, it has to be mentioned that since the corrosion layer was produced in soil, the excavation process changes dramatically the environmental conditions near the object. Therefore after the excavation the corrosion layer is no longer stable. In addition, for this type of objects another important issue is the presence of chlorine on the corrosion layer. In fact, when present in the burial site, this anion easily penetrates the corrosion layer reaching the interface between the

corrosion compounds and the metal core. (Scott and Eggert 2009). Its reaction with oxygen, water and iron leads to the formation of an acidic $FeCl_2$ solution (Selwyn 2004). This latter, trough oxidation and hydrolysis, creates iron oxyhydroxides and hydrochloric acid (eqs. 4 and 5). Both of these compounds are detrimental for the object. Indeed, iron oxyhydroxides are three times more voluminous than elemental iron and produce a mechanical pressure that lead to the formation of severe cracks and ruptures. Among iron oxyhydroxides, the formation of akaganeite [$FeO_{0.833}(OH)_{1.167}Cl_{0.167}$] (Turgoose 1982, Turgoose 1985, Ståhl, Nielsen et al. 2003) is particularly detrimental. This mineral is considered an unstable corrosion compound as it can release chloride ions in the presence of humidity and instigate further corrosion (Ståhl, Nielsen et al. 2003, Selwyn 2004).While, hydrochloric acid enhances the corrosion of the object by decreasing the pH and reactivating the reaction described in the equation 4 (Selwyn 2004). Chlorine is then responsible for a cyclic corrosion process that involves chemical, as well as mechanical damage of the iron objects.

$$2Fe + 4HCl + O_2 \rightarrow 2FeCl_2 + 2H_2O \qquad [4]$$

$$2FeCl_2 + 3H_2O + O_2 \rightarrow FeOOH + 4HCl \qquad [5]$$

The issues regarding submerged iron are really similar to the one already described for buried iron objects, the principal difference is that this type of objects develops a heavy concretionary corrosion due to a massive colonisation by marine organisms. In fact, this lead to the production of a corrosion layer composed of calcium carbonate containing ferrous as well as ferric ions (Scott and Eggert 2009).

In addition to the issue of chlorine contamination, iron surfaces exposed to atmosphere, are subjected to rainfall, solar radiation, wind, and pollution agents. In particular, four categories of atmospheric corrosion have been defined accordingly: rural, urban, industrial and marine (Scott and Eggert 2009). The atmospheric corrosion in rural environments is characterized by a low amount of pollutants in the air. The main elements responsible for this type of corrosion are moisture, small amounts of sulphur oxides, carbon dioxide and ammonia resulting from the employment of fertilizers in farming (Syed 2006). Objects exposed to this type of environment are subjected to a

corrosion rate between 4 and 65 µm/year (Scott and Eggert 2009). In urban environments air is more polluted due to domestic fuel consumption and vehicle emissions. These activities produce pollutants such as NO_x and SO_x that enhance iron corrosion by producing an acidic film on iron surfaces (corrosion rate 23-71 µm/year) (Scott and Eggert 2009). In industrial atmospheres, there is an extremely high concentration of pollutants such as SO_x, NO_x and chlorinated compounds resulting from the industrial activity. This atmospheric composition makes the industrial environment the most corrosive one (26-175 µm/year). Finally, marine environments are characterized by high moisture, severe atmospheric conditions, and the presence of chlorinated aerosols (26-104 µm/year) (Scott and Eggert 2009).

The presence of SO_2 as contaminant of air is known to be responsible of an acceleration of the deterioration and to cause cyclic corrosion process (Scott and Eggert 2009). In fact, as illustrated by equation 6, SO_2 is incorporated in the corrosion layer composed of iron oxides and hydroxides. Thereafter, water and oxygen allow the oxidation of iron that lead to the formation of $Fe_2O_3 \cdot H_2O$ and H_2SO_4 (eq. 7). The sulphuric acid produced during the latter reaction, instigates further corrosion establishing a cyclic corrosion process, shown in eq. 8 (Schikorr 1963, Scott and Eggert 2009).

$$Fe + SO_2 + O_2 \rightarrow FeSO_4 \quad [6]$$

$$4FeSO_4 + O_2 + 6H_2O \rightarrow 2Fe_2O_3 \cdot H_2O + 4H_2SO_4 \quad [7]$$

$$4H_2SO_4 + 4Fe + 2O_2 \rightarrow 4FeSO_4 + 4H_2O \quad [8]$$

1.3. Microbiologically-induced corrosion of metals

In general microbes are considered to be detrimental for iron surfaces. Several publications focus on the study of the negative interaction between microbes and iron and four different mechanisms concerning microbial induced corrosion of metals (MIC) have been reported: (i) differential concentration cell, (ii) production of corrosive substances, (iii) alteration of anion ratio and (iv) inactivation of corrosion inhibitors (Zarasvand and Rai 2014).

1.3.1. Differential concentration cell

When different areas of a metallic surface are exposed to different concentrations of the same solution corrosion can be enhanced. For instance, if the metal surface is exposed to different oxygen concentrations anodic and cathodic areas will be produced enhancing corrosion through an electron flux from the anodic to the cathodic area. Bacteria colonizing only a part of the iron surface can contribute to this phenomenon by creating heterogeneous surfaces (Zarasvand and Rai 2014).

1.3.2. Production of corrosive substances

Iron surfaces in an anoxic environment can be colonized by sulphur-oxidizing bacteria such as *Thiobacillus* species. This type of bacteria could produce sulphuric acid (H_2SO_4) through the oxidation of sulphides, accelerating the corrosion of iron surfaces. Another compound produced as a consequence of bacterial metabolism able to instigate iron corrosion is hydrogen sulphide (HS^-). This corrosive agent is produced by sulphate-reducing bacteria during the reduction of sulphates into sulphides that supplies energy to these bacteria. The latter reaction leads then to the formation of HS^- that reacting with the metal surface produces metal sulphides and atomic hydrogen that instigate further corrosion (Zarasvand and Rai 2014). Fungi are also microbes involved in iron corrosion. In fact, it has been reported that *Fusarium*, *Penicillium*, and *Hormoconis* species can enhance corrosion of metal surfaces by producing organic acids like citric, formic and acetic acids (Little and Staehle 2001).

1.3.3. Alteration of anion ratio

Sulphates are reported to have a repressive effect on the corrosion of aluminium alloys in presence of chlorides (Wu and Wu 1995, Zarasvand and Rai 2014). In fact, sulphates competing with chlorides for the absorption site in metal surface will decrease the content of chloride anions in the corrosion layer. Nevertheless, a high amount of sulphates could enhance corrosion. Therefore, a key factor parameter on metal corrosion is also the ratio between anions and not only their presence or absence. Then, since microbes through their metabolism can change this ratio they are considered to have a great influence on the corrosion rate of metals (Little and Staehle 2001, Zarasvand and Rai 2014).

1.3.4. Inactivation of corrosion inhibitors

The formation of biofilms on the object avoids the contact of the corrosion inhibitors with the metal surface enhancing their corrosion. Another mechanism able to inactivate the corrosion inhibitors is the bacterial consumption of these compounds. In fact several studies have reported the ability of bacteria to use this type of compound as nutrient source. Bacteria can then reduce the efficiency of corrosion inhibitors by avoiding the contact of these substances with the metal surface or by direct use of them as a nutrient source (Lundgren and Krikszens 1959, Zarasvand and Rai 2014).

1.4. Current methods for iron stabilisation

Nowadays, several techniques are employed to stabilize archaeological iron objects regardless of their provenience. In fact, the methods used for buried and submerged iron are the same. Archaeological iron objects are usually so badly encrusted with irregular corrosion layer, that these types of objects are barely recognisable just after their recovery. Then, the first step is a mechanical or chemical removal that will reveal some information about the original shape of the object. Usually this procedure is performed with sandblasting (using powder composed of aluminium dioxides, glass beads, or walnut shells particles), or through laser cleaning. Both techniques rely on the experience of the restorer that has to be really precise and great attention has to be dedicated to prevent removal of any part of the metal core (Scott and Eggert 2009). Cleaning can also be performed using acidic solution such as, vinegar, lemon juice, or oxalic acid. After the first cleaning, three techniques are currently employed in order to stabilize archaeological iron objects. Those focus on a passive removal of chloride ions by increasing the porosity of the corrosion layer (Selwyn 2004). The first is electrolytic reduction. This desalination method consists of immersion of the artefacts (acting as cathode) in an alkaline electrolyte containing an electrode (acting as anode). The voltage that is then applied causes the reduction of the Fe^{3+} oxyhydroxides into magnetite or Fe^{2+} oxyhydroxides, increasing the porosity of the corrosion layer and enhancing chloride ions diffusion (Selwyn 2004). The main disadvantage of this treatment is that it has to be carefully monitored because it causes the production of extremely explosive H_2 by the electrolysis of water. Moreover, the production of bubbles of H_2 can also lead to uncontrolled surface damage (Selwyn 2004), which is a concern in the context of archaeological objects, particularly, of small objects.

Therefore, this method is exploitable only for large marine findings (Scott and Eggert 2009).

Second, reactive hydrogen plasma (a highly reactive gas containing partially ionized hydrogen molecules and atoms) is also used to reduce the corrosion compound to a lower oxidation state. However, in order to prevent the alteration of the metallurgical structure, the temperature is maintained below 400 °C during the treatment. Due to the fact that experimental evidence proved that chlorides removal is not effective at this low temperature (Schmidt-Ott 1997), plasma treatment is usually employed only as a pre-treatment before alkaline sulphite desalination (detailed below) since it causes cracks and fissures improving the diffusion of chloride ions (Scott and Eggert 2009).

Finally, the most common used treatment for archaeological iron objects is the aqueous reduction in alkaline sulphite solution (0.001 mol/L of NaOH and 0.05 mol/L of Na_2SO_3) (Schmidt-Ott 2006). During the treatment oxyhydroxides are reduced into magnetite, a stable mineral with a smaller molar volume than the previous compounds. This will increase the porosity of the corrosion layer allowing the potentially present chloride ions to diffuse out of the corrosion layer into the alkaline solution (Selwyn 2004, Scott and Eggert 2009). However, the alkaline sulphite solution needs to be changed regularly and at the end of the treatment, the used solution requires specific waste neutralization. Moreover, the effective chlorides removal is difficult to assess without destructive analysis (Selwyn 2004, Rimmer, Wang et al. 2010).

On the other hand, the issue of the protection of outdoor iron surfaces is completely different from the one of archaeological iron. In fact, these infrastructures need a treatment able to protect them against sever atmospheric conditions, such as wind, acidic rain and pollution; while archaeological iron objects, once stabilized are kept under controlled conditions (constant low humidity and temperature). Stabilisation techniques for objects susceptible to atmospheric corrosion consist on the application of substances that will produce a physical barrier between the metal surface and the atmosphere, such as coating and the utilisation of corrosion inhibitors. Nowadays, two types of coating are employed: organic, such as waxes, resins, oil, and practically every other class of polymers; and inorganic, like nickel, chromium, metallic oxides, cements, and enamels. Both these types of coating have several disadvantages; indeed, their application drastically changes the appearance of the iron surface (colour and texture).

In addition, they are not so efficient on already corroded surfaces, and after few years they start to crack, exposing again the metal surface to oxygen and moisture instigating further corrosion (Scott and Eggert 2009). The second approach to protect these types of exposed objects is the use of corrosion inhibitors. These substances will decrease the corrosion rate of the iron surface by delaying the electrochemical interaction of the metal surface with the atmosphere. Nevertheless, the substances used for this purpose are frequently hazardous, such as chromates, benzoates, and nitrites, and then have to be manipulated with precaution.

Due to the caveats of the available methods to stabilize corroded iron, scientists agree with the fact that new technologies should be developed (Zarasvand and Rai 2014). Microorganisms are currently being considered for the development of alternative treatments in conservation-restoration (Bosch-Roig and Ranalli 2014). For example, nitrate- and sulphate-reducing bacteria have been used to remove black crust on stone artworks (Ranalli, Alfano et al. 2005, Cappitelli, Zanardini et al. 2006). Endospore-forming bacteria of the genus *Bacillus* have been recently reported as self-healing agents in concrete structures (Jonkers 2011), and biogenic carbonated phases have been employed for improving the durability of buildings (Dhami, Reddy et al. 2013). Likewise, *Pseudomonas* spp. has been reported to be more effective than enzymes in the removal of organic matter from frescoes (Ranalli, Alfano et al. 2005). Finally, the fungal production of copper oxalates has been exploited to produce a passivation layer on copper-based alloys (Joseph, Cario et al. 2012, Joseph, Simon et al. 2012, Joseph, Letardi et al. 2013). Iron has not yet been the target of such a biotechnological approach, but the multitude of microbial metabolisms affecting iron opens up the possibility of a microbiological method for the stabilization of iron artefacts.

1.5. Microbial metabolic abilities regarding iron

1.5.1. Bacteria

Oxidation

Microbial iron oxidation is a capacity detected in different types of bacteria, including autotrophs, heterotrophs, phototrophs, and chemotrophs. However, since Fe(II) oxidizes readily in presence of oxygen at neutral pH, bacterial iron oxidation occurs mainly in environments having an acidic pH or low oxygen content (Bird, Bonnefoy et al. 2011).

Under acidic conditions, chemolithotrophic Fe(II) oxidation is reported to be the main microbial strategy to reduce iron. Microbes having this metabolism are able to obtain energy from the oxidation of Fe(II) coupled with the reduction of oxygen under acidic conditions. A model microorganism for this metabolism is the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* (Valdés, Pedroso et al. 2008). On the other hand, in environments where the partial pressure of oxygen is lower than the atmospheric pressure, microaerophilic bacteria can oxidize iron by two different mechanisms. The first is photoautotrophic Fe(II) oxidation. Bacteria having this metabolic ability, such as *Chlorobium ferrooxidans*, are capable of oxidizing Fe(II) using light to fix CO_2 in their biomass. Second, experimental evidence suggests that, in absence of light, some bacteria such as *Ferroglobus placidus* couple iron oxidation with nitrate reduction (Weber, Achenbach et al. 2006).

Reduction

Iron reduction is a common metabolic reaction among anaerobic bacteria. It is important for the oxidation of organic matter in anoxic environments, and it directly impacts the production and weathering of minerals (Lovley 1991, Fredrickson, Zachara et al. 1998, Frankel and Bazylinski 2003, Dalla Vecchia, Suvorova et al. 2014). The most studied example of microbial iron reduction is iron respiration, where Fe(III) is used as a terminal electron acceptor by anaerobic iron-reducing bacteria. These bacteria are generally able to reduce both soluble iron and solid iron phases, such as iron oxyhydroxides (Dalla Vecchia, Suvorova et al. 2014). While the reduction of soluble iron phases occurs inside a cell compartment (i.e., cytoplasm or periplasm), the reduction of solid iron phases implies the extracellular transfer of electrons. Different strategies have been described for model microorganisms such as *Geobacter sulfurreducens* PCA and *Shewanella oneidensis* MR-1 (Weber, Achenbach et al. 2006, Dalla Vecchia, Suvorova et al. 2014). The first step in the electrons' outward movement is their transfer to the outer membrane via multiple c-type cytochromes (Schröder, Johnson et al. 2003, Shi, Squier et al. 2007, Dalla Vecchia, Suvorova et al. 2014). Once they are in the outer membrane, the electrons can reach a solid iron phase in three different ways: first, by direct contact of an external c-type cytochrome with the solid phase (Lower, Hochella et al. 2001, Gralnick and Newman 2007, Shi, Richardson et al. 2009, Inoue, Leang et al. 2011, Dalla Vecchia, Suvorova et al. 2014); second, via an electron-conductive pilus; and third, by a soluble electron carrier (such as a flavin) able

to transfer electrons to the solid phase (Reguera, McCarthy et al. 2005, Gorby, Yanina et al. 2006, Dalla Vecchia, Suvorova et al. 2014). Iron reduction can also be the result of fermentation. A recent study has shown a mechanism by which Fe(III) reduction enables the use of Fe(III) as an electron sink during pyruvate fermentation in *Desulfotomaculum reducens* strain MI-1 (Dalla Vecchia, Suvorova et al. 2014). Biogenic iron minerals can be produced as the result of iron reduction. Several studies reported the production of biogenic magnetite (Fe_3O_4), siderite ($FeCO_3$), vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$], and mackinawite (FeS) (Moskowitz, Frankel et al. 1989, Bazylinski and Frankel 2000, Frankel and Bazylinski 2003).

1.5.2. Fungi

Redox active reactions

Fungal iron oxidation is a recurring process in soil due to fungal metabolic activity and the oxidative conditions present in this type of environment. Indeed, fungal dissolution of minerals produces free Fe^{3+} and Fe^{2+}: these ions are subsequently exposed to oxidative conditions due to the presence of H_2O and O_2, but also to the protons and oxidative exo-enzymes produced by fungi (Guillén, Martínez et al. 2005). This leads to a rapid oxidation of iron in soil near fungal biomass.

Little is known about fungal iron reduction activity. Some research, focusing on yeast and human pathogens, demonstrated the presence of an iron reductase and oxidase coupled with a permease that allows iron to penetrate into fungal cells (Kosman 2003). However, reduced iron is not only relevant during animal infection, but also in several other fungal metabolisms, such as wood degradation. The production of free oxygen radicals that allows the degradation of lignin and cellulose wood compounds is caused by the interaction between Fe^{2+} and H_2O_2. Even if the process is still not completely elucidated, a Fenton reaction-like model has been widely accepted. In order to start this reaction (Eq. [1]), Fe^{2+} is required. Nevertheless, in soils and wood, the main fraction of available iron is in its trivalent oxidation state (Fe^{3+}). Therefore, in order to start the Fenton reaction, wood decay fungi have to be able to produce Fe^{3+}-reductive enzymes or low molecular weight Fe^{3+}-reducing compounds able to convert Fe^{3+} into Fe^{2+} (Shah, Schwenk et al. 2015).

$$Fe^{2+} + H_2O \rightarrow Fe^{3+} + .OH + -OH \quad [1]$$

Fungi can produce several compounds for this purpose, and a general enzyme or Fe^{3+}-reductant used by the majority of wood decay fungi does not exist. In most cases, these compounds are organic acids, phenol or alcohols, and each fungus possesses its own set of Fe^{3+}-reductants (Arantes, Milagres et al. 2011). Recently, this type of reactive molecule has also been described in mycorrhizal fungi. In fact, Shah, Schwenk et al. (2015) demonstrated that the ectomycorrhizal fungus *Paxillus involutus* produces involutin, a molecule able to reduce Fe^{3+} during Fenton-based decomposition of organic matter.

Although free Fe^{2+} is required for the Fenton reaction and wood degradation, an excessive quantity of this ion can be detrimental. Indeed, excessive quantities of toxic oxygen free radicals produced as result of the Fenton reaction can lead to degradation of the fungal biomass. In order to overcome this issue, fungi have developed several strategies to maintain the needed ionic equilibrium. Precipitation, complexation, adsorption (binding to cell walls) and active uptake are the most frequently described mechanisms (Gadd 1993).

Uptake

As previously described, iron is an essential element for fungal growth, and the ability to acquire and accumulate this ion in their biomass allows fungi to survive under iron-limiting conditions. Fungi have developed different strategies to facilitate their access to insoluble iron. Two of the most relevant strategies are the production of low molecular weight organic acids and the production of specific chelating compounds. Siderophores are an important family of the latter type of molecules able to chelate metals, which is produced under iron-limiting conditions (Neilands 1995). Either of these mechanisms enhances fungal iron uptake, allowing fungi to compete more efficiently for this important limiting growth factor. However, once inside the cell, free intracellular iron may become extremely cytotoxic, and in order to avoid cellular damage efficient immobilization and storage mechanisms are required. The mechanisms of iron

immobilization involve adsorption in the cell wall and active uptake followed by intracellular iron storage. Those mechanisms will be presented in the following section.

The first site of direct interaction between fungi and metals is the cell wall. The fungal cell wall is a multilayered structure composed by chitin, β-1,3/1,6-glucan, and an external layer of mannoproteins that offers the fungal cell both a mechanical and a chemical protection (Eisenman and Casadevall 2012). Under stress conditions, some fungi produce also melanin, which can be synthetized within the cell wall (over the layer of mannoproteins) or produced extracellularly (Gadd 1993, Philpott 2006). Due to its complex composition, the cell wall can act as a protective barrier against toxic metals. Indeed, it is widely recognized that melanin, mannoproteins and chitin have metal-binding properties (Gadd 1993, Philpott 2006) that allow fungi to adsorb not only toxic metals, but also beneficial ones such as iron.

Melanin is a fungal pigment, synthetized in response to different stress conditions such as ultraviolet radiation (Eisenman and Casadevall 2012). Furthermore, it has been shown that the presence of copper in the culture medium of *Gaeumannomyces graminis* is associated with an enhancement of the melanin content in the cell wall (Caesar-Tonthat, Van Ommen et al. 1995). In another study, it was demonstrated that the melanin from *Aureobasidium pullulans* has the ability to bind significant amounts of copper as well as iron (Senesi, Sposito et al. 1987, Gadd and de Rome 1988). Chitin and chitosan are also considered to be important metal bio-sorbent molecules, binding several metals on their amine nitrogen groups (Gadd 1993). Experiments conducted by Franco, Maia et al. (2004) demonstrate that chitin has also the ability to chelate iron.

The diversity of molecules involved in metal sorption in the fungal cell wall has a clear biotechnological interest. Metal biosorption abilities of fungi have been studied in order to decontaminate water and soil from toxic metals. Experimental evidence demonstrates that both living and dead biomass can be exploited as bio-sorbent and the efficacy of this approach has been proven also for a mixed solution of several toxic metals (Paknikar, Puranik et al. 1998, Bishnoi and Garima 2005, Mali, Pandit et al. 2014). Despite the fact that toxic metals have received more attention, cell wall compounds are also reported to be able to bind iron (Murugesan, Sathishkumar et al. 2006). Therefore these adsorption mechanisms have to be considered when studying fungal interactions with iron not only *in vitro* but also in the environment (e.g. in soils).

While the mechanisms described previously are based on the passive adsorption of metals in the fungal cell wall, fungi also display active iron uptake, which is metabolism-dependent and occurs only in living cells (Kosman 2003, Bishnoi and Garima 2005, Philpott 2006). In biosorption by living biomass, various parameters such as pH of the culture, temperature, composition of the growth medium, and especially culture age, all influence metal uptake. Experimental evidence suggests the active uptake of Cu, Cd, Ni, Zn, Co, Mn, Sr, Mg and Ca.

Active uptake of iron has been widely studied in yeast and pathogenic fungi, as this is an important virulence factor of human pathogens (Ruddat, Kokontis et al. 1991). *S. cerevisiae* has been reported as being able to retain a substantial amount of iron in the cell wall (Philpott 2006). Experimental evidence suggests that under iron-depletion conditions this yeast over-expresses genes for the synthesis of mannoproteins responsible for the retention of siderophore-iron complexes in the cell wall, called facilitator of iron transport proteins (FITs) (Protchenko, Ferea et al. 2001, Philpott 2006). Less information is available in the case of filamentous fungi. The mechanisms described until now are: (i) extracellular reduction of Fe^{3+} and uptake via a ferrous permease; (ii) extracellular oxidation of Fe^{2+} and uptake via a ferric permease; (iii) uptake of Fe^{3+} and Fe^{2+} respectively through a ferric and ferrous permeases; and, (iv) uptake of Fe^{3+}-siderophore complexes via a siderophore permease (Kosman 2003, Philpott 2006) (Figure 2).

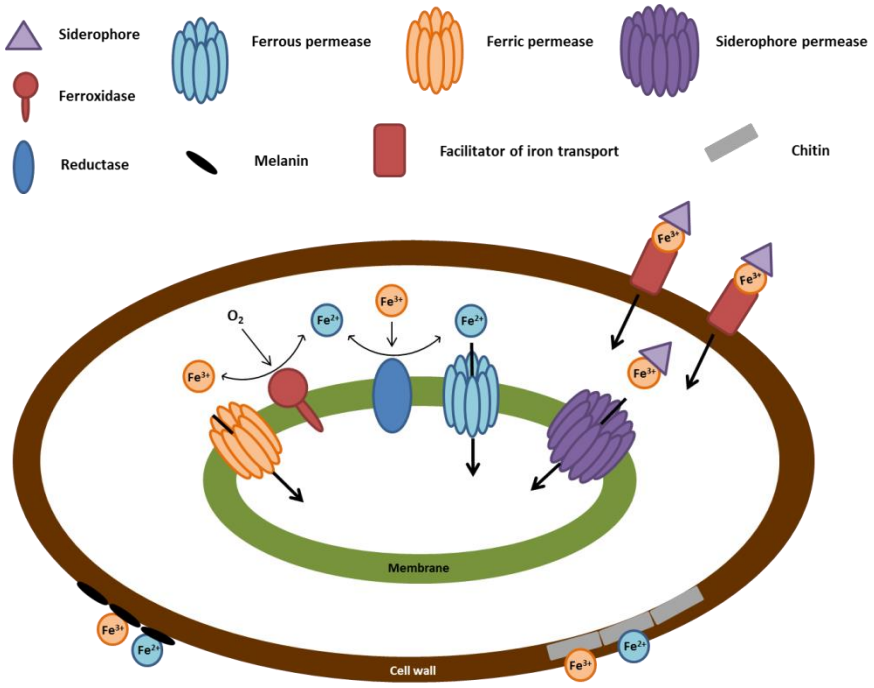


Figure 2: Schematic representation of possible mechanisms of iron uptake in fungi. Adapted from Kosman, 2003 and Philpott, 2006.

Due to its high reactivity, iron is rarely found free inside the cell. Fungi have developed several iron storage molecules for intracellular storage similar to siderophores, as well as proteins such as ferritins and metallothioneins, and also polyphosphates (Matzanke 1994, Howard 1999).

Siderophores are low molecular weight Fe^{3+} chelating molecules produced under iron-limiting conditions (Neilands 1995). With a few exceptions fungal siderophores identified so far are of the hydroxamate-type and can be classified into three structural families: fusarinines, coprogenes and ferrichromes (Renshaw, Robson et al. 2002). Iron acquisition by siderophores is an energy-dependent mechanism that can occur via four different ways: (i) shuttle transport mediated by penetration of the iron-siderophore complex in the fungal cell and cleavage of these two compounds by an intracellular reductase; (ii) taxicab transport mediated by the production of a siderophore that chelates iron and remains outside the membrane, while iron is transferred through ligand exchange to an internal siderophore pool (Van der Helm and Winkelmann 1994, Renshaw, Robson et al. 2002); (iii) hydrolic acquisition mediated by the penetration of the iron-siderophore complex into the fungal cell and subsequent cleavage of the complex by intracellular reductive and degradative reactions (Van der Helm and

Winkelmann 1994, Renshaw, Robson et al. 2002); and, (iv) reductive acquisition mediated by the production of a siderophore that chelates iron and remains outside the fungal cell, followed by iron reduction near the membrane and uptake of Fe^{2+} (Van der Helm and Winkelmann 1994, Renshaw, Robson et al. 2002).

For some species, siderophore production is an essential virulence factor that regulates pathogenicity. In fact, mutants of the plant pathogen *Microbotryum violaceum* producing a reduced amount of siderophores showed lower or no pathogenicity (Ruddat, Kokontis et al. 1991). Interestingly, it has been reported that fungi unable of producing siderophores have the ability to use these molecules when secreted by other microbes (Howard 1999). The use of heterologous siderophores by non-pathogenic fungi can then suppress the growth of phyto-pathogenic fungi, and fungi able to do this are then reported to be beneficial for plant health (Campbell, Renwick et al. 1986, Renshaw, Robson et al. 2002). There is also evidence that plants can exploit microbial siderophores in order to obtain iron (Crowley, Reid et al. 1988). The metal chelating capabilities of siderophores are studied and exploited in many biotechnological fields such as medicine, reprocessing of nuclear fuel, remediation of metal contaminated fields and treatment of industrial waste compounds. These possible applications are described exhaustively (Renshaw, Robson et al. 2002). Even if siderophores are considered to be one of the most important mechanisms developed in order to acquire iron under limiting conditions, they also play an important role in intracellular iron storage in fungal cytoplasm and spores (Winkelmann 1992).

Ferritin-like molecules are a broad superfamily of iron-rich proteins used for iron storage in different organisms. Three types are reported in the literature: (i) ferritin; present in animals (Carrano, Böhnke et al. 1996), (ii) phytoferritin; present in plants, and (iii) bacteriferritin found in bacteria. These types of iron-rich proteins are reported to be present only in Zygomycetes. Three types of ferritin-like molecules have been discovered in fungi: (i) mycoferritin, similar to ferritin present in animals; (ii) zygoferritin, found only in Zygomycetes; and, (iii) bacterioferritin, discovered in *Absidia spinosa* (Carrano, Böhnke et al. 1996). Other proteins able to bind metals in the fungal cytosol are metallothioneins and phytochelatins, two families of low-molecular weight cysteine-rich proteins containing sulphur-based clusters able to bind metals (Gadd 1993, Kameo, Iwahashi et al. 2000, Bellion, Courbot et al. 2006).

Polyphosphates are molecules present in many microorganisms. Their role is still not clear but there is some evidence suggesting that they could play a role in the storage of Fe^{2+}. Experimental evidence reveals accumulation of other divalent cations bound to polyphosphates in microbes. For instance, Ca^{2+} in yeast (Ashford, Vesk et al. 1999), Mn^{2+} in the bacterium *Lactobacillum plantarum* (Archibald and Fridovich 1982), and Al^{2+} in the cyanobacterium *Anabaena cylindrica* (Pettersson, Kunst et al. 1985). (Böhnke and Matzanke 1995) suggest that polyphosphates could play the role of Fe^{2+} storage molecules in *Escherichia coli*. It can then be hypothesised that polyphosphates may be used as iron storage molecules in the fungal vacuole as well.

Translocation

Fungal translocation is a phenomenon frequently discussed in relationship to fungal nutrient cycling. Indeed, it is widely recognized that fungi have the ability to transport carbon sources from the substratum to another distal location through their mycelial network. Through the use of radioactive isotopic tracers several studies have demonstrated the ability of fungi to translocate phosphorus (Lindahl, Finlay et al. 2001) or amino acids (Tlalka, Watkinson et al. 2002). This ability allows fungi to colonize environments with low resource availability. Translocation has been exhaustively described by Lindahl and Olsson (2004). Translocation and accumulation of toxic metals from polluted soils into the fruiting body of edible fungi is a well-known mechanism that can cause serious human health issues (Brown and Hall 1990, Gadd 1993). Even if metal translocation through the mycelial network has rarely been proved *in vitro*, the accumulation of heavy metals in the fruiting body demonstrates that fungi have the ability to translocate these. Studies by Furuno, Foss et al. (2012) and Schamfuß, Neu et al. (2013) proved the translocation of toxic polycyclic aromatic hydrocarbons (PAH) in the fungal-like mycelial network of the oomycete *Pythium ultimum*. These researchers suggested that translocation of PAH pollutants could be an interesting ability to improve degradation of toxic substances by increasing the dispersal and the bioavailability of the pollutant into the soil matrix in order to enhance bacterial degradation.

Further work is still required to elucidate the mechanisms and the molecules involved in the translocation of iron. Nonetheless, fungal iron translocation can be used to provide trace elements to plants. For example, a microcosm experiment has demonstrated that

the arbuscular mycorrhizal fungus *Glomus mosseae* is able to mobilize, uptake and translocate iron from soil to the shoots of sorghum plants (Caris, Hördt et al. 1998). That study shows that fungi are also able to translocate beneficial trace elements such as iron and not only amino acids or toxic metals.

Solubilisation/weathering

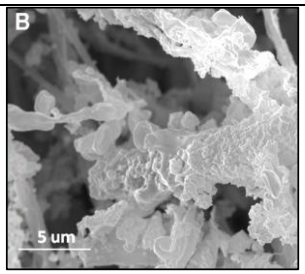
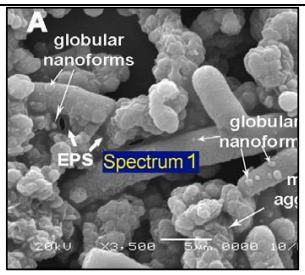
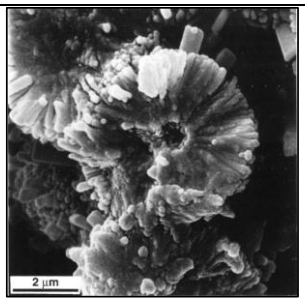
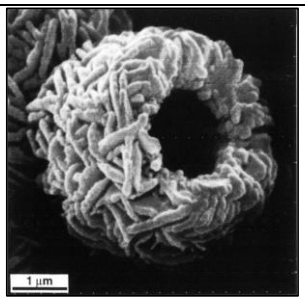
In natural environments, iron bioavailability is poor. To overcome this issue, fungi have developed different strategies to acquire this element from insoluble minerals. The first is the enhancement of mineral solubilisation via acidification of the environment near their mycelial network. This occurs either by proton secretion or by producing extracellular low molecular weight organic acids (LMWOA) (Gadd 1993, Gadd 1999, Fomina, Hillier et al. 2005, Ehrlich 2006). LMWOA are important extracellular metabolites that promote the solubilisation of unavailable ions from mineral substrata by decreasing the pH of the microenvironment, as well as by chelating the ions released. This is one of the reasons why fungi are considered important geological weathering agents (Sterflinger 2000). Several types of fungal organic acids have been reported: citric acid, gluconic acid, itaconic acid, L-lactic acid, fumaric acid, L-malic acid, and succinic acid. However the most representative is oxalic acid (Magnuson and Lasure 2004). This compound is also considered as an important virulence factor, since it is responsible for host tissue acidification and sequestration of important ions like Ca^{2+}, Mn^{2+}, Mg^{2+} and $Fe^{2+/3+}$ (Godoy, Steadman et al. 1990). Finally, citric and oxalic acid have also the property to chelate divalent and trivalent metals (Gadd 1993, Gadd 1999, Fomina, Hillier et al. 2005). The ability of several fungi to produce organic acids with chelating properties is considered an important extracellular resistance mechanism against heavy metals. Indeed, as a result of the interaction between oxalic acid and cations, crystals of metal oxalates will be produced. This important property allows fungi to decrease the concentration of toxic metals in solution producing secondary biogenic minerals, like metal-oxalates (Gadd 1993).

Biogenic mineral production

A direct consequence of fungal solubilisation mechanisms such as organic acid production and their interaction with the environment (soil, rock, wood, animal or vegetal tissues) is the precipitation of secondary biogenic minerals. Bacterial iron mineral production is a domain studied in detail. Since bacteria are able to grow under

both aerobic and anaerobic conditions they are able not only to produce Fe^{3+} minerals, such as hematite and goethite (Frankel and Bazylinski 2003) but also, as a consequence of iron reduction, Fe^{2+} minerals like magnetite, siderite, pyrite, and vivianite, among others (Frankel and Bazylinski 2003). Biogenic production of iron minerals by fungi is less studied probably because of their supposed inability to grow under anaerobic conditions. In fact, iron chemistry in an aerobic system is circumscribed (Kosman 2003). Fe^{2+} rapidly oxidizes to its trivalent state in contact with the oxygen contained in the atmosphere. This Fe^{3+} further hydrolyses, producing insoluble ferric hydroxide species. However fungi usually induce drastic changes in the microenvironment near their hyphal network (Robson, Prebble et al. 1996). For instance, a modification of pH is an essential environmental factor influencing the redox state and the stability of iron ions, as well as its solubility. In fact Fe^{2+}, which is stable under anaerobic conditions, is also stable under alkaline conditions (Selwyn 2004). Nevertheless, few studies have focused on fungal biogenic formation of iron minerals. However, experimental studies have provided evidence for the production of iron oxalate (FeC_2O_2) (Joseph, Cario et al. 2011), iron oxyhydroxides such as hematite (Fe_2O_2) and goethite (α-FeO(OH)) (Feldmann, Neher et al. 1997) and sulphates like jarosite ($KFe_3(OH)_6(SO_4)_2$) (Oggerin, Tornos et al. 2013) (Table 2).

Table 2: Type and Scanning Electron Microscopy picture of biogenic iron minerals produced by several fungi in different environments.

Fungal species	Biogenic mineral	Electronic Microscopy	Reference
Beauveria bassiana	Iron oxalate $FeC_2O_4 \cdot 2\ H_2O$		Joseph et al. (2011)
Purpureocillium lilacinum	Jarosite $KFe^{3+}_3(SO_4)_2(OH)_6$		(Oggerin et al., 2013)
Unidentified basidiomyces	Goethite (α-FeO(OH))		(Feldmann et al., 1997)
Unidentified basidiomyces	Hematite (Fe_2O_3)		(Feldmann et al., 1997)

The most studied secondary weathering products are metal oxalates. The interaction between oxalate anions and divalent and trivalent cations leads to the formation of metal oxalates. Fungi are reported to be able to produce oxalate salts mainly with calcium, copper, magnesium, and strontium.

However many other oxalate minerals exist (Fomina, Hillier et al. 2005, Gadd 2007). For instance, iron oxalate crystals have been found on sheeted hyphae of the entomopathogenic fungus *B. bassiana* (Joseph, Cario et al. 2011) (Table 2). However, the stability of these crystals is not completely understood. Indeed, Eckhardt (1985) and Varadachari, Barman et al. (1994) reported that oxalic acid produces insoluble salts only reacting with divalent cations such as Ca^{2+} or Cu^{2+}, while oxalates resulting from the reaction with trivalent cations are soluble salts. Experimentally, it is suggested that only Fe^{3+} is able to react with oxalic acid (Eick, Grossl et al. 1996), and this could be the reason why fungal iron oxalate is rarely found in nature, even though fungi produce high amounts of oxalic acid and iron is the most abundant metal on Earth. Further research is required to better understand fungal iron oxalate production and the stability of the resulting minerals.

Poorly ordered iron oxides and oxyhydroxydes are frequently reported in association with lichens and are the result of the primary oxidation of iron-bearing rocks induced by the production of organic acids by fungi. The subsequent release and hydrolysis of Fe^{2+} and Fe^{3+} leads to the precipitation of secondary biogenic minerals on rock surfaces (Siever and Woodford 1979, Eick, Grossl et al. 1996). An example of this mechanism has been described by Adamo, Colombo et al. (1997). These authors show the presence of poorly crystallized ferrihydrite in the interaction zone between a volcanic rock and the thallus of *Stereocaulum vesiuvianum*. Fungal erosion of a quartzite deposit in a Swiss Alpine environment has been studied by Feldmann, Neher et al. (1997). Their work revealed the presence of crystalline goethite and hematite spherulites around fungal hyphae produced by an unidentified basidiomycete (Table 2).

Recently, Oggerin, Tornos et al. (2013) were able to isolate a strain of the fungus *Purpureocillium lilacinum* from the acidic environment of Río Tinto. An in-depth study of its biomineralization abilities revealed that this fungus was able to produce biogenic jarosite (Table 2). Well-crystallized jarosite was produced by living and dead fungal biomass. The biomineralization process started in both cases in the cell wall, at the outer part of the fungal cell. However living and dead fungal biomass performed differently in terms of bimineralization efficiency. Indeed, only 0.5 g/L of jarosite were produced in cultures inoculated with dead cells compared to 2 g/L in living cultures. The authors explain this difference by the presence of extracellular polymeric substances (EPS) associated with living fungal biomass.

They conclude that jarosite formation is a process independent from active metabolism and that requires fungal biomass and EPS as nucleation sites. The biogenic mineral formation starts in the fungal cell wall and EPS probably enhances the cohesion of newly formed jarosite crystals, allowing the formation of larger slime-like aggregates (Oggerin, Tornos et al. 2013, Oggerin, Rodríguez et al. 2014).

1.6. Fungi and biotechnology

In the last few decades, biotechnology has become a field of increasing interest. Researchers are developing sustainable and environmentally friendly technologies exploiting the physiological capabilities of plants and microbes in order to decrease the footprint of humans on earth. In this field fungi have a remarkable potential, as many of them exhibit a filamentous and modular structure called a mycelium in which they are able to translocate, accumulate, volatilize and transform substances. Ultimately some compounds can also be transferred into macroscopic structures such as the carpophores formed in higher fungi. Due to these metabolic abilities, fungi are employed in several domains such as agriculture, pharmaceutical and food industries, bioremediation, and also more recently in conservation-restoration science (Broadbent 1966, Feng, Poprawski et al. 1994, Zouboulis, Matis et al. 2003, Joseph, Cario et al. 2011).

For example, in the case of agriculture, *Beauveria bassiana* is currently used as a biological alternative to chemical insecticides (Feng, Poprawski et al. 1994). Besides this, arbuscular mycorrhizal fungi are reported as being able to improve the growth of plants in saline soils by increasing their tolerance to osmotic stress induced by salts (Feng, Zhang et al. 2002). In addition, fungi can control the development of phytopathogens. For example, fungal endophytes isolated from the plant *Artemisia annua* are reported to have antifungal activities against phytopathogens such as *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium satium*, *Fusarium graminearum*, *Gerlachianialis,* and *Phytophthora capsici* (Liu, Zou et al. 2001). In the pharmaceutical industry, since the discovery of penicillin, great attention has been given to the discovery of fungal antibacterial substances. In addition to those, research into the function of fungal extracellular polymeric substances (EPS) is starting to emerge. In fact, these fungal metabolites are considered as high value biomacromolecules with multiple applications (Mahapatra and Banerjee 2013). For instance, experimental evidence demonstrates that EPS from *Aureobasidium pullulans*

can be used as thickener in the production of biodegradable and edible plastics (Paul, Morin et al. 1986, Mahapatra and Banerjee 2013). Moreover, fungal β-glucans are an EPS class studied in human health promotion for its ability to confer a protective behaviour against mutation, microbial infections and diabetes (Chen and Seviour 2007, Mantovani, Bellini et al. 2008). In addition, EPS are also employed as biosorbant matrixes in wastewater treatment because of their ability to chelate toxic metals like lead, zinc and copper (Moon, Park et al. 2006, Yin, Hu et al. 2011). Finally, another fungal ability exploited in order to treat polluted water or soil is the biosorption of metals into fungal biomass (Gadd 1993, Singh 2006, Mali, Pandit et al. 2014). More recently, the production of biogenic oxalates minerals by the entomopathogenic fungus *B. bassiana* has been exploited in order to develop a sustainable conservation-restoration method for copper-based alloyed artefacts (Joseph, Cario et al. 2011, Joseph, Simon et al. 2012, Joseph, Letardi et al. 2013).

Iron is an essential trace element in the microbial metabolism. It is an important co-factor of many enzymes (Howard 1999) and it is essential during DNA synthesis (ribonulceotide synthetase) and cleavage (endonuclease III) (Matzanke 1994). This metal is also crucial for some wood decay fungi since it is involved in the non-enzymatic degradation of lignocellulosic polysaccharides and lignin. In fact, Fe^{2+} is necessary to start the Fenton-based reactions leading to the production of oxygen free radicals that cause the non-enzymatic degradation of wood components (Arantes, Milagres et al. 2011). Despite its ubiquitous involvement in physiology, microorganisms face a conundrum to obtain iron: the main reservoirs of this metal in nature are not bioavailable, and microbes, and in particular fungi, have developed several strategies to overcome this. Two of those strategies are the production of siderophores (Neilands 1995, Philpott 2006) and organic acid over-excretion (Fomina, Hillier et al. 2005, Gadd 2007). The multitude of roles of iron and its low environmental bioavailability explain the importance of this element in fungal physiology. Therefore, iron is often considered as a limiting factor for fungal growth and development.

Even if iron is an essential element for fungal physiology and in soil dynamics, at present the general knowledge of possible fungal reactions involving iron is incomplete. For instance the role of filamentous fungi in the geochemical cycling of iron is still poorly documented (e.g. (Kosman 2003)). In order to develop new biotechnological approaches related to iron, the first step is to understand fungal abilities regarding this

metal. Therefore, the aim of this review is to present an overview of the known fungal physiological capabilities in relationship to iron cycling and their possible application in biotechnology.

1.7. Fungal biotechnology involving iron

1.7.1. Biosorption

Even though iron is not considered a conventional toxic metal, high amounts can lead to a reduction of soil fertility and human health issues. In fact, the maximum concentration of iron recommended by the Food and Agriculture Organization of the United Nations (FAO) in irrigation water is 5 mg/L. A higher amount of iron is not toxic to plants in aerated soils, but can contribute to soil acidification and a loss of availability of essential nutrients such as phosphorus and molybdenum (Board 1972). For drinking water 0.3 mg/L is the limit recommended by the FAO in order to avoid staining of laundry and sanitary ware. Indeed, studies revealed that an excessive accumulation of iron in the body could cause haemorrhagic necrosis and sloughing of areas of the mucosa in the stomach. The lethal dose for humans is 200–250 mg/kg of body weight, but death has occurred following the ingestion of doses as low as 40 mg/kg of body weight (World Health Organization 2004). Sources of iron contamination of water are mainly industry, metallurgic manufacturing, mining, and in some cases the iron-based flocculants used in waste water treatment plants. Often in these cases, iron is not the only metal pollutant present in water, and studies have focused on the use of dead fungal biomass in order to remove metals in a non-selective way (Zouboulis, Matis et al. 2003, Ahluwalia and Goyal 2007, Mali, Pandit et al. 2014). For instance, experimental evidence demonstrated arsenic and iron removal abilities of autoclaved fungal biomass from contaminated groundwater. 100% of As(III), As(V) and Fe(II) was removed by dead fungal biomass after 30 minutes of contact (Murugesan, Sathishkumar et al. 2006).

1.7.2. Bioremediation of asbestos using fungi

Asbestos is a group of minerals composed of fibrous silicates producing hazardous airborne particles. Reaching the respiratory system they can cause pulmonary fibrosis, pleural or peritoneal mesothelioma and lung carcinoma. The toxicity of these fibres relies on the iron associated with asbestos that produces free hydroxyl radicals and

consequently DNA damage (Weitzman and Graceffa 1984, Hardy and Aust 1995, Fubini 1997, Daghino, Turci et al. 2006). Due to this, the use of asbestos has been banned in several countries, but this compound is still present in contaminated soils or near abandoned mines. Remediation strategies involving soil removal are not suitable in this case because of the production of airborne fibres of asbestos. Therefore *in situ* treatments involving fungi have been studied in order to bioremediate these contaminated environments. Experimental evidence shows that iron removal with fungal organic chelators and siderophores decreases the toxicity of these compounds (Weitzman and Weitberg 1985, Chao and Aust 1994, Martino, Prandi et al. 2003). A study by Daghino, Turci et al. (2006) demonstrated that *Verticillium* sp. and *Paecilomyces* sp. were able to remove iron from asbestos fibres. They demonstrated through *in vitro* tests of DNA damage, that fungal-treated asbestos causes less DNA damage effects. Their study concludes that fungi can be appropriate candidates to develop bioremediation strategies for asbestos-contaminated environments by reducing the amount of iron present in this compound.

1.7.3. Biopassivation on iron objects

In order to develop an eco-friendly conservation-restoration method for archaeological iron artefacts, Joseph, Cario et al. (2011) studied the biogenic mineral formation abilities of the fungus *B. bassiana*. That study aimed at producing a protective layer of iron oxalate on the surface of the objects through the conversion by the fungus of the existing corrosion products into less soluble and more stable compounds. In solid media culture amended with different iron sources, no crystal formation was observed. However, in liquid cultures, hyphae on the surface of immerged objects were completely encrusted with biogenic oxalate crystals as detected by optical microscopy and Raman spectroscopy. It was concluded that *B. bassiana* could be considered a good candidate for the development of new conservation-restoration methods for archaeological iron artefacts.

1.8. Inhibition of metal corrosion by bacteria

Given the large metabolic diversity found within the bacterial world, an increasing number of studies focus on the exploitation of bacterial metabolisms for the protection of metal surfaces. This section summarises the main bacterial mechanisms reported to inhibit metal corrosion.

1.8.1. Use of nitrate-reducing bacteria

Experimental evidence demonstrated that the injection of nitrate near a metal surface can decrease the above-mentioned detrimental metabolic activity of sulphate-reducing bacteria. This substrate will enhance the growth of heterotrophic nitrate-reducing bacteria and the sulphur-oxidizing nitrate-reducing bacteria that will affect negatively the metabolic activities of sulphate-reducing bacteria by essentially three mechanisms (Zarasvand and Rai 2014). First, heterotrophic nitrate-reducing bacteria are able to compete more efficiently for the same carbon source than sulphate-reducing bacteria. In fact, the energy obtained from nitrates reduction is greater that the energy acquired from the reduction of sulphates (Thauer, Jungermann et al. 1977, Zarasvand and Rai 2014). In presence of nitrates, heterotrophic nitrate-reducing bacteria will decrease metal corrosion by overcoming sulphate-reducing bacteria. Second, sulphur-oxidising nitrate-reducing bacteria will decrease metal corrosion through the oxidation of sulphuric acid (produced by sulphate-reducing bacteria) into sulphates. Finally, the production of nitrites by the nitrate-reducing bacteria in general, inhibits the dissimilatory sulphite reductase enzyme, responsible for the reduction of sulphites to sulphides, during sulphate reduction. Without this enzyme the growth of sulphate-reducing bacteria will be inhibited, and the corrosion decreased (Bødtker, Thorstenson et al. 2008, Zarasvand and Rai 2014). However, it has to be mentioned that the enhancement of a particular metabolic pathway is complicated while really sensitive to ions ratio. Then, even if this technique is really promising in laboratory, its application in field could be problematic due to the rapid change in the ionic composition near outdoors metal surfaces.

1.8.2. Formation of a protective layer

Bacteria are reported to produce a protective layer against corrosion. In fact the formation of biofilms on the surface of metals represses the charge transport between cathodic and anodic sites on the metal surface (Videla and Herrera 2009). In addition, the production of extracellular polymeric substances (EPS) is also reported to decrease the corrosion rate of metals by the formation of an iron-EPS complex (Chongdar, Gunasekaran et al. 2005). Finally, the production of a layer of biogenic iron phosphates (vivianite) on the surface of mild and carbon steel with *bacteria* was reported to reduce the corrosion rate of this metal surface (Volkland, Harms et al. 2000, Cote, Rosas et al. 2015).

1.8.3. Production of antimicrobial compounds

Experimental evidence suggested that iron surfaces could be protected via the production of antimicrobial agents that will decrease the amount of corrosion-causing bacteria, such as sulphate-reducing bacteria. Zuo and Wood (2004) demonstrated that the corrosion rate of mild steel exposed to the sulphate-reducing bacterium *Desulfosporosinus orientis* and the iron-oxidising bacterium *Leptothrix discophora* decreased 20-fold in presence of a *Bacillus brevis* able to produce the antibiotic gramicidin S.

1.8.4. Production of corrosion inhibitors

Another interesting bacterial ability exploitable for the protection of metal surfaces is the production of compounds able to inhibit corrosion. Örnek, Jayaraman et al. (2002) proved that corrosion of aluminium was reduced by 90 % when exposed to bacterial cultures of an engineered *Bacillus subtilis* able to produce polyaspartate. This compound is in fact considered to be an inhibitor of corrosion that reacts with metals to form a metal-peptide complex that passivates the metal surface.

1.9. Aim of this project

Almost all the biotechnological approaches for the protection of iron previously described involve only bacteria, and focus on the protection of bare iron surfaces before their exposure to outdoor environment. Not a single study was carried out on already corroded samples or on archaeological iron objects. Then, this study aims at investigating the potential of microbes; bacteria, as well as fungi, for the development of new biotechnological approaches for the stabilization of already corroded iron (archaeological objects and outdoor surfaces). Since the most problematic compound is chlorine, in this study two different approaches were used to remove chlorine from the object: indirect chlorine extraction and direct chlorine extraction (Figure 3).

Regarding indirect chlorine extraction, two strategies were adopted. First, exploiting the microbial ability to reduce iron under anoxic conditions was studied in order to convert part of the original corrosion layer into more stable biogenic minerals containing iron.

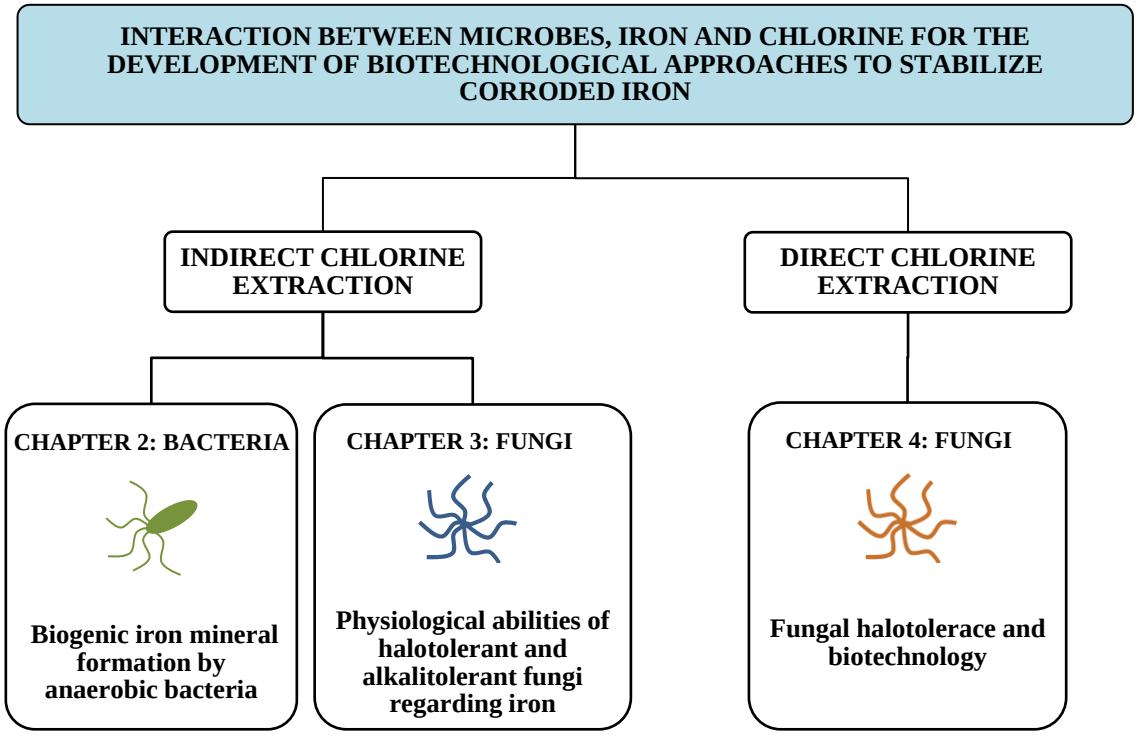


Figure 3: Graphic summary of the overall structure of the present study.

This conversion will allow a passive diffusion of chlorine from the corrosion layer into the culture medium. Second, the interaction between fungi and iron was studied aiming to acquire more knowledge about this topic, and to investigate the potential exploitation of fungi for the stabilization of corroded iron. Alkalitolerance was an important prerequisite for the fungal strains, since in presence of oxygen iron rapidly oxidizes at neutral pH, while under alkaline conditions iron is passivated (Supplementary figure 1). In this section, biogenic mineral formation, iron uptake, and reduction were studied in selected fungal species.

The second approach investigated in this study aiming to remove chlorine from iron artefacts was direct chlorine extraction with fungi. In this part the resistance

mechanisms against chlorine as well as a preliminary screening of the potential ability of fungi to uptake or volatilize chlorine from the corrosion layer of iron objects was investigated.

1.10. Principal microorganisms studied

1.10.1. *Desulfitobacterium hafniense*

Desulfitobacterium spp. are strict anaerobic bacteria with a highly versatile metabolism. Representative of this genus are able to use a large variety of electron acceptors, such as nitrates, sulphites, metals, humic acids, and halogenated organic compounds (Villemur, Lanthier et al. 2006). This genus belongs to the phylum of Firmicutes, class of Clostridia, order of Clostridiales and family Peptococcaceae. The first representative of the genus was isolated from sites contaminated with halogenated organic compounds. They are currently used to develop biotechnological strategies to remediate these toxic substances exploiting their ability to degrade halogenated organic compounds through reductive dehalogenation (Villemur, Lanthier et al. 2006). Six species have been proposed for this genus up to now; *D. hafniense, D. chlororespirans, D. dehalogenans, D. dichloroeliminans, D. metallireducens* and *D. aromaticivorans* (Futagami and Furukawa 2016). The most frequently isolated species in environments contaminated with chlorinated organic compounds is *D. hafniense*. Nine strains of this species have been reported so far: DP7, TCE1, Y51, GBFH, TCP-A, PCP-1, G2, DCB-2, and PCE-S. Individual cell size ranges between 2 and 6 µm, cells are motile and possess a flagellum. In addition, except for the strains TCE1 and DP7, bacteria belonging to this species are reported to produce endospores. Finally, the optimal temperature for growth is between 34 and 40°C, and the optimal pH is reported to be between 7.2 and 7.5 (Madsen and Licht 1992, Bouchard, Beaudet et al. 1996, Christiansen and Ahring 1996, Gerritse, Drzyzga et al. 1999, Breitenstein, Saano et al. 2001, Niggemyer, Spring et al. 2001, van de Pas, Harmsen et al. 2001, Shelobolina, VanPraagh et al. 2003, Villemur, Lanthier et al. 2006) Experimental evidence suggests the capabilities of the strains of *D. hafniense* to reduce iron (Villemur, Lanthier et al. 2006). However, contradictory results have been reported, suggesting that not all the strains of *D. hafniense* have this ability (Gerritse, Drzyzga et al. 1999, Luijten, Weelink et al. 2004). In fact, experimental

evidence demonstrated that while the strain TCE1 was able to use iron as final electron acceptor, this ability is lost during sequential transfers of strain DP7 (Luijten, Weelink et al. 2004). In the present study iron reduction in the strains TCE1 and LBE of *D. hafniense* was assessed as a way to convert a part of an unstable iron corrosion layer into more stable layer composed of biogenic iron minerals.

1.10.2. *Beauveria bassiana*

The genus *Beauveria* belongs to the phylum of Ascomycota, class of Sordariomycetes, order of Hypocreales and family Cordycipitaceae. Colonies of the species representative of this genus display white, later yellowish, or occasionally reddish colonies, and flask-shaped conidiogenous cells. Conidia are typically smooth-walled, hyaline, 1.5-5.5 μm large, and globose to cylindrical or vermiform. The specific morphology of these asexual spores is the key parameter for the morphological differentiation between *Beauveria* species (Rehner, Minnis et al. 2011).

Species of this genus are arthropod pathogens, but can also survive as saprophytes or endophytes of plants (Vega, Posada et al. 2008, Rehner, Minnis et al. 2011). Several species are reported for this genus, but the best-studied species are *B. bassiana*, *B. brognartii*, and *B. caledonica*. Since the first isolation, occurred nearly 100 year ago, these entomophatogenic fungi have been considered in the development of mycopesticides for the biological control of pest insects (Zimmermann 2007, Rehner, Minnis et al. 2011). In addition, *Beauveria* spp. are reported to be able to produce large amounts of oxalic acid. This ability allows fungi belonging to this genus to be highly resistant to heavy metals. In fact, as previously described in the section *1.5. Metabolic activities regarding iron*, oxalic acid can chelate metals to produce insoluble metal-oxalates complexes decontaminating the fungal growth environment (Gadd 1993). Exploiting this ability, Joseph, Cario et al. (2011) developed an innovative stabilisation methods for the protection of copper-based alloys. The main idea was to convert a part of the unstable corrosion layer of outdoor monuments and archaeological objects into more stable biogenic copper oxalate with the fungus *B. bassiana* and creating then a protective layer of stable crystals. After the promising results that led to the production of a kit for the conservation-restoration of corroded copper-based surfaces, the same research group tested the efficacy of *B. bassiana* for the stabilisation of iron-based surfaces through the production of biogenic iron oxalates. First experimental evidence

suggested that this fungus, in certain conditions, was able to produce hyphae coated with iron oxalates. For this reason in the present study, the ability to produce iron biogenic minerals was investigated in *B. bassiana*, and the potential of this fungus for the development of stabilisation methods for corroded iron was assessed.

1.10.3. *Alternaria* sp.

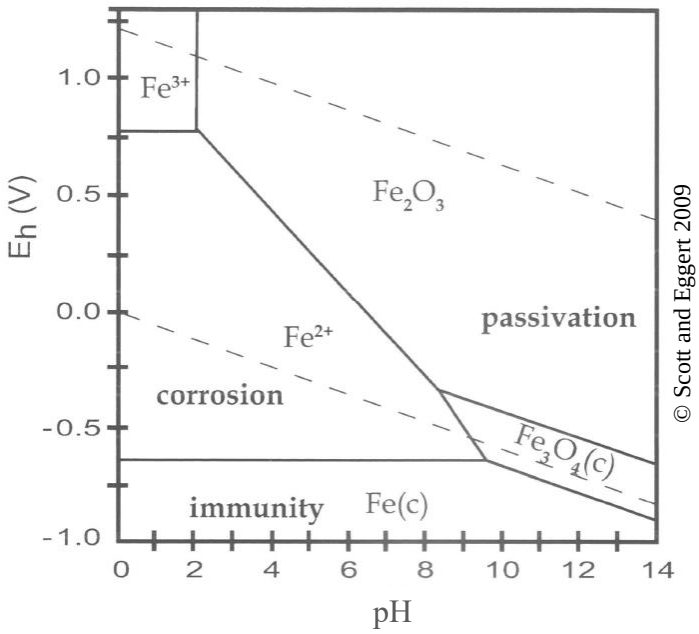
The genus *Alternaria* genus belongs to the phylum of Ascomycota, class of Dothideomycetes, subdivision of Pezizomycotina, order of Pleosporales and family Pleosporaceae. Over hundred species of the genus *Alternaria* have been described (Simmons 1992). The key characteristic of this genus is the presence of multicellular and melanised conidia presenting transvers and longitudinal septa. These asexual spores are produced in single branched chains by erected conidiophores (Thomma 2003).

Fungi belonging to the genus of *Alternaria* are mainly saprophytes, considered as agents of decay able to colonise different types of environment, such as soil, wood, and rocks (Sterflinger 2000). Nevertheless, most of the species of the genus *Alternaria*, such as *A. alternata* and *A. brassicicola* are considered to be also phytopathogens, causing economically significant diseases like leaf spot or leaf blight on different types of important agronomic plants (e.g. vegetables, fruits, oilcrops, and cereals). In addition, these fungi are also considered as post-harvested pathogens, degrading fruits, vegetable and crops during storage. Finally, *Alternaria* spores are frequently reported as airborne allergens, and in immune-compromised patients this fungus can cause severe infections (Thomma 2003).

Alternaria species are reported to produce high amounts of melanin. This compound is a dark pigment with a high molecular weight, produced by different kinds of organisms, such as animals, plants, and also microorganisms by an oxidative polymerisation of phenolic or indolic compounds (Bell and Wheeler 1986, Thomma 2003). This pigment has multiple roles in fungal resistance. In fact, several studies have demonstrated that melanin has a protective effect against different harsh environmental conditions, such as extreme temperature, UV-radiation, compounds secreted by microbial antagonists, and metal contamination (Lockwood 1960, Gadd and de Rome 1988, Rehnstrom and Free 1996, Kawamura, Tsujimoto et al. 1999, Thomma 2003). Indeed, as previously described (*1.5. Metabolic activities regarding iron*) melanin is a pigment with metal

chelating properties. Then, the ability of *Alternaria* sp. to adsorb iron was evaluated with the aim of developing a biocleaning assay for corroded iron.

1.11. Supplementary information



Supplementary figure 1: Pourbaix diagram based on the total concentration of iron ions ar 1.00 µmol/L in water at 25 °C. The areas of passivation, corrosion, and immunity of iron to corrosion under these condition are shown on the diagram (generated using MEDUSA). Ions concentration and temperature are important factors influencing the stability of the different phases present. Each purbaix diagram is defined for a specific temperature an ions concentration.

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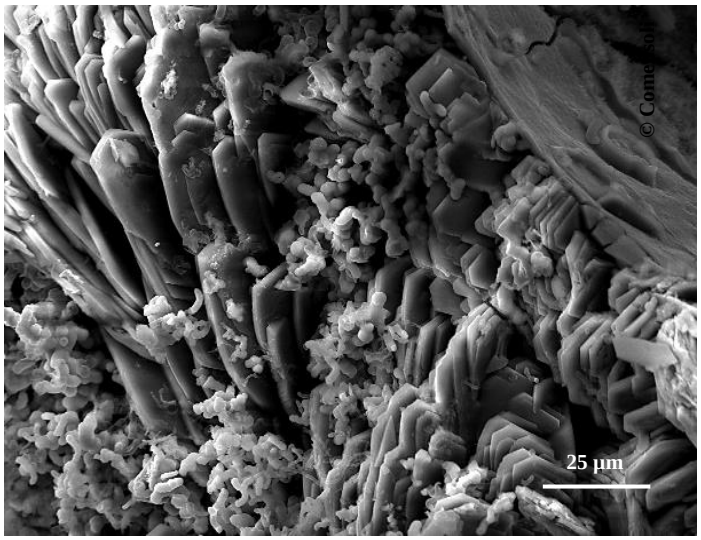
CHAPTER 2

Use of bacteria to stabilize archaeological iron



Biogenic crystals produced by *D. hafniense*

This chapter is based on the results of the following accepted article:

Comensoli L., Maillard J., Albini M., Sandoz F., Junier P. & Joseph E. (2017). Use of bacteria to stabilize archaeological iron. *Applied and Environmental Microbiology*. doi:10.1128/AEM.03478-16

and on the following articles in preparation:

Comensoli L., Kooli W., Albini M., Maillard J., Woerle M., Junier P. & Joseph E. Evaluation of the performance of a biotechnological treatment on corroded iron artworks. *Microchemical Journal 2017*. In preparation.

Kooli W., Comensoli L., Maillard J., Gelb A., Albini M., Junier P. & Joseph E. Production and control of biogenic minerals for the protection of corroded iron objects. *Nature communication* 2017. In preparation.

2.1. Abstract

Although bacterial iron reduction and biogenic iron mineral production have been studied extensively (Fredrickson, Zachara et al. 1998, Frankel and Bazylinski 2003), only a few studies have focused on the exploitation of this ability to protect iron surfaces (Volkland, Harms et al. 2000, Cote, Rosas et al. 2015), without the consideration of the stabilization of archaeological iron objects. Archaeological objects are extremely fragile and require specific conservation treatments aiming to remove chloride ions contained in the corrosion layer, as well as to passivate the unstable metallic substrate. In this chapter, the feasibility of a bacterial treatment for the stabilization of archaeological iron objects with the train TCE1 and LBE of *Desulfitobacterium hafniense* is presented. Our hypothesis is that bacterial iron reduction could be successfully used for the conversion of unstable corrosion products into stable biogenic minerals having a stabilisation effect. The validation of the hypothesis was made with the following steps summarized in the Figure 4. First, an in-deep investigation of the ability of the chosen strains to reduce soluble and solid iron phases was carried out. Iron reduction was observed for soluble Fe(III) phases as well as for akaganeite, the most troublesome iron compound in the corrosion layer of archaeological iron objects. In terms of biogenic mineral production, differential efficiencies were observed in assays performed on corroded iron coupons. Strain TCE1 produced a homogeneous layer of vivianite covering 80% of the corroded surface, while on the coupons treated with strain LBE, only 10% of the surface was covered by the same mineral. In addition, an attempt to reduce iron on archaeological objects was performed with strain TCE1, which led to the formation of both biogenic vivianite and magnetite on the surface of the artefacts. Finally, cross-section investigation of the newly formed biogenic layer allowed evidencing a potentially detrimental layer containing sulphur between the original corrosion layer and the newly formed biogenic crystals. This study demonstrates that microbial biogenic mineral production is a promising alternative to develop new methods for the stabilisation of corroded iron. However, great attention has to be dedicated to the medium composition, which not only influences the type of biogenic mineral produced, but also contaminates the corrosion layer with undesired compounds able to instigate further corrosion, such as sulphur-containing compounds. At the end of this chapter results obtained through a

collaborative research on the evaluation of the performance of a second bacterial treatment are briefly presented.

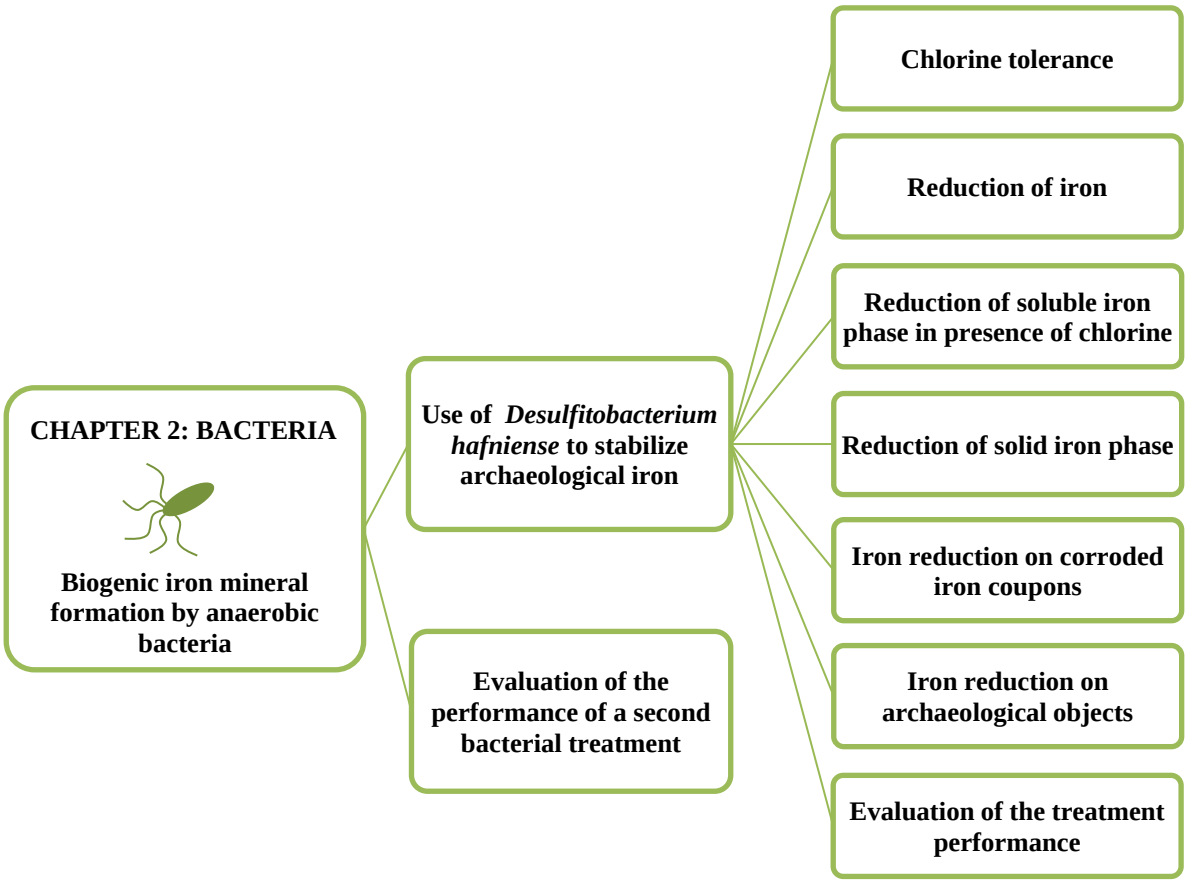


Figure 4: Graphic summary of the overall structure of the chapter 2: Biogenic iron minerals production by anaerobic bacteria

2.2. Introduction

Since the Iron Age, iron has been a fundamental material for the building of objects used in everyday life. However, due to its reactivity, iron can be easily corroded, and the physical stability of the object built is at risk. This is particularly true for archaeological objects on which a potentially unstable corrosion layer is formed during the time the object is buried. After excavation, changes in environmental conditions (e.g., higher oxygen concentration or lower humidity) alter the stability of the corrosion layer and can lead to the total destruction of the object. In this study, we demonstrate the feasibility of an innovative treatment based on bacterial iron reduction and biogenic mineral formation to stabilize the corrosion layer and protect these objects.

Recently, increasing attention has been given to the development of biotechnological solutions involving fungi and bacteria to preserve metal surfaces. For example, the fungus *Beauveria bassiana* has been employed to passivate corroded copper based alloys for outdoor monuments and archaeological objects (Joseph, Simon et al. 2012). Experimental evidence demonstrated that respiration of aerobic bacteria attached to copper surface in freshwater and seawater reduces the corrosion rate by decreasing the oxygen content nearby the metal (Zarasvand and Rai 2014). In addition, several studies reported the protective behaviour of specific biofilms for submerged carbon steel pipelines by different mechanisms (Volkland, Harms et al. 2000, Mansfeld, Hsu et al. 2002, Zuo, Örnek et al. 2004, Chongdar, Gunasekaran et al. 2005). One of them consists of the production of a protective coating of iron phosphates by exploiting the bacterial ability to produce biogenic minerals on iron surfaces. For instance, electrochemical impedance spectroscopy demonstrated that the presence of a biogenic layer of vivianite produced by *Geobacter sulfurreducens* on the surface of carbon steel coupons has a protective behaviour against corrosion after exposure to oxygen (Cote, Rosas et al. 2015).

The coupling of iron reduction with the biogenic formation of biogenic mineral phases was evaluated in this study as an alternative method to stabilize the corrosion layer of archaeological iron artefacts. The anaerobic Firmicute *Desulfitobacterium hafniense* was selected due to the ability of the members of this genus to use a large spectrum of electron acceptors, including nitrate, sulphite, metals, humic acids, and halogenated organic compounds (Villemur, Lanthier et al. 2006). In addition, previous studies

reported the ability of various members of the *Desulfitobacterium* genus to reduce ferric iron (Christiansen and Ahring 1996, Gerritse, Drzyzga et al. 1999, Niggemyer, Spring et al. 2001, Villemur, Lanthier et al. 2006). Finally, this bacterial species is not reported as a human pathogen (biosafety risk group 1 [Federal Institute for Occupational Safety and Health (2006)]). In this study, we first evaluated iron reduction in the presence of chloride ions. Second, we assessed the potential of *D. hafniense* to reduce the corrosion layer of corroded iron coupons and archaeological iron artefacts containing chlorine. Finally, we characterized the biogenic minerals produced, and we evaluate the efficiency of the treatment through stratigraphic analyses on cross-sectioned samples.

2.3. Material and methods

2.3.1. Bacterial strains and growth conditions

D. hafniense strains TCE1 (DSM 12704) and LBE were used in this study. Strain TCE1 was isolated from enriched cultures dechlorinating tetrachloroethene (Gerritse, Drzyzga et al. 1999), while strain LBE was isolated as contaminant from a *Dehalobacter*-containing culture at the Laboratory of Environmental Biotechnology (LBE), EPFL, Switzerland. The draft genome of strain LBE is available in the repository of DOE Joint Genome Institute (http://genome.jgi.doe.gov/DesspLBE_FD/DesspLBE_FD.info.html). To verify the identity of the strain, an Average Nucleotide Identity analysis (ANI) was performed using the complete genome of *D. hafniense* strain DCB-2 as a reference (GenBank accession number NC_011830.1). The results show a mean ANI of 97.99% with the reference genome confirming the identity of the LBE strain (Supplementary figure 2). The entire 16S rRNA gene sequence of the strain has been submitted to GenBank under accession number KY554954. *S*trains TCE1 and LBE were cultivated under anaerobic conditions (500 mL serum bottles capped with butyl rubber stoppers filled with 200 mL anaerobic medium) in a standard mineral medium as previously described (Prat, Maillard et al. 2011). The exact composition of the medium is provided as supplementary information. For the experiments the medium contained 45 mM lactate and 20 mM fumarate, as electron donor and acceptor respectively. This standard medium was modified for the iron reduction experiments as described below. All the cultures were incubated at 30°C under agitation at 100 rpm until reaching an OD_{600} between 0.100 and 0.17 for strain TCE1 and 0.0.09 and 0.13 for strain LBE. The pH of the cultures was buffered at a value of 7.3 with a combination of phosphates (4 mM

K_2HPO_4 and 1 mM NaH_2PO_4), carbonates (54 mM $NaHCO_3$ and 6 mM NH_4HCO_3), and N_2/CO_2 (80%/20%) in the headspace.

2.3.2. Chlorine tolerance

In order to test the chlorine tolerance of strains TCE1 and LBE, the standard medium was amended with the following NaCl concentrations: 0, 0.2, 0.5, 1, 1.5 and 2% [wt/wt] in deionised water (Sigma-Aldrich, USA). Bacterial growth was evaluated by measuring optical density at 600 nm with a UV-Vis spectrophotometer (Thermo Scientific, USA) after 0, 1, 2, 3, 4, 6, and 7 days of incubation at 30°C. To evaluate iron reduction cultures with and without fumarate were performed.

2.3.3. Iron reduction test

Soluble iron reduction was assessed by adding Fe(III)-citrate (Fluka Analytical, Germany) at a final concentration of 10 mM to bacteria cultivated for 3 days at 30°C in standard medium. Aliquots of 0.5 mL were collected at 0, 6, and 16 days after the addition of iron citrate. The timing of the sampling was decided in accordance to the visual inspection of the cultures. The samples were filtered at 0.22 µm and the total dissolved iron concentration was measured in the filtrate using a multiparameter ion specific meter (C211 HANNA INSTRUMENT, Portugal). This approach was selected as a precipitate was formed during incubation with Fe(III)-citrate (presumably solid Fe(II)) and thus measuring the total dissolved iron concentration was indicative of the dissolved Fe(III) concentration still in solution. In order to evaluate the amount of Fe(III) reduced to Fe(II), the relative amount of Fe(III) was expressed as the percentile ratio between the remaining and the initial dissolved Fe(III) concentration. Non-inoculated medium was included as abiotic control. In order to study bacterial metabolism during iron reduction, additional samples were collected at 0, 6, and 16 days. In these samples lactate, acetate, fumarate, and succinate were measured by High-Performance Liquid Chromatography (HPLC) in 0.5 mL samples after protein precipitation by adding 75 µL of $ZnSO_4$ 0.15 M in deionised water (Fluka Chemika, Switzerland) and 75 µL $Ba(OH)_2$ 0.15 M in deionised water (Fluka analytical, Germany) and filtering at 0.22 µm, as described previously (Lochmatter, Maillard et al. 2014). Standard solutions of individual organic acids were used to establish respective calibration curves.

2.3.4. Reduction of soluble iron phase in presence of chlorine

Soluble iron reduction was assessed by adding Fe(III)-citrate (final concentration 10 mM) (Fluka Analytical, Germany) to 20 mL of a bacterial culture incubated 1 day in standard medium at 30°C. In order to avoid changes in the pH of the cultures the pH of the Fe(III)-citrate solution was adjusted to 7 by adding drops of 10 mM NaOH. In order to assess the influence of chlorine in iron reduction, cultures were also amended with 0.2 % and 0.5 % of NaCl (Fluka Analytical, Germany). Abiotic controls without bacteria were also prepared. Aliquots of approximately 0.5 mL were collected at 0, 1, and 2 days after the addition of iron citrate. These samples contained the bacterial cells as well as the black precipitate produced during incubation. Iron reduction was evaluated by the quantification of Fe(II) with the Ferrozine® assay as previously described (Stookey 1970) with a few modifications. Briefly, to measure the total concentration of Fe(II), 450 µL of the previous sample was mixed with 50 µL of 5 M HCL to solubilize Fe(II) present in solid phase (black precipitates). 10 µL of this solution were transferred in a microplate containing 90 µL of 2 mM ferrozine® reagent (Sigma-Aldrich, Germany) prepared in 100 mM HEPES solution (Sigma-Aldrich, United States). The absorbance was measured within 2 minutes at 562 nm with a UMV-340 microplate reader (Asis Hitech, Austria). The amount of Fe(II) was calculated through a calibration curve with known concentrations of ferrous ammonium sulfate as Fe(II) standard. All the experiments were conducted in triplicate.

2.3.5. Reduction of solid iron phase

Considering that the majority of iron compounds found in the corrosion layer of archaeological iron objects are not soluble in aqueous solutions, iron reduction was also studied by analysis of solid iron phases. For this, the standard medium described above was amended with synthetic akaganeite ($FeO_{0.833}(OH)_{1.167}Cl_{0.167}$) at a concentration of approximately 10 mM. Synthetic akaganeite was prepared following a published procedure (Schwertmann and Cornell 2008). In order to confirm the purity of the synthetic akaganeite, Raman spectroscopy was conducted on the mineral before the experiment (Supplementary figure 3). Abiotic controls without bacteria but containing akaganeite were also prepared. Akaganeite-containing solution was added to 20 mL of a 1-day bacterial culture obtained in standard medium. Homogeneous samples of the culture, containing bacterial cells, as well as the black precipitate produced during

incubation, were collected after 0, 1, and 2 days of incubation, and the Fe(II) concentration was determined with the Ferrozine® assay as described in the previous section. All the experiments were conducted in triplicate.

2.3.6. Iron reduction on corroded coupons

In order to evaluate iron reduction of a real object, steel coupons presenting a natural corrosion layer produced after outdoor exposure in the city of Zurich were used as an iron source. Before the incubation the coupons were sterilized by spraying a solution of ethanol 70 % w/w in deionized water, followed by exposure to UV radiation (20 min each side). Next, each sample was placed in an anaerobic serum bottle. Anoxic conditions were obtained by replacing the aerobic atmosphere with N_2. After a second sterilization through autoclaving (120°C, 20 min), 20 mL of the cultures of strains TCE1 and LBE, as well as the abiotic control were added to the serum bottle containing the sterile coupons. All the experiments were conducted in triplicate.

Cultures containing the coupons were incubated at 30 °C for 7 days. After the incubation coupons were washed with a solution of ethanol 70 % w/w in deionized water and exposed to UV radiation (20 min each side), in order to remove remaining bacteria. Scanning Electron Microscopy coupled with Energy Dispersive X-Ray Spectroscopy (SEM-EDS) was carried out in order to evaluate the habit (size and shape of the crystals), the distribution, and the elemental composition of the newly formed biogenic minerals. Coupons were directly analysed simply by positioning them inside the microscope chamber without preparation. A Philips ESEM XL30 FEG environmental scanning electron microscope equipped with an energy-dispersive X-ray analyser was used. The samples were observed in secondary electrons mode at an acceleration potential of 10–25 keV and a working distance of 10 mm. The homogeneity of the biogenic layer was evaluated through an estimation of the surface covering by stereoscopic observations (Nikon SM218, Japan). To study the composition of the corrosion layer before and after the bacterial treatment, non-destructive Raman Spectroscopy was performed directly on the surface of the coupons. The analysis was carried out with a Horiba-Jobin Yvon Labram Aramis microscope equipped with a Nd:YAG laser of 532 nm at power lower than 1 mW (600 gr/mm). The spectral interval was 100 and 1600 cm^{-1} and the measurement conditions were 1000 µm hole, 100 µm slit and 5 accumulations of 100 s. The spectra recorded were corrected (automatic

baseline correction) by using LabSpec NGS Spectral software. Reference spectra were used for the identification of the compounds present.

2.3.7. Iron reduction on archaeological objects

Archaeological iron nails provided by a metal conservator-restorer were used for the treatment of real objects. These nails have been recovered from the Mediterranean Sea and presented a typical submarine corrosion layer. After sandblasting the artefacts were prepared as described above for the iron coupons. After autoclaving 20 ml of 1-day cultures of strain TCE1 as well as 20 ml of medium without bacteria as an abiotic control were added to the anaerobic flasks containing the nails. The cultures were incubated for 3 additional days. The sterilization process after the bacterial treatment was performed as described above for the iron coupons. All the experiments were conducted in triplicate. Nail surfaces were studied before and after the treatment by SEM-EDS and Raman spectroscopy as described for the iron coupons. Microsampling of the crystals was required for the Raman analyses.

2.3.8. Evaluation of the treatment performance

One sample for each conditions tested (untreated, bacterial treatment, and abiotic control) was entirely embedded in methacrylate resin employing the EpoFix Kit (resin and hardener), Struers. Cross-polishing was performed using successive silicon carbide abrasive papers with 250, 500, and 1000 grit and Micro-Mesh abrasive cloths 1800, 2400, 3200, 3600, 4000, 6000, 8000, and 12000 grades. In order to assess the efficiency of the bacterial treatment, cross-sectioned samples were analysed with optical and scanning electron microscopy, as well as with Raman spectroscopy (Figure 5).

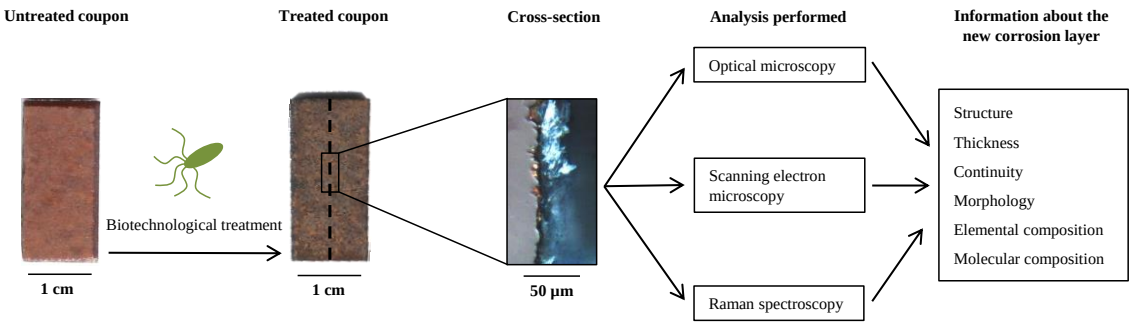


Figure 5: Schematic representation of the analytical methods performed during this study.

Microscopic observations were performed on the cross-sectioned samples in order to study the appearance and the thickness of the corrosion layer, as well as the continuity of the layer of biogenic crystals. To verify the continuity of the layer composed of newly formed biogenic minerals, an estimation of the percentage of original corrosion layer covered by the biogenic crystals was extrapolated from the microscopic images. Observations were carried out with a Reichert Jung Polyvar MET optical microscope, and data were collected with Axio Vision LE software.

SEM-EDS mapping was carried out to evaluate the distribution, and the elemental composition of the newly formed biogenic minerals. Coupons were directly analysed simply by positioning them inside the microscope chamber without preparation. A Philips ESEM XL30 FEG environmental scanning electron microscope equipped with an energy-dispersive X-ray analyser was employed. The samples were analysed in secondary electrons mode at an acceleration potential of 10 to 25 keV and a working distance of 10 mm. For elemental mapping a resolution of 64x50 pixels and a dwell of 1000 were employed.

To study the molecular composition of the corrosion layer before and after the bacterial treatment, non-destructive Raman spectroscopy was performed directly on the cross-sectioned samples. The analysis was carried out with a Horiba-Jobin Yvon Labram Aramis microscope equipped with a Nd:YAG laser of 532 nm at a power lower than 1 mW. The spectral interval analysed was between 100 and 1600 cm^{-1}. Single points analyses were carried out with the following conditions: 400-µm hole, 200-µm slit, and 10 accumulations of 10 s. Raman mapping was performed in selected areas of cross-sectioned samples with a step size of 2.5 µm in x and y directions. The conditions previously described were used also for these analyses. The spectra recorded were corrected (automatic baseline correction) using LabSpec NGS spectral software. Reference spectra were used for identifying the compounds present and for elaborating chemical maps.

2.4. Results and discussion

The conservation-restoration methods for archaeological iron objects currently available present multiple disadvantages. To provide a sustainable and ecologically friendly alternative, we studied iron reduction in the presence of chlorine with *D. hafniense* strains TCE1 and LBE. We also examined biogenic mineral production as the result of iron reduction on archaeological objects.

2.4.1. Chlorine tolerance

Optical density measurements in the cultures amended with different concentrations of NaCl allowed to study the behaviour of the strains TCE1 and LBE of *D. hafniense* in presence of chloride ions. It appears that the growth of both strains was not affected up to a concentration of 0.5% of NaCl (Figure 6). Previous studies have shown that an archaeological object may contain an average chlorine concentration of 50 mM, which corresponds to ~0.3% [wt/wt] in deionised water (Rimmer, Watkinson et al. 2012). Therefore, these results suggest that both TCE1 and LBE strains are potential candidates for a bacterial treatment aiming to preserve archaeological iron in objects contaminated with chlorine.

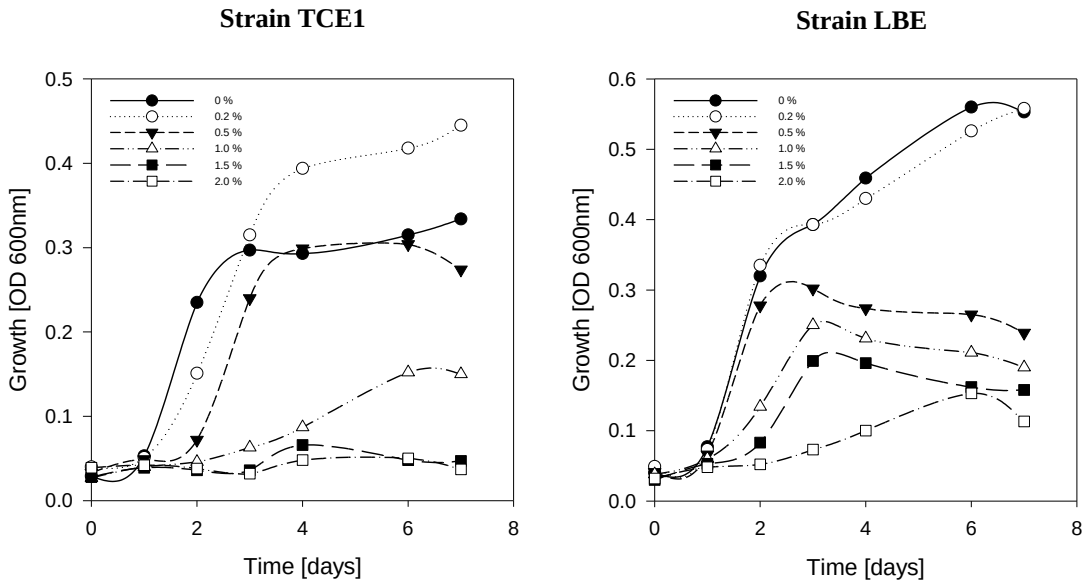


Figure 6: Growth curves of the strains TCE1 and LBE of *D. hafniense* exposed to different concentrations of NaCl (0, 0.2, 0.5, 1.0, 1.5, and 2.0 %).

2.4.2. Iron reduction

Several *Desulfitobacterium* species are reported to be able to reduce Fe(III) as a result of anaerobic respiration (Villemur, Lanthier et al. 2006). The capability of *D. hafniense* (strains TCE1 and LBE) to reduce soluble iron was assessed here. A black precipitate was observed after six days of incubation with Fe(III)-citrate in the cultures with strains TCE1 and LBE cultivated in standard medium containing lactate and fumarate. The culture medium of strain TCE1 became completely transparent, while the one of strain LBE remained brown. On the contrary, the abiotic control remained orange and no black precipitates were observed (Figure 7).

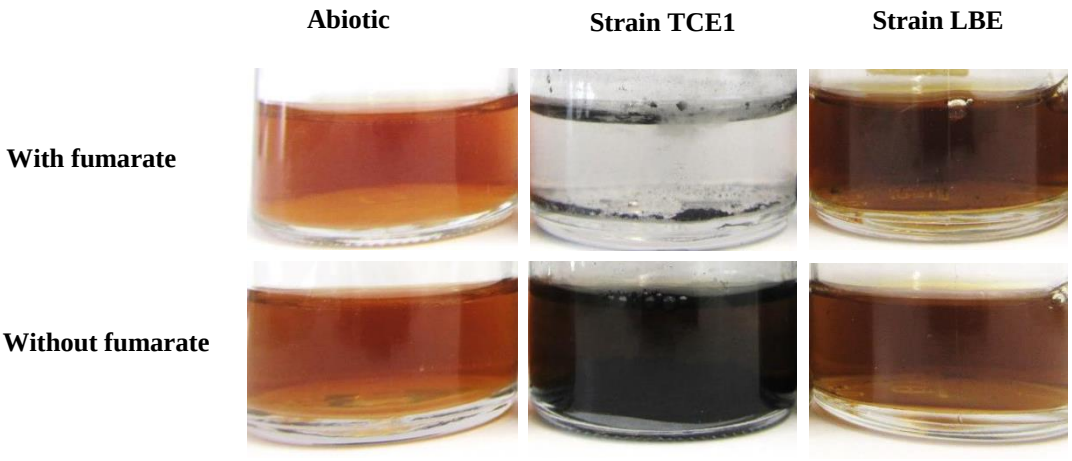


Figure 7: Appearance of the abiotic control as well as the culture of the strains TCE1 and LBE, with and without fumarate, after 6 days of incubation at 30 °C.

In order to better understand the metabolism involved in iron reduction and in particular the role of fumarate, incubation experiments were performed in presence and absence of this electron acceptor (Table 3). Considering the fact that a precipitate has formed in the active cultures, iron reduction was measured and expressed as the concentration of Fe(III) remaining in solution. A different behaviour was observed between TCE1 and LBE cultures in presence of fumarate. At day 6, 61% of the initial iron content was reduced in the TCE1 cultures, contrary to LBE cultures where only 7% of the initial iron was reduced. However at day 16, iron content in LBE cultures reached similar value as for TCE1. These results suggested a higher rate of iron reduction for strain TCE1.

Table 3: Estimation of iron reduction and lactate oxidation during incubation of strains TCE1 and LBE in absence or presence of fumarate. Standard deviation is calculated in % of averaged values.

	WITH FUMARATE					
	Strain TCE1			Strain LBE		
Time [Days]	**0**	**6**	**16**	**0**	**6**	**16**
Iron reduction [%]	0 ± 7	61 ± 2	63 ± 3	0 ± 0	7 ± 3	56 ± 2
Lactate oxidation [%]	4 ± 1	21 ± 3	76 ± NA	2 ± 0	2 ± 0	89 ± 56
	WITHOUT FUMARATE					
	Strain TCE1			Strain LBE		
Time [Days]	**0**	**6**	**16**	**0**	**6**	**16**
Iron reduction [%]	0 ± 7	8 ± 12	67 ± 1	0 ± 8	0 ± 19	29 ± 26
Lactate oxidation [%]	0 ± 12	0 ± 9	61 ± 0	0 ± 0	0 ± 0	86 ± 68

Iron reduction appeared to be less efficient for both the strains without fumarate. In fact, for TCE1 a similar amount of iron (67%) was reduced but only after 16 instead of 6 days. The absence of fumarate had an even more drastic effect for LBE cultures as only 29% of the initial iron content was reduced after 16 days of incubation, compared to 56% with fumarate. While for TCE1, iron reduction was coupled to lactate oxidation for LBE strain no meaningful conclusion can be drown due to a very high standard deviation. Nevertheless, this suggests that iron reduction is probably not directly coupled to respiratory activity in any of the two strains. In this case, iron could be used as an electron sink or can be reduced by other redox active molecules present in the bacterial membrane such as cytochromes (Park, Kim et al. 2001). Furthermore, the high standard deviations of the samples of the LBE strain after 16 days of incubation are due to a different behaviour between the replicates. This difference can be explained by the fact that iron respiratory and reduction abilities can easily be lost during sequential cultures (Luijten, Weelink et al. 2004). This suggests that LBE is not the best candidate because its metabolic activities are not predictable.

2.4.3. Reduction of soluble iron phase in the presence of chlorine

As chlorine is the most problematic compound present in the corrosion layer of archaeological objects (Selwyn 2004), iron reduction was studied in liquid cultures amended with this element. Quantification of Fe(II) revealed some chemical reduction of iron in the abiotic controls (Figure 8). This was probably due to the reductant (Na_2S) added in the culture medium to remove oxygen, which likely reduced iron, as well. However, in addition to this abiotic reaction, both strains clearly displayed iron reduction abilities. In fact, the amounts of Fe(II) measured after 2 days of incubation were significantly higher in the TCE1 and LBE cultures than the amount of Fe(II) measured in the abiotic control. For example, in the experiment without additional chlorine, the amounts of Fe(II) measured in the cultures of strains TCE1 and LBE after 2 days of incubation (10.9 and 11.1 mM, respectively), corresponded to the reduction of the entire iron source added (when taking into account potential pipetting errors). By contrast, for the same incubation time, only 5.2 mM Fe(II) was measured in the abiotic control.

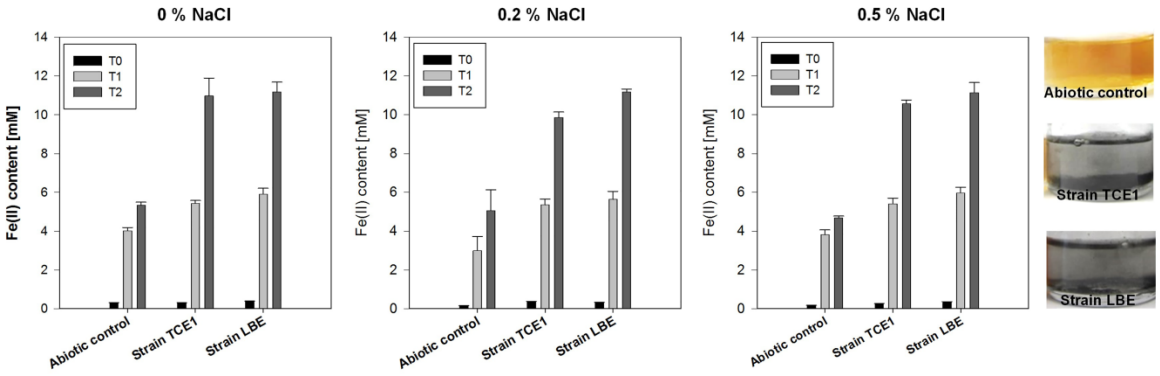


Figure 8: Reduction of iron citrate (10 mM initial concentration; dashed line in the graphs) in cultures of *Desulfitobacterium hafniense* strains TCE1 and LBE in the presence of chlorine. Three concentrations of NaCl (0, 0.2, and 0.5 %) were compared. Iron reduction was evaluated by the production of Fe(II) at 0, 1, and 2 days of incubation after the addition of the Fe(III) source. Abiotic controls were performed for each NaCl concentration tested. Fe(II) values correspond to the average and standard deviation of three independent replicates. A change in the appearance of the TCE1 and LBE cultures compared to the abiotic control is shown on the right hand side of the figure (example for the cultures at 0% NaCl after 2 days of incubation).

Moreover, a change in the aspect of the cultures was observed in contrast to the abiotic control. In the latter, the solution remained orange, while in the cultures, a black precipitate was observed (Figure 8). Overall, TCE1 and LBE strains performed similarly in terms of iron reduction. In the case of the development of a novel treatment

for stabilizing archaeological iron artifacts, iron reduction is the major prerequisite. However, the effect of chlorine on the bacterial activity must also be considered. In fact, a Cl^- concentration of up to 50 mM (~ 0.3% [wt/wt] in deionized water) has been reported for dissolved archaeological nails (Rimmer, Watkinson et al. 2012). In this study, the abilities of strains TCE1 and LBE to reduce iron in the presence of chlorine were evaluated at 0.2 and 0.5% NaCl. These values corresponded to the Cl^- concentration measured in archaeological iron artefacts (Rimmer, Watkinson et al. 2012). The results showed that up to 0.5% of NaCl did not affect bacterial iron reduction, as Fe(III) was also fully reduced in the cultures amended with NaCl (Figure 8).

2.4.4. Reduction of solid iron phases

To evaluate the reductive dissolution of iron present as a solid phase, synthetic akaganeite was added to the cultures. Fe(II) concentration was measured at regular time intervals (Figure 9). Within the first day, reductive dissolution was observed in the treatment with akaganeite. Fe(II) concentrations reached a maximum of 1.3 mM for strain TCE1 and 1.6 mM for strain LBE after 2 days of incubation. In contrast to the results obtained when reducing soluble Fe(III) phase, in the abiotic controls, Fe(II) increased only slightly and the maximal Fe(II) concentration measured was 0.4 mM (Figure 9). During incubation with akaganeite, a change in colour from yellow-green to black was also observed in the cultures.

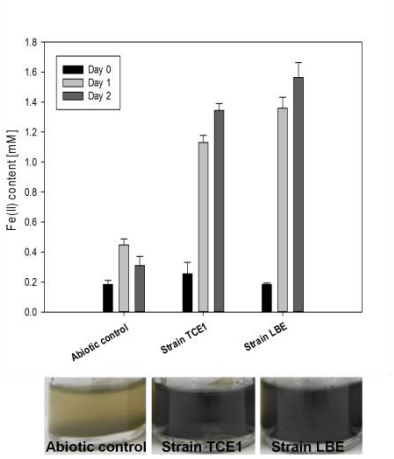


Figure 9: Reduction of akaganeite in cultures of *Desulfitobacterium hafniense* strains TCE1 and LBE. Reduction was measured by the quantification of Fe(II) after 0, 1, and 2 days of incubation in the presence of akaganeite. Abiotic controls were also performed. Fe(II) values correspond to the average and standard deviation of three independent replicates. A change in the appearance of the TCE1 and LBE cultures compared to the abiotic control is shown at the bottom of the figure.

By contrast, the appearance of the abiotic controls remained unchanged (Figure 9). With the observed production of Fe(II), we demonstrated that strains TCE1 and LBE reduced solid iron phases, particularly, akaganeite. As akaganeite represents the most problematic iron corrosion compound found on archaeological objects, its reduction by *D. hafniense* is very promising for the development of an alternative stabilization method to preserve iron. The role of biological reduction in the transformation of iron oxides such as akaganeite into crystalline Fe(II)-containing phases has already been studied using *S. oneidensis* strain MR1, *Shewanella pealeana* strain W3-7-1, and *Thermoanaerobacter ethanolicus* strain TOR-39 (Roh, Zhang et al. 2003, Emmerich, Bhansali et al. 2012). The results have underpinned the contribution of iron-reducing microorganisms in the geological formation of Fe(II)- and Fe(III)-bearing minerals in sediments and their role in the biogeochemical cycles of iron and carbon. Our results suggest that *D. hafniense* might be involved in the transformation of iron-bearing minerals in the environment. Regarding the mechanism of iron reduction, we hypothesize that Fe(III) acts as an electron sink, as described for the reduction of insoluble Fe(III) by *D. reducens* strain MI-1 during pyruvate fermentation (Dalla Vecchia, Suvorova et al. 2014).

2.4.5. Iron reduction on corroded iron coupons

Although the reduction of akaganeite is promising, the composition of the corrosion layer of archaeological iron objects is not homogeneous and varies according to the burial environment. Therefore, to validate bacterial iron reduction and biogenic mineral production on real artefacts, naturally corroded iron coupons were used. The corrosion layer was composed of a mixture of iron oxides and oxyhydroxides. This composition corresponds to what it is commonly considered as urban corrosion layer. The untreated coupons presented a homogeneous red-brown colour (Figure 10) and scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDS) revealed that iron and oxygen were the main elements found, while silicon, carbon, aluminium, and calcium were found as secondary elements (Table 4). After a 7-day incubation period with the two strains, changes in the colour, surface aspect, and its elemental composition were observed on the coupons (Figure 10 and Table 4).

The coupons used in the abiotic control turned to black-yellow, and spherical 1 µm aggregates covering 40% of the surface were observed. Besides iron and oxygen, carbon was also detected as a major element. In addition, several elements originating from the growth medium were detected as trace elements (Table 4).

Table 4: Elemental composition of the iron coupons.

	Elements identified			
	Before treatment	After treatment		
Composition category[a]		Abiotic control	Strain TCE1	Strain LBE
Main elements (>10%)	Fe, O	Fe, O, C	Fe, O, C	Fe, O, C
Secondary elements (between 10 and 1%)	Si, C, Al, Ca	Si, Al	P, S, Mg, Ca	P, S, Si, Ca, Al
Trace elements (<1%)	K, Mg, P	Ca, Na, K, Mg, S, P, Cl	Si, Al	Mg, K

[a]The percentages reported correspond to the atomic percentages.

The coupons treated with strain TCE1 turned grey, and rosette-like crystals composed of iron, oxygen, and carbon (as major elements), phosphorus, sulphur, magnesium, and calcium (as secondary elements), covered 80% of the surface. The same types of crystals were also detected on the coupons treated with strain LBE. However, the layer produced was not as homogeneous and covered only a small proportion of the surface.

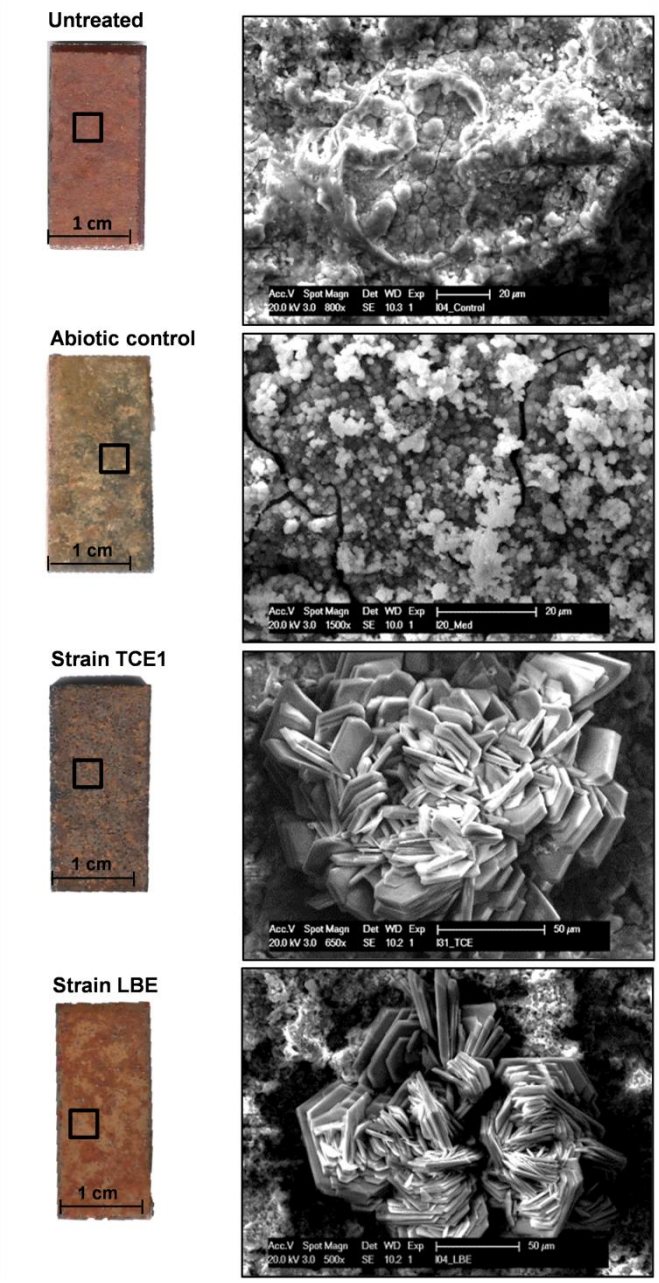


Figure 10: Iron reduction tests on corroded iron coupons treated with cultures of *Desulfitobacterium hafniense* strains TCE1 and LBE. On the left the appearance of the untreated coupon, the abiotic control and the coupons treated with the strains TCE1 and LBE, is shown. On the right the corresponding SEM images of the area indicated by a black square inside the coupons are presented.

Indeed, the coupons presented a rusty aspect in contrast to the coupons treated with strain TCE1. Likewise, rosette-like crystals with a similar elemental composition were also observed on the coupons of the abiotic control; however, they covered a minor proportion of the surface (Supplementary figure 4).

The biogenic minerals produced were identified by Raman spectroscopy. Before treatment, the corrosion layer of the coupons was mainly composed of lepidocrocite (Figure 11) (Monnier, Bellot-Gurlet et al. 2011). After the treatment, new minerals were detected on all the coupons. On the coupons of the abiotic control, small aggregates were observed with SEM and identified as a mixture of mineral sulphur (α-S_8) and poorly crystallized mackinawite [Fe(II)/Fe(III)S] (Rémazeilles, Tran et al. 2013). The additional large Raman shift between 980 and 1100 cm^{-1} and the presence of carbon as a major element in the EDS spectrum suggested the formation of amorphous siderite. The crystals on the coupons treated with strain TCE1 were identified as mainly vivianite [$Fe_3^{2+}(PO_4)_2 \cdot 8H_2O$] (Frost, Martens et al. 2002) (Figure 11), as were the crystals on the coupons treated with strain LBE. Nevertheless, other corrosion compounds, such as lepidocrocite, were also detected. In addition, small white-yellow aggregates were found and identified as elemental sulphur (Figure 11). Crystals found on the coupons used for the abiotic control were less abundant but similar to those on the coupons treated with strain TCE1 and also identified as vivianite (Supplementary figure 4). The partial abiotic reduction of the corrosion layer is likely the result of the reductant employed in the medium as indicated previously for the reduction of soluble Fe(III). On the basis of these results and regarding the treatment of a complex corrosion layer, we concluded that strains TCE1 and LBE have different efficiencies in terms of iron reduction on corroded coupons. With the aim of developing a biotechnological stabilization method for archaeological iron, it seems *D. hafniense* strain TCE1 would be more suitable as it produced a homogeneous layer of biogenic crystals of vivianite, a chemically stable iron phosphate compound.

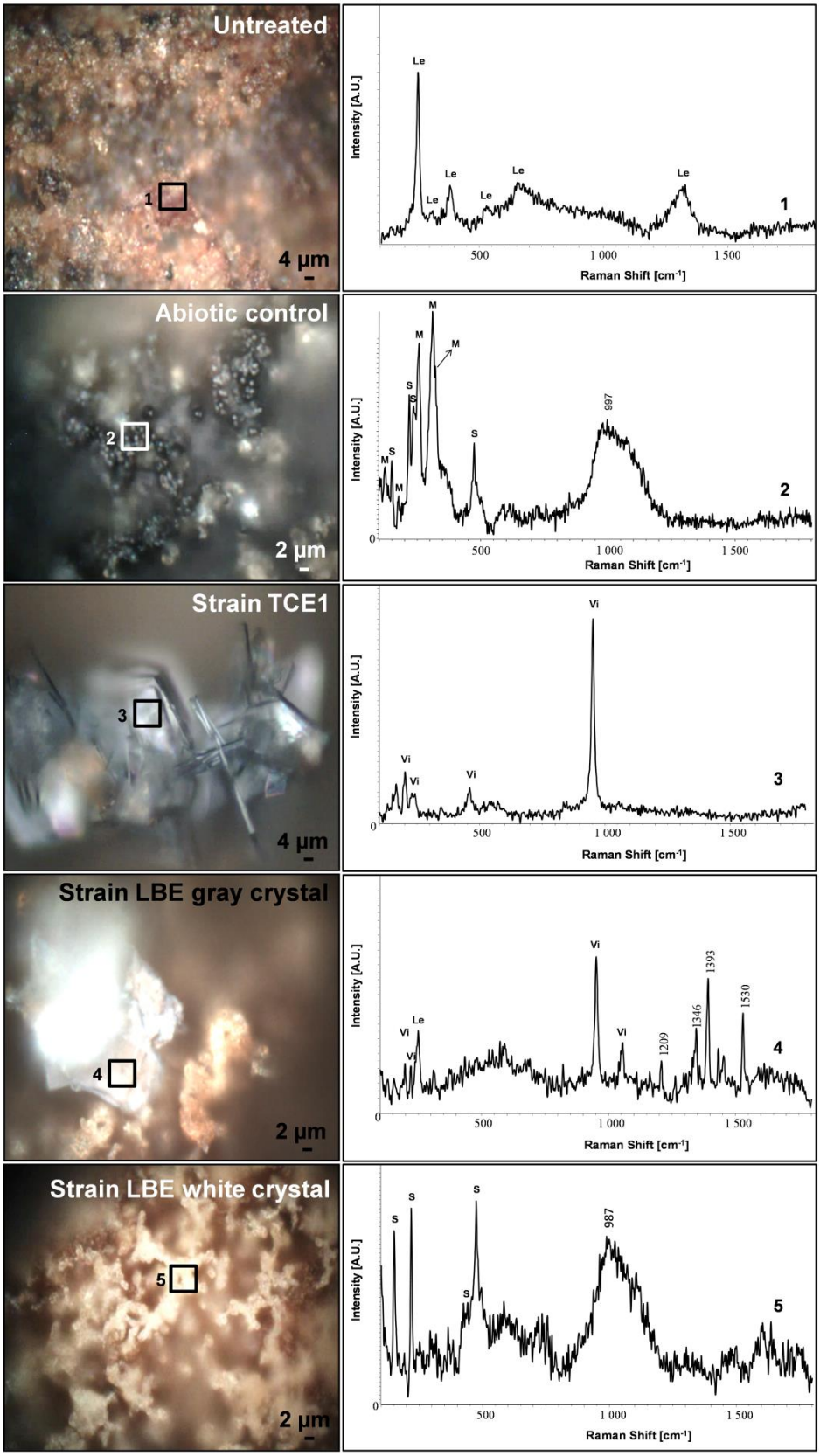


Figure 11: Identification of the iron mineral phases produced on corroded iron coupons after the treatment with strains TCE1 and LBE of *D. hafniense.* On the left, images of the area analysed by Raman spectroscopy (black squares) (original magnification 1000x); on the right, corresponding Raman spectra identified as; 1: Lepidocrocite (Le), 2: Mixture of poorly crystallized makinawite (M) and elemental Sulphur (S), 3: Vivianite (Vi), 4: Mixture of vivianite (Vi) and lepidocrocite (Le) and 5: Elemental Sulphur (S).

2.4.6. Iron reduction on archaeological objects

According to the above results on corroded iron coupons, only strain TCE1 was used for the evaluation of bacterial iron reduction on archaeological iron objects. First, the surfaces of nails were prepared by sandblasting according to the protocol traditionally employed for any conservation-restoration intervention. To ascertain the chemical composition of its corrosion layer, each object was characterized by non-invasive analytical techniques before and after bacterial treatment. Before treatment, the nails' surfaces presented a brown-red colour with orange spots (Figure 12). According to SEM-EDS analyses, crystals were not observed on the surfaces of the objects before incubation (Figure 12). Oxygen and iron were detected as the main elements, while aluminium was found as a secondary element (Table 5).

Table 5: Elemental composition of the corroded iron nails

	Element(s) identified			
	Abiotic control		Strain TCE1	
Composition category[a]	Before treatment	After treatment	Before treatment	After treatment
Main elements (>10%)	Fe, O	Fe, O	Fe, O	Fe, O, P
Secondary elements (between 10 and 1%)	Al	C	Al, Si	C
Trace elements (<1%)	Si, Ca	Al, Si, P, Ca	Ca, P	

[a]The percentages reported correspond to the atomic percentages.

After 3 days of incubation, compared with the results for corroded coupons, the nails used for the abiotic control remained unchanged; neither a colour change nor crystal formation were detected (Figure 12). This is probably due to the difference, in terms of cohesion, between the corrosion layers on the coupons and the nails. In fact, the coupons presented a powdery corrosion layer that had been formed recently and hence, is much more reactive and unstable than the corrosion layer found on archaeological objects aged for centuries. The aspect of the nails treated with cultures of strain TCE1 changed dramatically during incubation and their surfaces turned dark-grey. Moreover, the formation of biogenic minerals presenting a bladed cluster habit was identified during SEM observations (Figure 12).

EDS analyses on biogenic mineral indicated the presence of oxygen, phosphorus, and iron as the main elements, and carbon as a secondary element. No phosphorus was detected on the surface of the nails used for the abiotic control (Table 5).

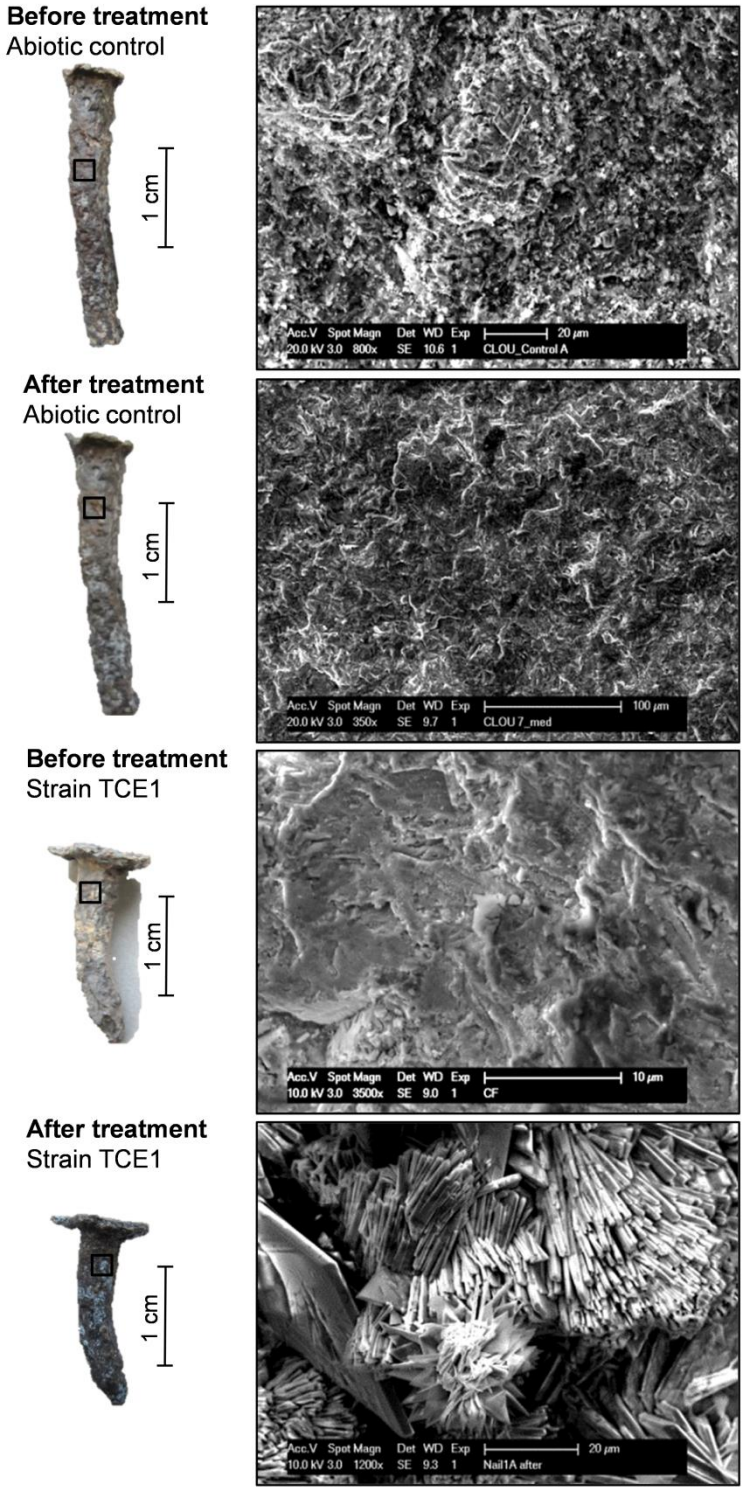


Figure 12: Iron reduction on archaeological iron nails using strain TCE1. On the left, the appearance of the abiotic control and the nails treated with strain TCE1 before and after the treatment is shown; on the right, the corresponding SEM images of the area indicated by black square inside the nails are presented.

Raman spectroscopy was carried out to identify the newly formed biogenic crystals. The corrosion compounds present before treatment were characterized as a mixture of goethite γ-FeOOH and lepidocrocite α-FeOOH (Figure 13). The same compounds were detected on the surface of the nails used for the abiotic control (Supplementary figure 4). Raman analysis on microsamples identified these biogenic minerals as magnetite (Fe_2O_4) and vivianite [$Fe_3^{2+}(PO_4)_2 \cdot 8H_2O$) (Figure 13). The ability of bacteria to produce vivianite is well documented (Fredrickson, Zachara et al. 1998, Glasauer, Weidler et al. 2003, O'Loughlin, Boyanov et al. 2013, Rothe, Kleeberg et al. 2016) and is generally linked to the activity of dissimilatory metal-reducing bacteria that couple organic carbon oxidation and Fe(III) reduction. However, in our study, vivianite production is not likely to be the consequence of iron respiration but rather of Fe(III) used as an electron sink. The production of iron phosphates might be the result of the presence of phosphate buffer in the culture medium, as it is known that the medium composition can affect the type of biogenic mineral formed (Bell, Mills et al. 1987, Roh, Zhang et al. 2003). The colour and appearance of the artefact is also an important criterion in conservation-restoration, and the black appearance obtained with the formation of magnetite would be accepted according to conservation ethics. By contrast, the formation of blue stains related to the precipitation of vivianite should be avoided.

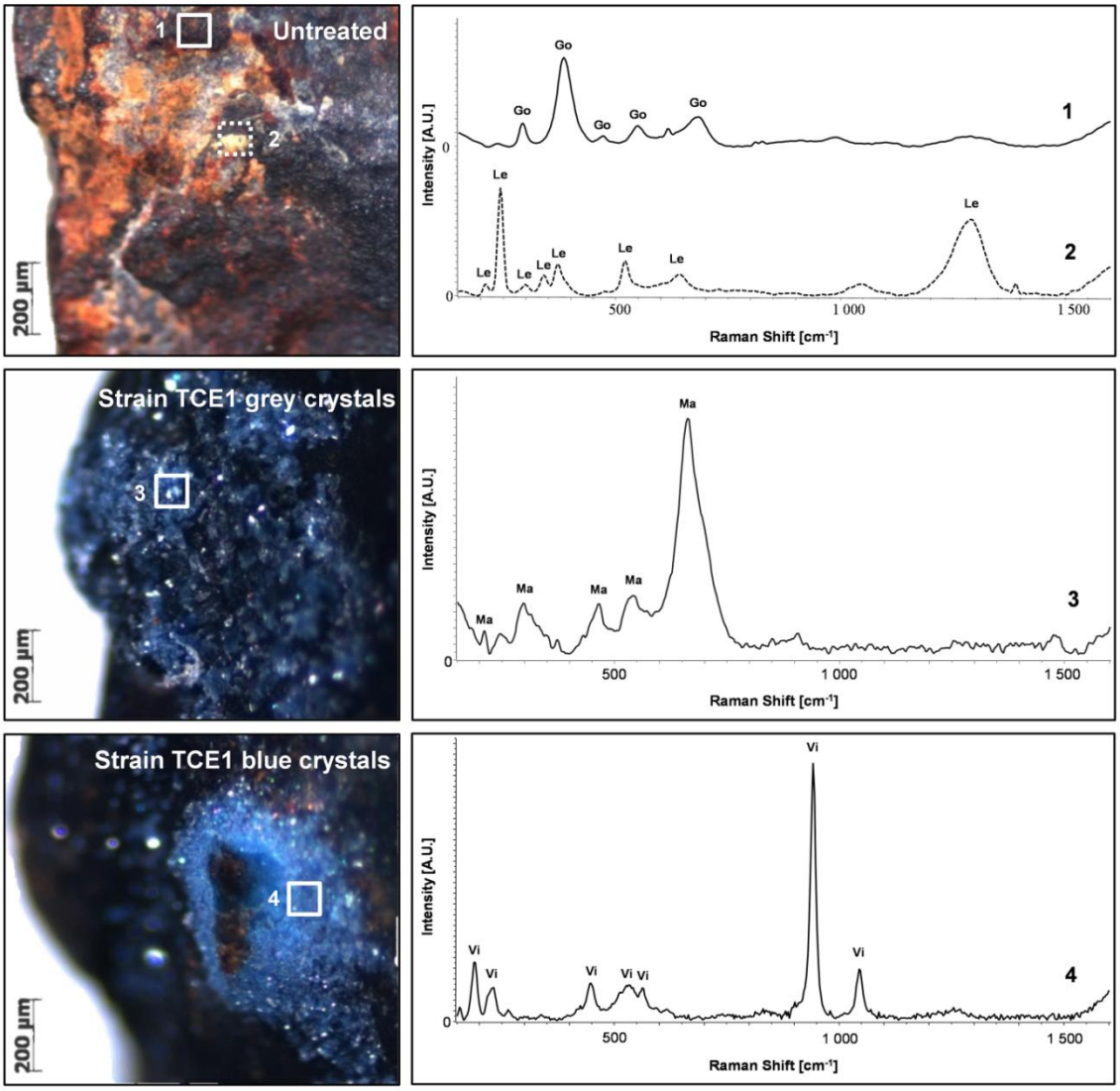


Figure 13: Raman analysis of the archaeological nails before and after the treatment with strain TCE1. On the left, stereoscope images of the area sampled for the analysis (original magnification 20x); on the right, the corresponding Raman spectra identified as; 1: Goethite (Go), 2: Lepidocrocite (Le), 3: Magnetite (Ma) and 4: Vivianite (Vi).

2.4.7. Evaluation of the treatment performance

Structure, thickness and continuity of the corrosion layer

After bacterial treatment the coupons' colour was completely different from the untreated one. Indeed, microscopic observations of untreated samples revealed a corrosion layer with brown, red and orange colours. The corrosion layer of the abiotic control coupons after the incubation had similar thickness and colour of the untreated one. On the outer part of this sample, some blue areas were also detected; however, those did not form a continuous layer (Figure 14). The formation of blue crystals in the abiotic control samples was probably the consequence of an interaction between iron corrosion products and the reducing agents present in the bacterial culture medium. On the other hand, after the bacterial treatment the original corrosion layer had almost disappeared, and a continuous layer of blue biogenic crystals was observed (Figure 14).

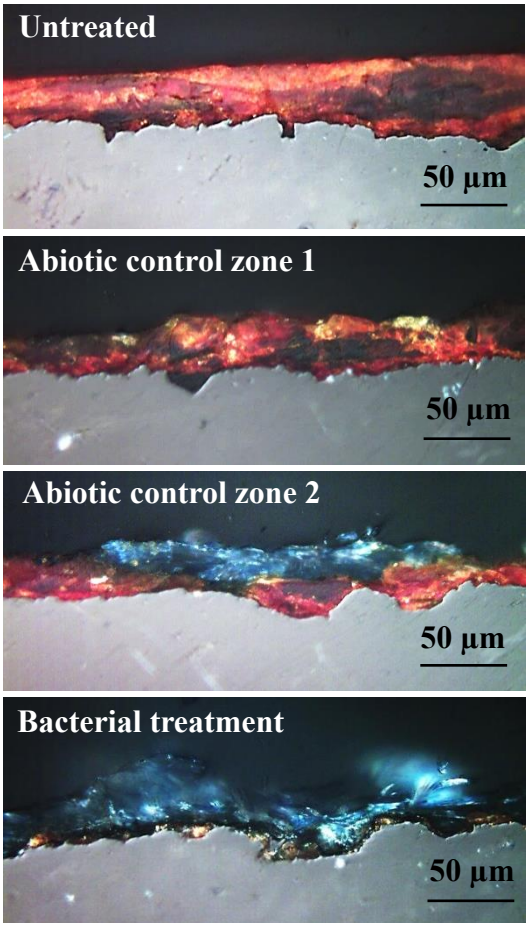


Figure 14: Microscopic images of the untreated, two contrasting zones in the abiotic control, and bacterially treated samples treated with the strain TCE1 of *D. hafniense*. Original magnification 500x.

Even if, the thickness of a natural corrosion layer is generally not homogenous, an overall decrease of the thickness of the original iron oxyhydroxides corrosion layer was observed in the samples treated with bacteria (Figure 14 and Figure 15 a and b). In fact, the average value of the thickness of the original corrosion layer, decreased from nearly 28 µm in the untreated samples, to about 7 µm in the treated coupons. The decrease of the thickness of the original corrosion layer is due to dissolutive microbial reduction of solid-iron phases composing the original corrosion layer. As a result part of the Fe^{3+} oxyhydroxides is converted into Fe^{2+} biogenic minerals (Figure 15 a and b). In this case it can be affirmed that microbial activity did not induce corrosion by attacking the metal substrate, but rather converted a part of the original corrosion layer into stables iron compounds.

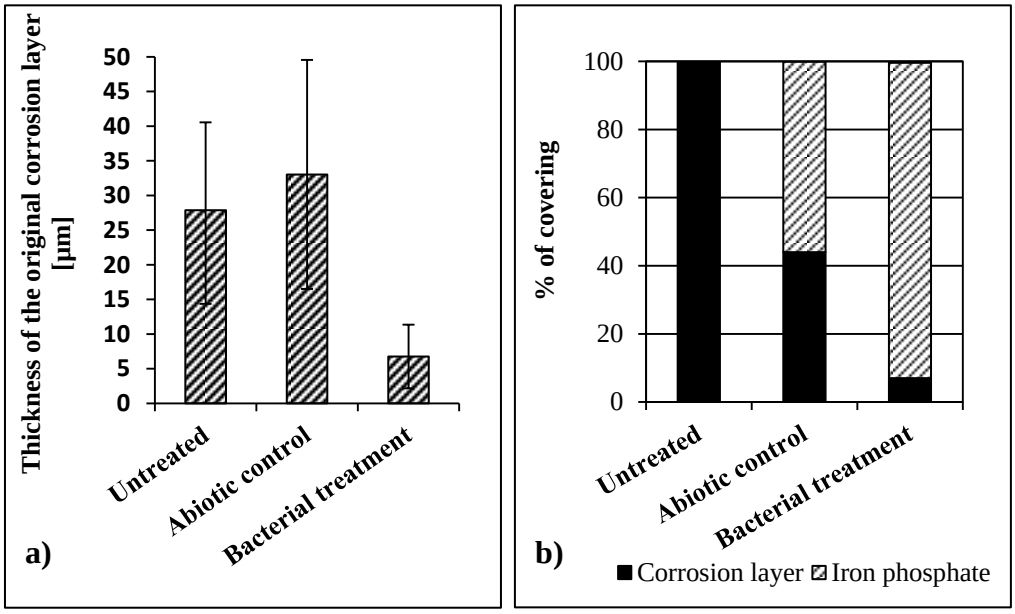


Figure 15: a) Graphic representation of the thickness of the corrosion layer of the untreated, abiotic control, and the bacterially treated samples with the strain TCE1 of *D. hafniense*. b) Estimation of the surface covered by newly formed crystals.

Another important characteristic analysed through cross-section observations was the continuity of the newly formed biogenic layer. Indeed, in order to be protective, this layer has to cover completely the remaining original corrosion layer, avoiding a maximum of future contact of the metal core with oxygen and moisture (Scott and Eggert 2009). Microscopic observations of the cross-sections demonstrated that after 7 days of incubation with bacteria, about 92 % of the surface analysed was covered by

biogenic crystals, while only 55 % of the original corrosion layer was covered by crystals in the abiotic control coupons (Figure 15 b). Therefore, it can be concluded that bacteria are needed to produce a homogeneous and continuous layer of biogenic minerals.

Elemental composition

The continuity of the newly formed biogenic minerals was confirmed also by SEM images. In fact, no discontinuity between crystals was observed on the areas analysed (Figure 16 a and b). Elemental mapping revealed that the corrosion layer after the bacterial treatment was mainly composed by iron (Fe), oxygen (O), sulphur (S), and phosphorus (P) (Figure 16). The same elements were detected on the corrosion layer of the abiotic control, while only Fe and O were found on the untreated samples (Supplementary figure 6 and Supplementary figure 7). In the coupons treated with bacteria, the layer (indicated as number 1 in Figure 16 b) on the metal core was mainly composed of Fe and O. This was the remaining part of the original corrosion layer (probably composed of iron oxyhydroxides) not converted into biogenic crystals. A second layer (indicated as number 2 in Figure 16 b) mainly composed of S was observed towards the external part of the corrosion layer. This layer was detected also on abiotic samples (Supplementary figure 7). Finally, a layer (indicated as number 3 in Figure 16 b) composed of Fe, O, and P, was detected and corresponded to area covered by biogenic crystals. The presence of a layer composed of sulphur under the biogenic crystals is an interesting result. In fact, a previous study from Comensoli, Maillard et al. (2017) showed the formation of elemental sulphur (S_8) and partially oxidized makinawite ($Fe^{2+}/Fe^{3+}S$) on the surface of coupons used as abiotic control. Whereas these compounds were not detected on the surface of the coupons incubated with bacteria. Analysing cross-sectioned samples, the current study demonstrates that a layer mainly composed of S is also present on the bacterially treated coupons, and is localised between the remaining original corrosion layer and the biogenic minerals. This layer is probably the results of an abiotic reaction between the Na_2S added to ensure anoxic conditions and the surface of the corroded iron coupons. Since, this layer is located under the biogenic crystals, and since formation of these latter imply the dissolutive reduction of iron compounds, it can be then assumed that this layer of sulphur was produced before the formation of biogenic crystals. This sulphur layer is already

reported for iron corrosion developed in anaerobic environment (Bellot-Gurlet, Neff et al. 2009). The effect of sulphur on the corrosion process of metals is still under evaluation. Nevertheless, experimental evidence suggests that elemental sulphur could increase the corrosion of metal objects. Indeed, this compound is known to be highly reactive, oxidizing organic and inorganic material regardless of the oxygen content (Little, Ray et al. 2000). In presence of humidity, a steel surface covered by a layer of elemental sulphur is corroded by an electrochemical reaction involving the reduction of elemental sulphur coupled with the oxidation of iron (Little, Ray et al. 2000). As a consequence the layer composed of sulphur detected by elemental analysis in our samples, and localized under the biogenic crystals, could be detrimental for the objects, and has to be avoided. It can be then concluded that, for future experiments Na_2S have to be replaced from the culture medium to be replaced by other reductive compounds containing less sulphur, such as cysteine.

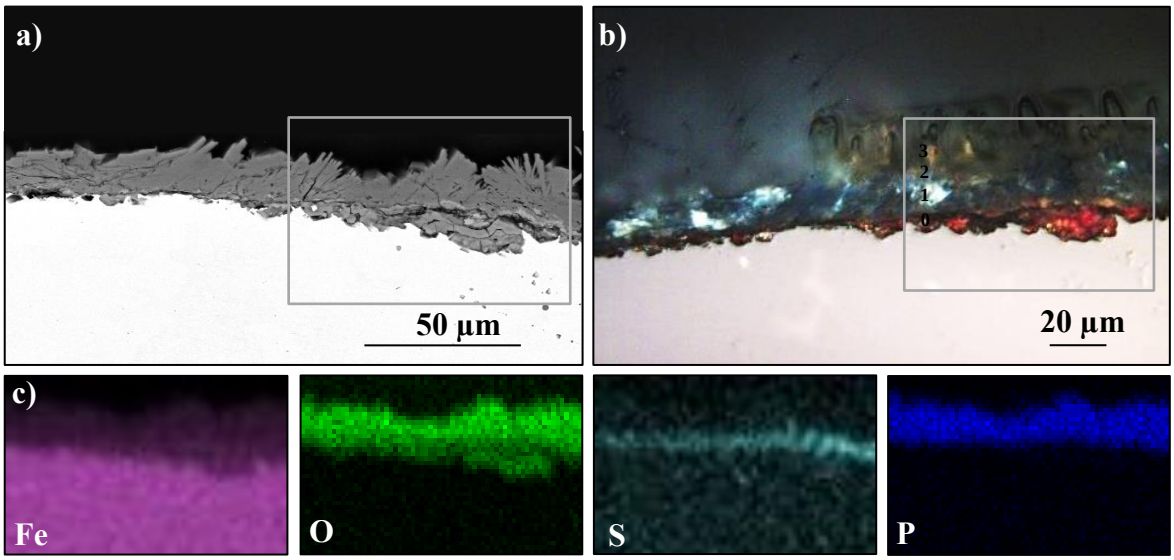


Figure 16: Elemental mapping performed on the cross-sectioned coupons treated with the strain TCE1 of *D. hafniense*. a) Scanning electron microscopy image of the cross-sectioned coupons after the bacterial treatment, b) Microscopic images of the same area with elemental mapping area indicated by a grey box (original magnification 500x), c) elemental mapping showing the presence of iron (pink), oxygen (green), sulphur (turquoise), and phosphorus (blue).

As previously reported, the formation of biogenic iron minerals containing phosphorus, can be attributed to the microbial iron reduction of solid-iron phases, and the reaction of Fe^{2+} ions with phosphates (PO_4^{3-}) added in the bacterial medium as a buffer and phosphorous source for bacterial growth (Comensoli, Maillard et al. 2017).

Molecular composition

Single points as well as area analysis with Raman spectroscopy allowed to identify lepidocrocite and goethite as main compounds in the corrosion layer of the untreated samples (Supplementary figure 8). This corrosion compounds were not detected in the samples treated with bacteria. In fact, the treatment decreased the thickness of the original corrosion layer making its detection limited by Raman spectroscopy (spatial resolution of about 1µm). However, as mentioned above, the original corrosion layer was converted into biogenic crystals. These newly formed minerals were identified as a mix of two different iron phosphates. The most abundant was vivianite $Fe^{2+}_3(PO_4)_2 \cdot 8H_2O$ (Figure 17). Vivianite spectrum displayed an intense vibrational peak at 949 cm^{-1} and two lower vibrational peaks at 1014 and 1052 cm^{-1} typical of the P-O stretching mode (Frost, Martens et al. 2002). In order to localise this compound the principal peak at 949 cm^{-1} was employed for chemical mapping (Figure 17). Interestingly another iron phosphate compound identified as barbosalite $Fe^{2+}Fe^{3+}_2(PO_4)_2(OH)_2$ was also detected. The same peaks of the P-O stretching mode were present but with different relatives intensities. In fact, the principal Raman shift was at 1015 and not at 949 cm^{-1}. Therefore the vibrational peak at 1015 cm^{-1} was chosen to elaborate the chemical mapping of barbosalite (Figure 17). Since barbosalite is composed of a mix of Fe^{2+} and Fe^{3+}, its formation could be the consequence of the interaction between free Fe^{2+} and Fe^{3+} ions present in the corrosion layer, Fe^{2+} ions produced from the microbial iron reduction, and the PO_4^{3-} from the buffer. On the abiotic control lepidocrocite and goethite were detected, however siderite and vivianite were also identified (Supplementary figure 9). Siderite was probably produced as the consequence of the interaction between corroded iron and the organic carbon source in the culture medium. The formation of vivianite on the abiotic control samples can be the result of two different phenomena. First, phosphates used as buffer in the culture media could have interact with the Fe^{2+} ions present in the original corrosion layer precipitating as iron phosphates. Second, biogenic Fe(II)-phosphates could have been

produced also as a consequence of an abiotic reduction of solid-iron corroded phases caused by some reductive molecules employed in the preparation of culture media for anaerobic bacteria, such as Na_2S. In fact, the reduction of iron in abiotic control has been already documented in similar conditions (Comensoli, Maillard et al. 2017). Finally Raman measurements did not allow the detection of a sulphur layer under the biogenic crystals, as revealed by SEM-EDS. This was probably due to the thickness of this layer, probably under the spatial resolution of Raman spectroscopy (about 1µm).

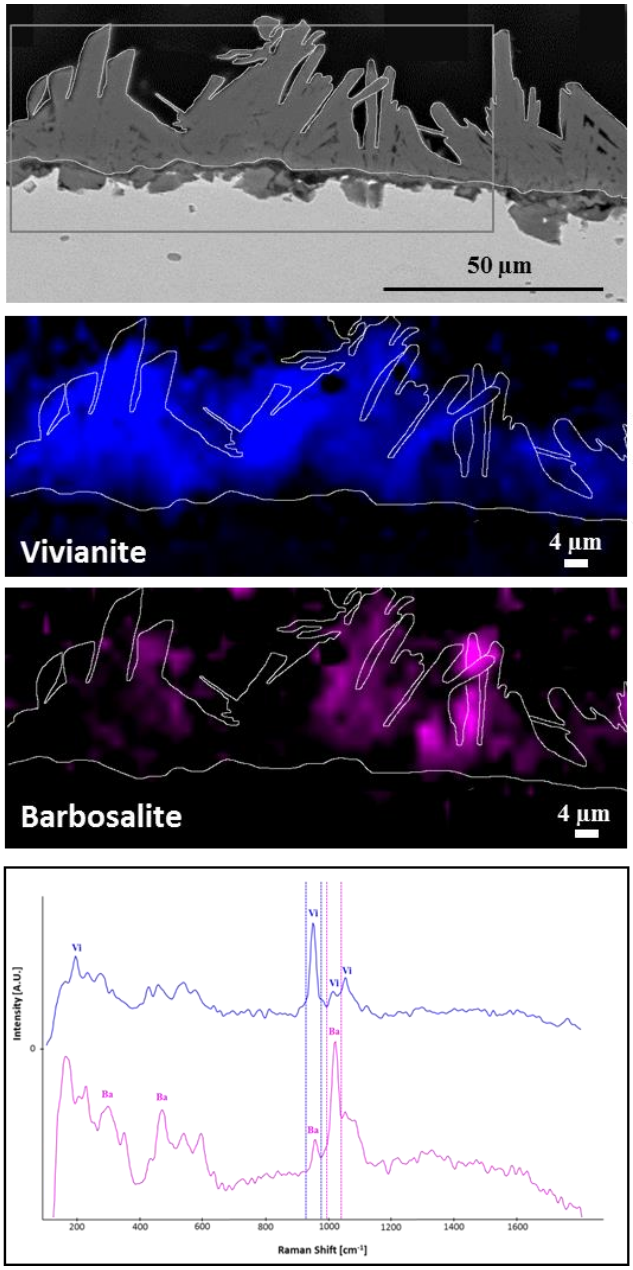


Figure 17: Molecular mapping performed on the coupons after bacterial treatment with the strain TCE1 of *D. hafniense*. From top, scanning electron microscopy image of the area analysed by Raman spectroscopy indicated by a square box (original magnification 400x), chemical maps of vivianite in blue (Vi), and of barbosalite in pink (Ba), representative extracted Raman spectra where vivianite and barbosalite were identified with the corresponding peaks selected for the elaboration of the respective chemical maps.

2.5. Conclusion

Currently, the negative aspects of the interaction between metallic objects and microbes have been highlighted in scientific research. Several studies have examined microbiologically induced corrosion of industrial materials such as steel (Li, Kim et al. 2001, Videla and Herrera 2005, Xu, Li et al. 2013, Valencia-Cantero and Peña-Cabriales 2014). Indeed, the development of bacterial biofilms on the surface of such objects induces changes in the electrochemical conditions (Videla and Herrera 2005), which leads to the formation of corrosion layers and produces structural damage to the metal core. In this study, we demonstrated that bacteria can be used to convert an existing layer of unstable corrosion on archaeological iron objects into more stable compounds. These data represent a demonstration of the potential to use bacteria to reduce iron on archaeological objects for conservation-restoration purposes. Indeed, biogenic crystals were produced directly on the objects' surfaces, suggesting that the bacterial treatment converted the corrosion products present into more stable compounds, thereby stabilizing the objects. The production of chemically stable iron compounds displaying a lower molecular volume than the compounds originally present in the corrosion layer enhances the porosity of the latter, facilitating the removal of chlorine by diffusion. Additionally, SEM observations showed that the newly formed biogenic mineral layer was homogeneous and appeared to completely cover the original corroded surface. This homogeneous layer of stable crystals was produced after 3 to 7 days of incubation with *D. hafniense* strain TCE1. This represents an improvement with respect to conventional methods (such as alkaline sulphide baths) where immersion for a period of several weeks is required for objects of the same size (Selwyn 2004).

Our study revealed that even though biogenic minerals formation under anoxic conditions is an easy and interesting method to protect iron, great attention has to be dedicated to the composition of the culture media used for iron reduction. In fact, through stratigraphic analysis a layer containing sulphur was detected under the biogenic crystal formation in the case of the urban corrosion products. This layer was not detected with surface analysis, but stratigraphic investigations allowed to conclude that to produce a stabilizing biogenic layer, soluble sulphur has to be removed from the culture media.

Further investigation on the long-term stability of the treatment and its efficiency on artefacts presenting different corrosion types should be carried out. However, the results obtained here are encouraging and represent a basis for the development and optimization of a sustainable and eco-friendly alternative conservation-restoration method for preserving archaeological iron.

2.6. Evaluation of the performance of a second bacterial treatment

In this section results obtained during a collaborative effort with the PhD student Kooli W. were presented. The aim of this study was to evaluate the potential of an alternative bacterium for the development of a bacterial treatment for corroded iron. Iron reduction abilities of the halophilic facultative anaerobe *Shewanella loihica* strain PV-4 (DSM 17748), and the abilities to produce stable biogenic iron minerals was investigated by this student (Kooli, Comensoli et al. in preparation). This strain was isolated from sea and, under anoxic conditions, is able to use iron as electron acceptor (Gao, Obraztova et al. 2006, Roh, Gao et al. 2006). During this study, reduction of soluble and corrosion layer of corroded iron coupons were performed and lead to the production of stable biogenic vivianite and siderite. Since this bacterium has the ability to growth in both oxic and anoxic conditions, during iron reduction test on corroded coupons bacterial growth and iron reduction was decoupled, allowing to obtain high amount of bacterial biomass in oxic conditions (Kooli, Comensoli et al. in preparation). My contribution to that study was the Raman investigation of the cross-sectioned samples as well as the interpretation of all the raw data on the cross-section analyses (optical-microscope, SEM-EDS). For the methods used during this analysis see the section *2.3.8. Evaluation of the treatment performance*. The following paragraphs resume the results obtained during this collaboration on the structure, thickness and continuity, as well as on the elemental and molecular composition of the corrosion layer before and after the treatment of corroded iron coupons with *S. loihica* strain PV-4.

Structure, thickness and continuity of the corrosion layer

Untreated samples displayed a corrosion layer with dark-brown, red and orange tonalities. The same was observed for the abiotic control samples (Figure 18). In contrast, a precipitation of grey-green biogenic minerals was observed on the outermost corrosion layer in the coupons treated with bacteria (Figure 18). As for the previous bacterial treatment an overall decrease on the thickness of the original corrosion layer was measured. Indeed, the values decreased from nearly 151 µm and 128 µm for the untreated and the abiotic control coupons, respectively, to about 44 µm and 67 µm after treatment with bacteria for 7 and 14 days respectively (Figure 19 a). The estimation of the continuity of the biogenic layer confirmed that bacteria produced a homogeneous layer of crystals on the top of the original corrosion layer (Figure 19 b). Indeed, while 100 % of the original corrosion layer was found to be exposed in the untreated samples as well as in the abiotic control samples, 71 % of the original corrosion layer was covered by biogenic crystals on plates incubated 7 days and 81 % on plates incubated for 14 days.

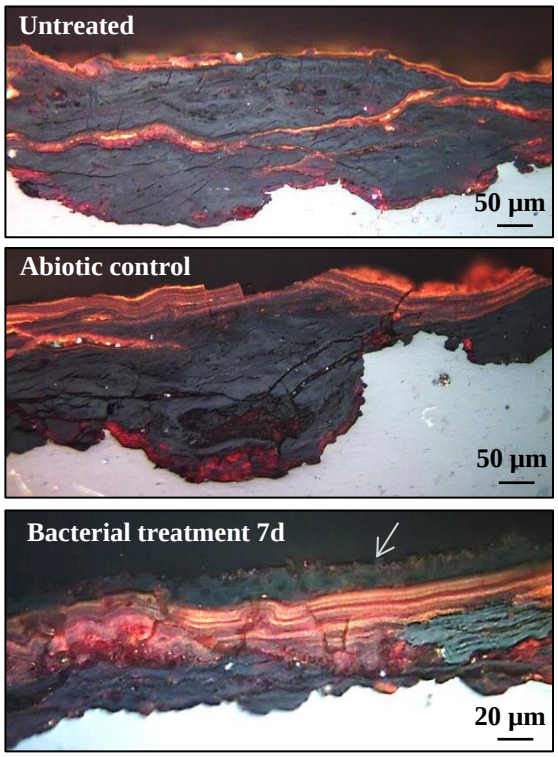


Figure 18: Microscopic images of the untreated, abiotic control, and bacterially treated samples with the strain PV-4 of *S. loihica*. Due to the difference of the thickness of the corrosion layer before and after the treatment, images were taken with different magnifications. Untreated and abiotic control samples: original magnification 200x, bacterially treated samples: original magnification 500x.

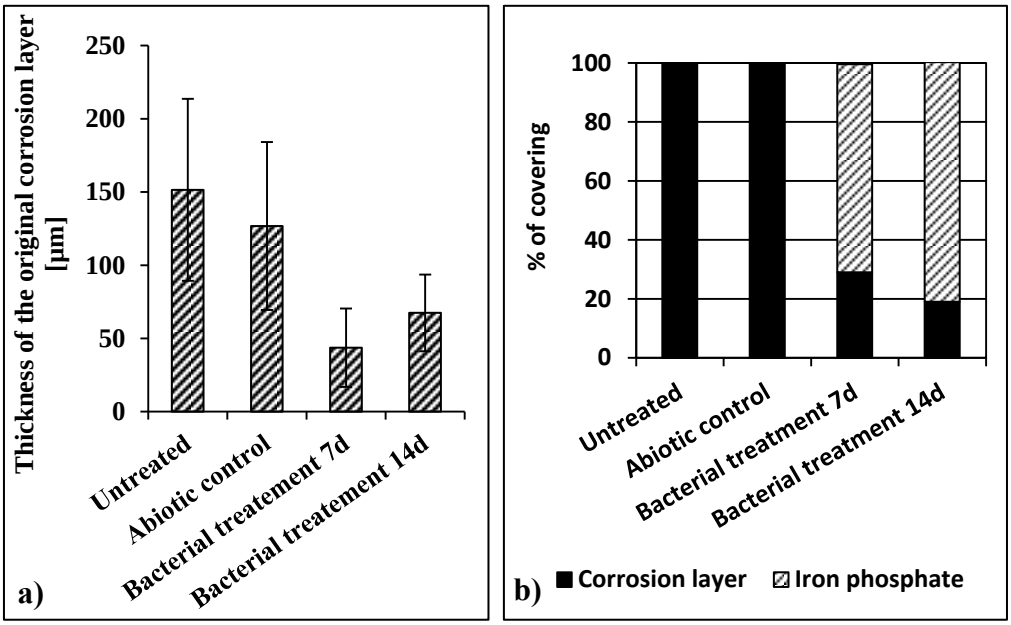


Figure 19: Graphic representation of the thickness of the original corrosion layer on the untreated, abiotic control, and the bacterially treated coupons treated with the strain PV-4 of *S. loihica*. b) Estimation of the covering of the original corrosion layer by newly formed crystals.

Elemental composition of the corrosion layer after the treatment

Untreated coupons had a marine atmospheric corrosion layer mainly composed of iron, oxygen and chlorine (Figure 20). After the bacterial treatment, Fe and O were found to be the main components of the corrosion layer; nevertheless, localized between the metal core and the corrosion layer some Cl was also detected. Interestingly, the comparison of these two images shows a smaller amount of chlorine in coupon after the treatment. This analysis showed also that the biogenic crystals were mainly composed of Fe oxygen and P (Figure 21). In this case a layer composed of S was not detected.

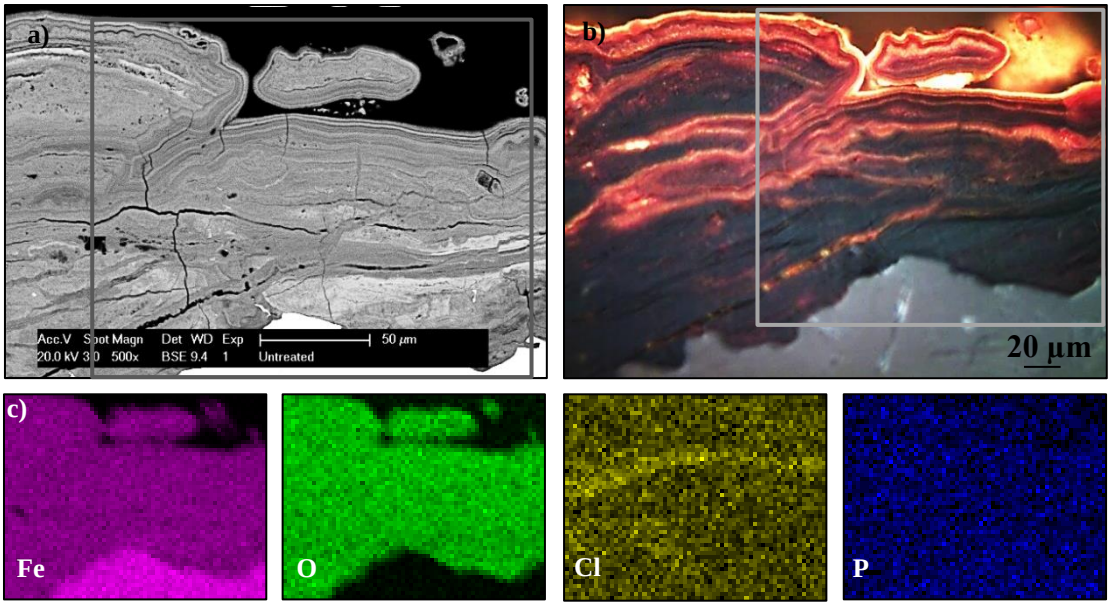


Figure 20: Elemental mapping performed on the untreated coupons. a) Scanning electron microscopy image of the untreated coupons, b) microscopic image of the same area, indicating the area where elemental mapping was performed by a grey box (original magnification 500x), c) elemental mapping showing the presence of iron (pink), oxygen (green), chlorine (yellow), and phosphorus (blue).

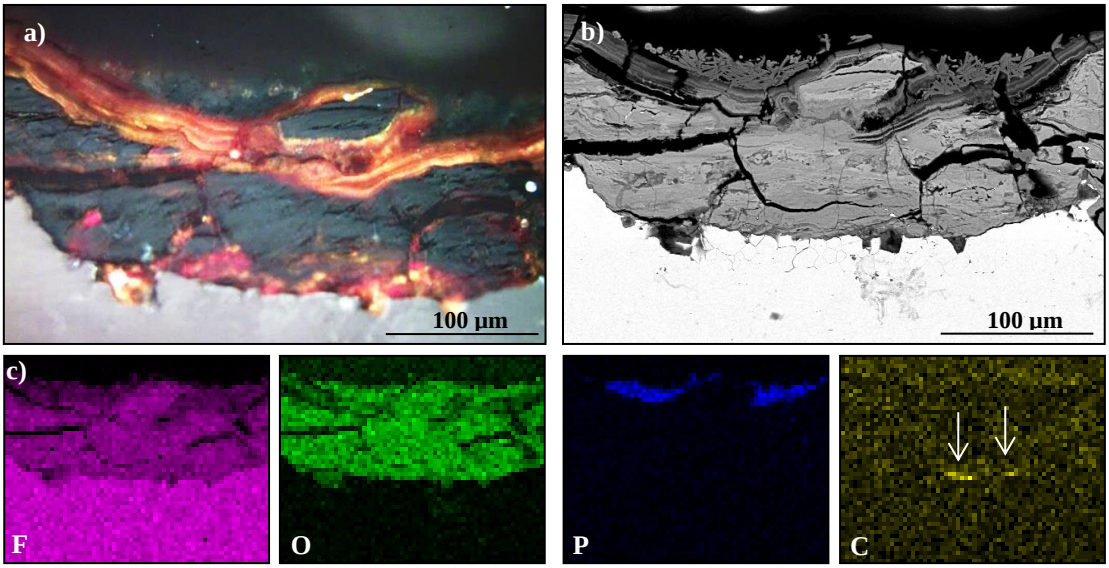


Figure 21: Elemental mapping performed on the bacterially treated coupons treated with the strain PV-4 of *S. loihica*. a) Microscopic image of the coupons, b) scanning electron microscopy image of the same area, c) elemental mapping of the corresponding area showing the presence of iron (pink), oxygen (green), phosphorus (blue), and chlorine (yellow). Mapping was performed on all surface represented on the SEM image. Arrows indicate the few amount of chlorine remained after treatment**.**

Molecular composition of the corrosion layer after the bacterial treatment

Goethite, lepidocrocite, and ferrihydrite were detected through Raman analysis on the untreated samples (Figure 22). Whereas, the analysis carried out on the biogenic crystals produced after the treatment, revealed that this layer was mainly composed of vivianite (principal vibrational peak at 959 cm^{-1} was used for the Raman chemical map) (Figure 23). Under this layer, the same compounds detected on the untreated samples were found and identified as goethite, lepidocrocite, and ferrihydrite. No chlorinated compounds were detected through Raman investigation, neither on the untreated nor on the treated samples. This was probably due to the fact that the amount of chlorine revealed by SEM-EDS mapping is under the detection limit of Raman spectroscopy. The production of biogenic vivianite in this case is the consequence of a mechanism already reported (Cosmidis, Benzerara et al. 2014, Kooli, Comensoli et al. in preparation). In fact, in the anaerobic culture media no phosphate was added. However, the strain PV-4 of *S. loihica* was found to be able to accumulate polyphosphates inside the cells, in order to ensure growth also in phosphorus-limiting conditions (Kooli, Comensoli et al. in preparation). Then we can conclude that in this experiment, biogenic vivianite production was the consequence of the interaction of PO_3^{2-} released by the bacterium in the culture medium with Fe^{2+} ions resulting from bacterial iron reduction.

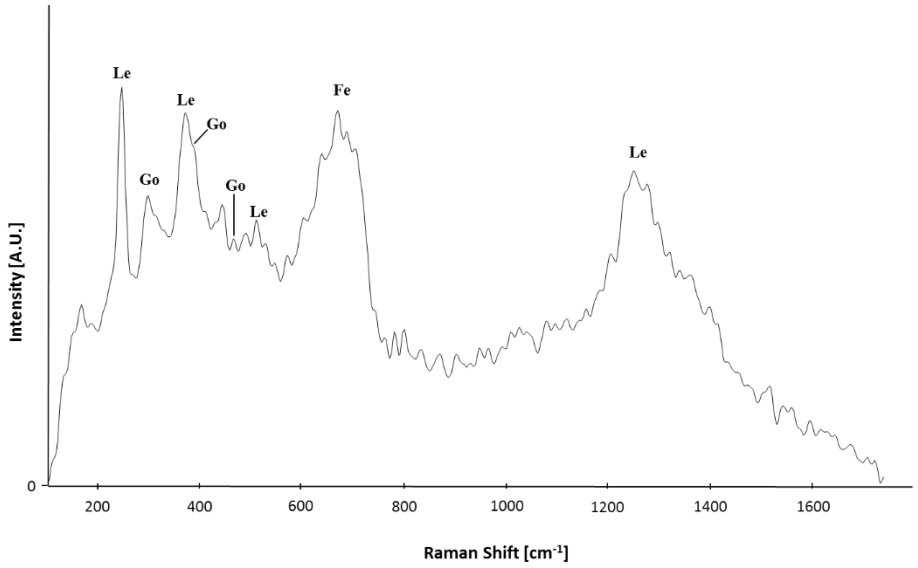


Figure 22: Raman investigation on the cross-sectioned untreated coupons. Corrosion compounds were identified as lepidocrocite (Le), goethite (Go), and ferrihydrite (Fe).

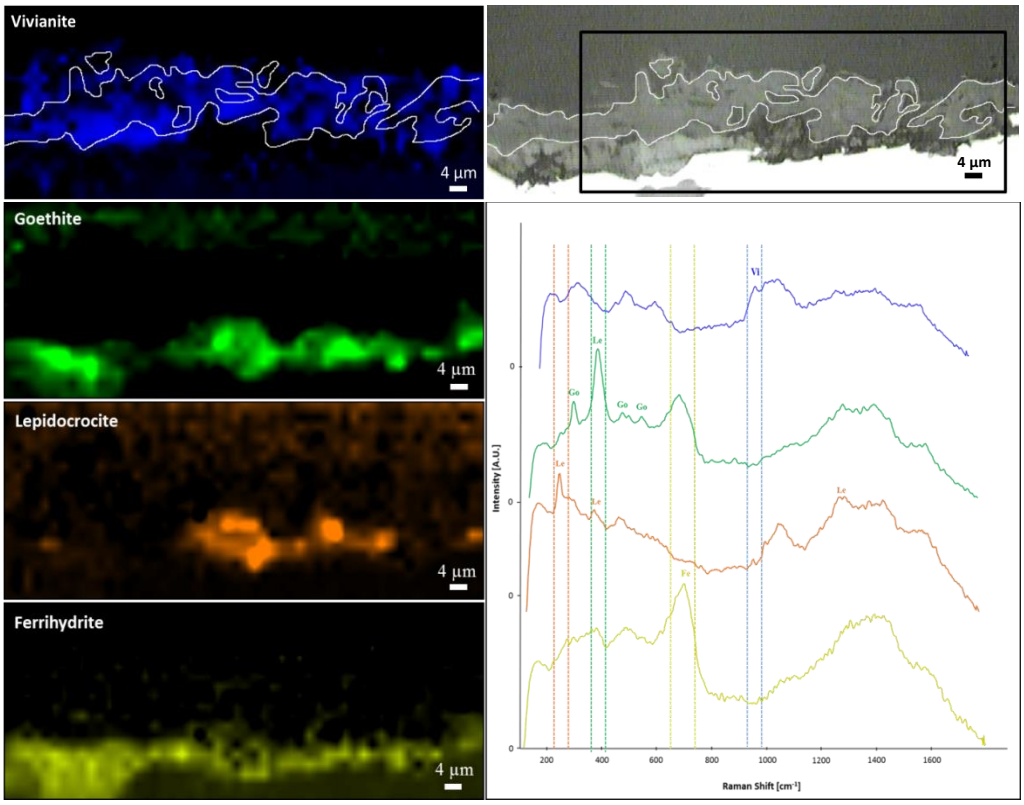
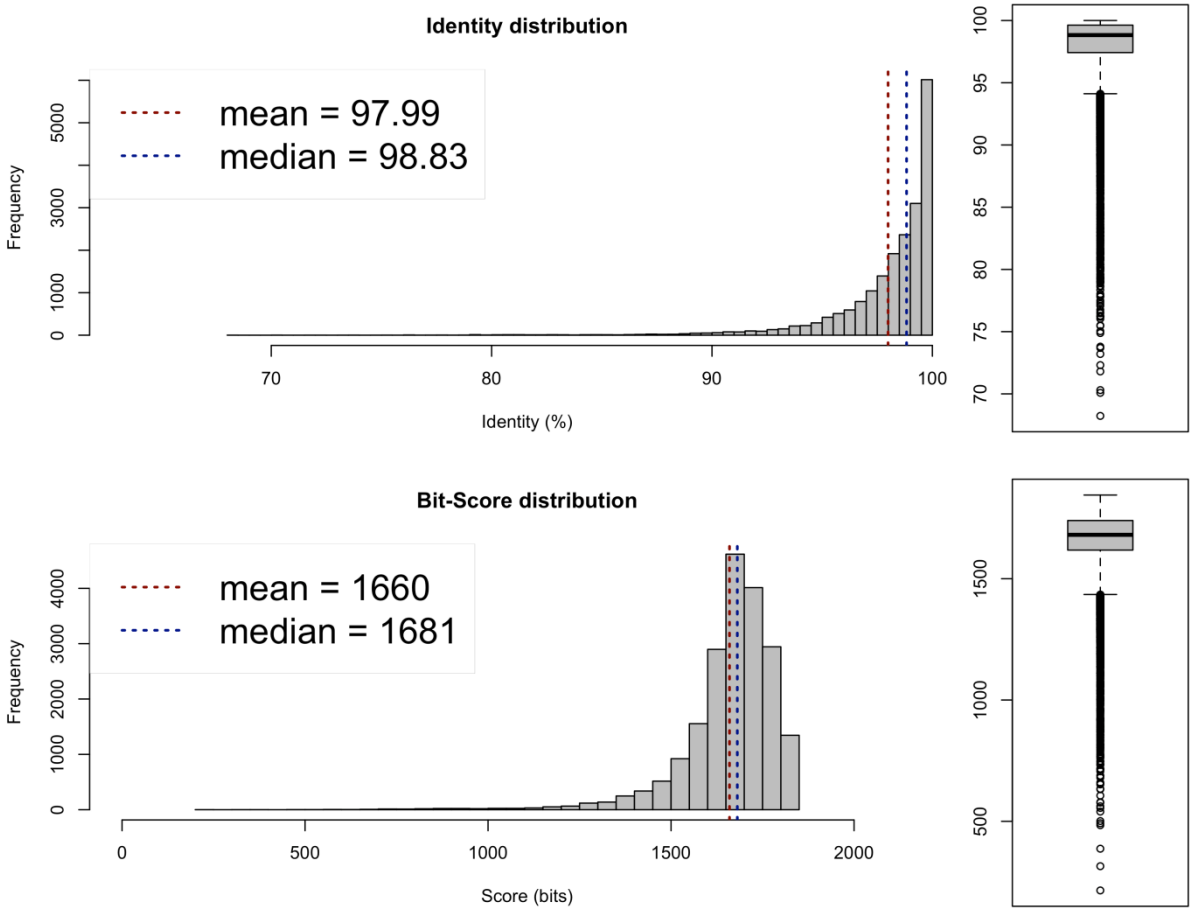
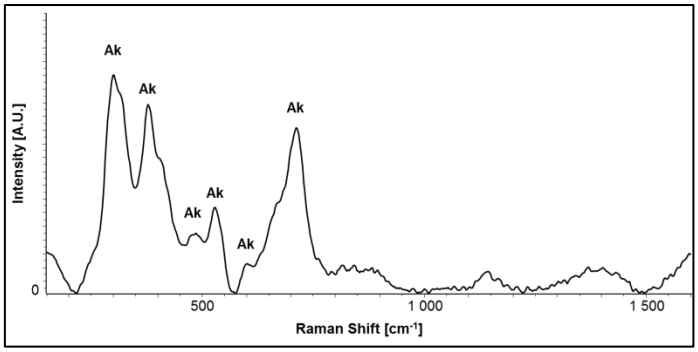


Figure 23: Molecular mapping performed on the coupon after bacterial treatment with the strain PV-4 of *S. loihica*. On the left; Raman mapping of vivianite in blue (Vi), of goethite in green (Go), of lepidocrocite in orange (Le), and of ferrihydrite in yellow (Fe). On the right; Scanning electron microscopy image with the area analysed by Raman spectroscopy indicated by a black box (original magnification 400x), as well as the representative extracted Raman spectra where vivianite, goethite, lepidocrocite, and ferrihydrite were identified, with their corresponding peaks selected for the elaboration of the respective chemical maps.

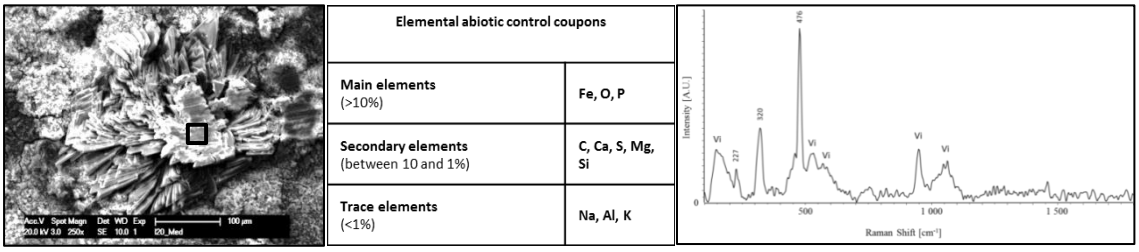
2.7. Supplementary information



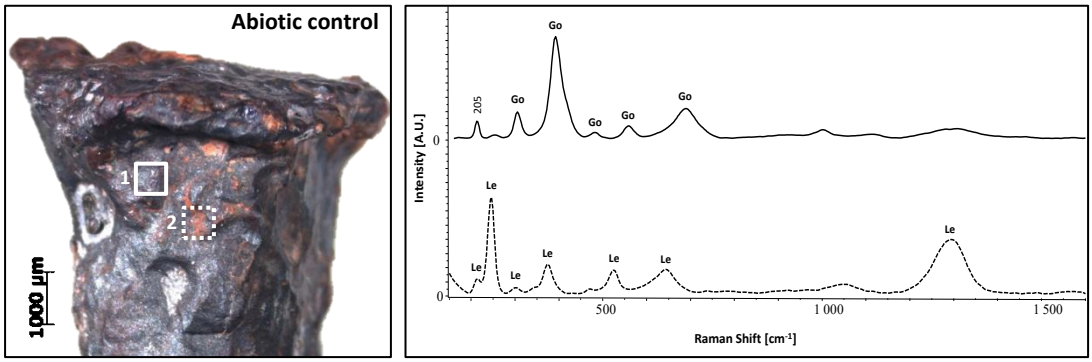
Supplementary figure 2: Results of the average nucleotide identity (ANI) analysis performed between *Desulfitobacterium hafniense* strain LBE and the reference genome of *D. hafniense* strain DCB-2 (GenBank accession number NC_011830.1). The analysis was performed using the on-line ANI calculator tool available at http://enve-omics.ce.gatech.edu.



Supplementary figure 3: Raman spectrum of the synthetic akaganeite. The analysis was carried out with a Horiba-Jobin Yvon Labram Aramis microscope equipped with a Nd:YAG laser of 532 nm at power lower than 1 mW (600 gr/mm). The spectral interval was 100 and 1600 cm^{-1} and the measurement conditions were 1000 µm hole, 100 µm slit and 5 accumulations of 100 s.



Supplementary figure 4: Secondary elemental image of the crystals found on the abiotic control coupons, elemental composition, and corresponding Raman spectra identified as vivianite (Vi).



Supplementary figure 5: Raman analysis of the abiotic control nail after the reduction test. On the left, the stereoscope images of the area sampled for the analysis; and, on the right, the corresponding Raman spectra identified as; 1: Goethite (Go), 2: Lepidocrocite (Le).

Supplementary material: Anaerobic standard medium for *Desulfitobacterium hafniense*

Standard minimal medium (MM) according to Prat L, Maillard J, Grimaud R, Holliger C. 2011. Physiological adaptation of *Desulfitobacterium hafniense* strain TCE1 to tetrachloroethene respiration. Applied and environmental microbiology **77:**3853-3859.

Mixture for 50 mL of culture, add aseptically with syringes the following solutions to 45 mL of **solution A**:

- 1.25 mL solution B
- 2.50 mL solution C
- 1.25 mL solution D
- 0.5 mL sodium lactate 40% (v/v)
- 1 mL disodium fumarate 16% (v/v)

Solutions

Solution A :

- $K_2HPO_4 . 3 H_2O$ 0.958 g/L
- $NaH_2PO_4 . 2H_2O$ 0.218 g/L
- Peptone 0.1 g/L
- Resazurin 0.5g/L 1 mL

➔ Boil, cool down under N_2/CO_2, distribute to anaerobic flasks, gas exchange for N_2/CO_2, autoclave.

Solution B :

To 20 mL of anaerobic sterile H_2O, add the following solutions:

- 1 mL solution IV, filter sterilize
- 1 mL solution V
- 1 mL solution VI
- 1 mL solution VII
- 1 mL solution VIII

Solution C :

To 49 mL of solution IX, add :

- 1 mL solution X

Solution D :

- $CaCl_2 \cdot 2\ H_2O$ 4.40 g/L
- $MgCl_2 \cdot 6\ H_2O$ 4.06 g/L

➔ Gas exchange for N_2, autoclave.

Solution IV : Trace elements

- EDTA 500 mg/L, dissolve in 900 mL H_2O, adjust the pH to 7.0 with HCl, then add:
- $FeCl_2 \cdot 4\ H_2O$ 2000 mg/L
- $MnCl_2 \cdot 4\ H_2O$ 100 mg/L
- $CoCl_2 \cdot 6\ H_2O$ 190 mg/L
- $ZnCl_2$ 70 mg/L
- $CuCl_2 \cdot 2\ H_2O$ 2.55 mg/L
- $AlCl_3$ 5.52 mg/L
- H_3BO_3 6 mg/L
- $Na_2MoO_4 \cdot 2\ H_2O$ 41.4 mg/L
- $NiCl_2 \cdot 6\ H_2O$ 24 mg/L, add to 1 L with H_2O

Solution V : Vitamins-1

- Biotin 50 mg/L
- P-aminobenzoate (sodium salt) 250mg/L
- Pantothenate (sodium salt) 50mg/L
- Folic acid$\cdot 2\ H_2O$ 20mg/L
- Lipoic acid 50mg/L
- Pyridoxine 100mg/L
- Nicotinic acid 550mg/L

➔ Filter sterilize in sterile anaerobic flasks, gas exchange for N_2.

Solution VI : Vitamins-2

- Thiamine-HCl 100 mg/L

➔ Filter sterilize in sterile anaerobic flasks, gas exchange for N_2.

Solution VII : Vitamins-3

- Riboflavine 50 mg/L

➔ Filter sterilize in sterile anaerobic flasks, gas exchange for N_2

Solution VIII : Vitamins-4

- Cyanocobalamin 250 mg/L

➔ Filter sterilize in sterile anaerobic flasks, gas exchange for N_2.

Solution IX :

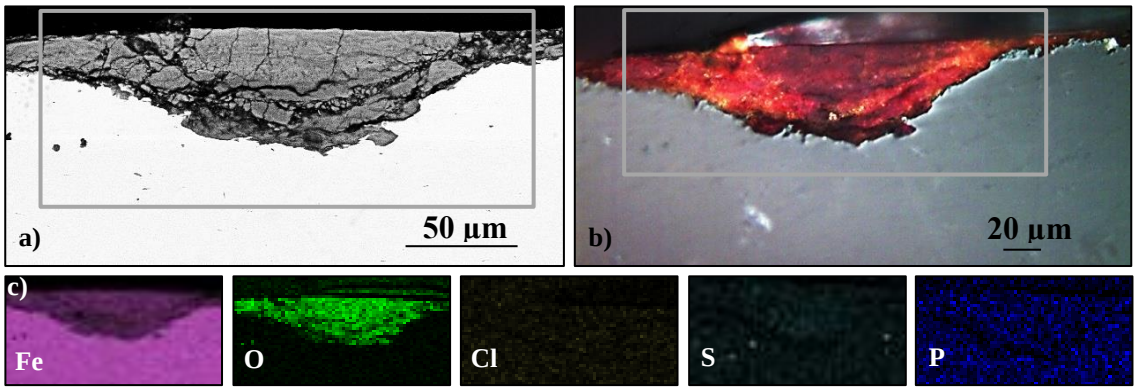
- NH_4HCO_3 9.01g
- $NaHCO_3$ 76.11 g/L

➔ Boil, cool down under N_2/CO_2, distribute 49 mL to anaerobic flasks, gas exchange for N_2/CO_2, autoclave.

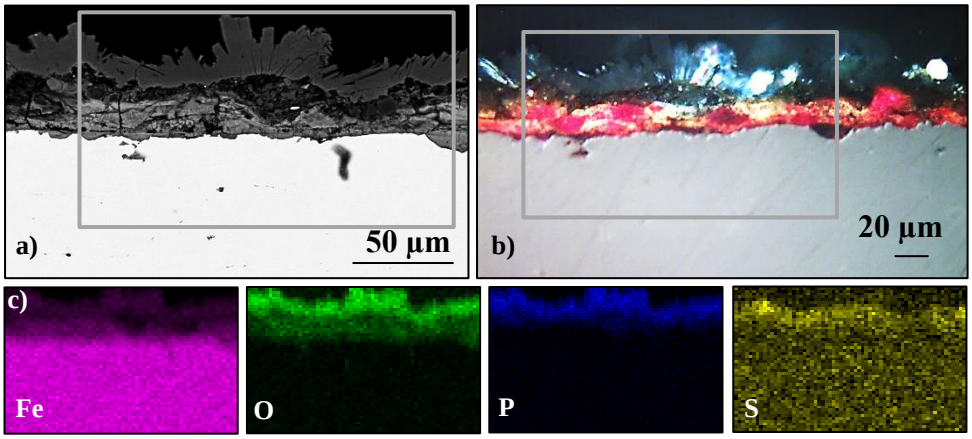
Solution X :

- $Na_2S \cdot 9H_2O$ 24.02 g in 100 mL

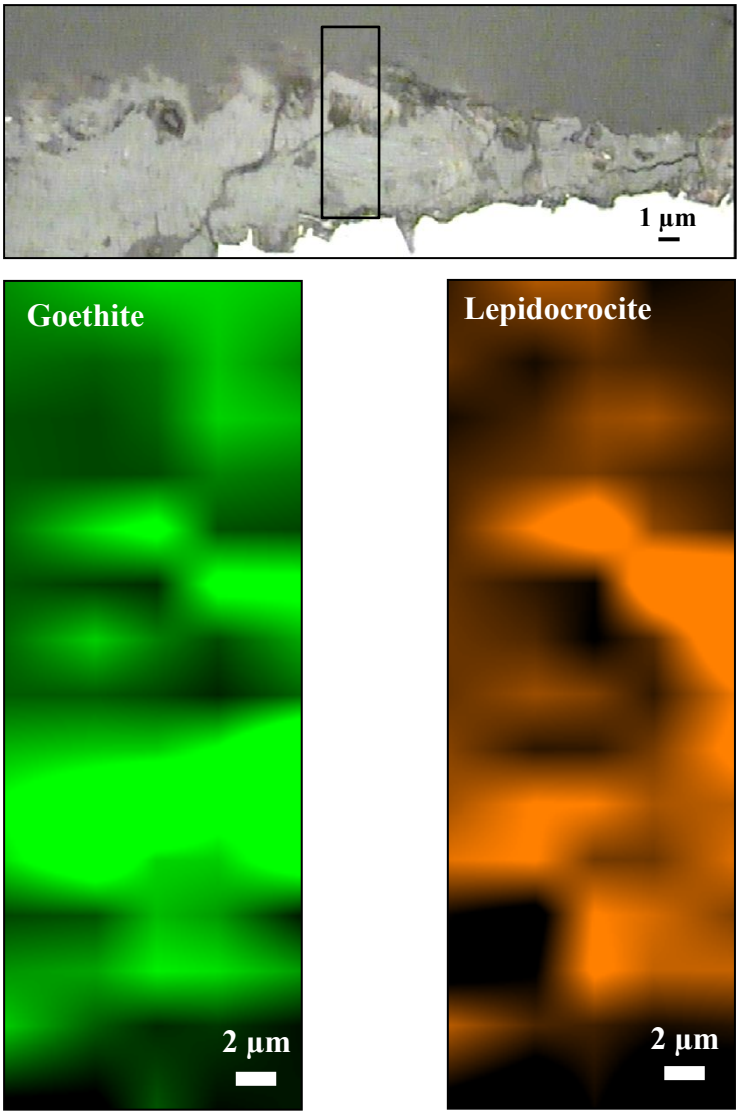
➔ Wash crystals with N_2-degased H_2O, weight, dissolve in degased H_2O, filter sterilize into anaerobic flasks, gas exchange for N_2.



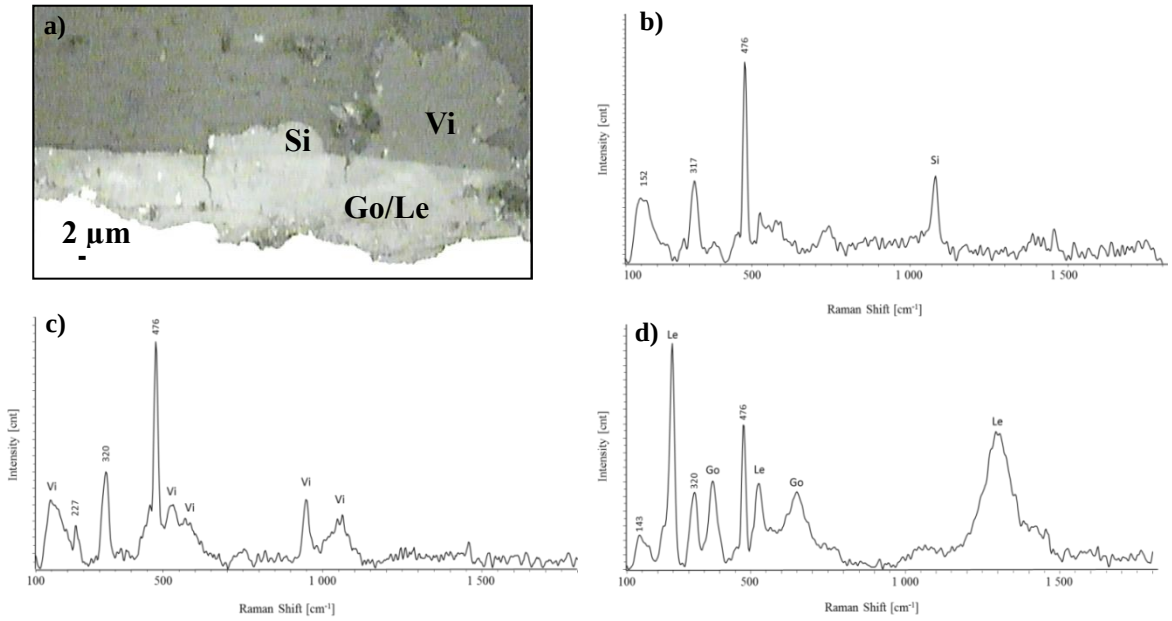
Supplementary figure 6: a) Scanning electron microscopy image of the cross-section of an untreated coupon presenting a urban atmospheric corrosion, b) Microscopic image of the same area with elemental mapping area indicated by a grey box, c) elemental mapping of the corresponding area showing the presence of iron (pink), oxygen (green), chlorine (yellow), sulphur (turquoise), and phosphorus (blue).



Supplementary figure 7: a) Scanning electron microscopy image of the cross-sectioned abiotic control coupon presenting an urban atmospheric corrosion, b) Microscopic image of the same area with elemental mapping area indicated by a grey box, c) elemental mapping of the corresponding area showing the presence of iron (pink), oxygen (green), phosphorus (blue), and sulphur (yellow).



Supplementary figure 8: Chemical maps of goethite (green) and lepidocrocite (orange), obtained by Raman spectroscopy on the untreated samples presenting a natural urban corrosion.



Supplementary figure 9: Raman investigation on the abiotic control samples presenting an urban atmospheric corrosion. a) Microscopic image of the area analysed with the compounds identified and indicated as, siderite (Si), Vivianite (Vi) and a layer composed of a mix of goethite (Go) and lepidocrocite (Le). b) Raman spectrum collected on the Si area where siderite was identified, c) Raman spectrum collected on the Vi area where vivianite was identified. d) Raman spectrum collected on the underneath corrosion layer where a mix of goethite and lepidocrocite was identified.

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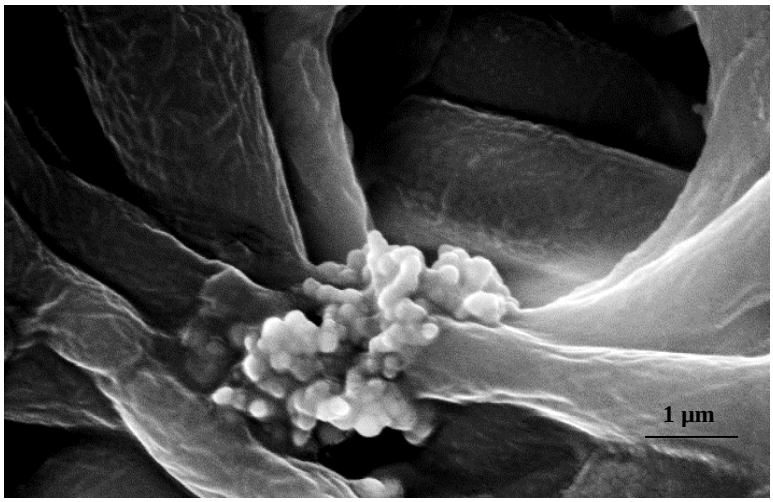
CHAPTER 3

Physiological abilities of alkalitolerant and halotolerant fungi regarding iron



Hyphae of *B. bassiana* with small precipitates

This chapter is based on the results of the following published article:

Comensoli L., Bindschedler S., Junier P., & Joseph E. (2017). Iron and Fungal Physiology: A Review of Biotechnological Opportunities. In S. Sariaslani, & G. M. Gadd (Eds.), *Advances in Applied Microbiology*, Volume 98, (pp. 31–60).

3.1. Abstract

Bacteria are not the only microorganisms able to interact with metals and produce biogenic minerals. For example, fungi are well known for their ability to produce several types of metal oxalates (Gadd 1993, Gadd 1999, Fomina, Hillier et al. 2005). Accordingly, the third chapter of this thesis explored the potential of fungi to develop biotechnological methods for the stabilisation of corroded iron. Our hypothesis is that several fungal metabolic activities regarding iron could be exploited for the development of protective treatments for corroded iron. In order to verify our hypothesis this study was divided in 4 sections, presented in the Figure 24. First, a screening of selected fungal species was carried out to identify alkalitolerant and halotolerant species (*3.1. Selection of fungal species with suitable metabolisms*). Results revealed that *Agaricus lignicola* was able to reduce iron, while *Alternaria* sp. adsorbed nearly all $FeCl_3$ added in the culture medium after already 4 days of incubation. In addition, biogenic iron crystals and EPS chelating iron were also observed. Second, two fungal species were individuated from the screening and their ability to produce biogenic minerals and to uptake/adsorb iron was studied further in order to develop biotechnological methods stabilizing or cleaning corroded iron, respectively (*3.2. Fungal treatment of corroded iron coupons*). Even though no biogenic crystals were produced after up to 4 weeks of incubation, Fourier transformed infrared spectroscopy (FTIR) allowed observing the production of EPS on the corroded iron surfaces. Regarding the cleaning method, after 4 weeks of incubation, the fungus used in this experiment successfully removed the main part of the corrosion layer. Third, in order to enhance the production of biogenic fungal minerals containing iron, the effects of the oxidation state of the iron source used and of manganese were evaluated (*3.3. Enhancing of the ability to produce biogenic iron mineral of selected fungi*). Unfortunately, even if some crystals were detected, none of these strategies allowed to increase the production of biogenic crystals. Finally, a section presenting a biotechnological application to exploit fungal adsorption of iron is presented in the fourth section (*3.4. Fungal iron adsorption and biotechnology, what else?*). Quantification of pyoverdine after one-day incubation of a bacterial culture with the fungal biomass chelating iron, proved that *Pseudomonas fluorescens* was able to use the iron chelated in the fungal biomass of *Alternaria* sp. as a source of bioavailable iron. Overall, this study shows that fungal biogenic minerals production is not at this stage a suitable approach to develop stabilisation methods for corroded iron. In contrast iron adsorption could be successfully

exploited for a biocleaning procedure. In addition, some metabolic activities regarding iron, potentially influencing iron cycling in soils were also revealed.

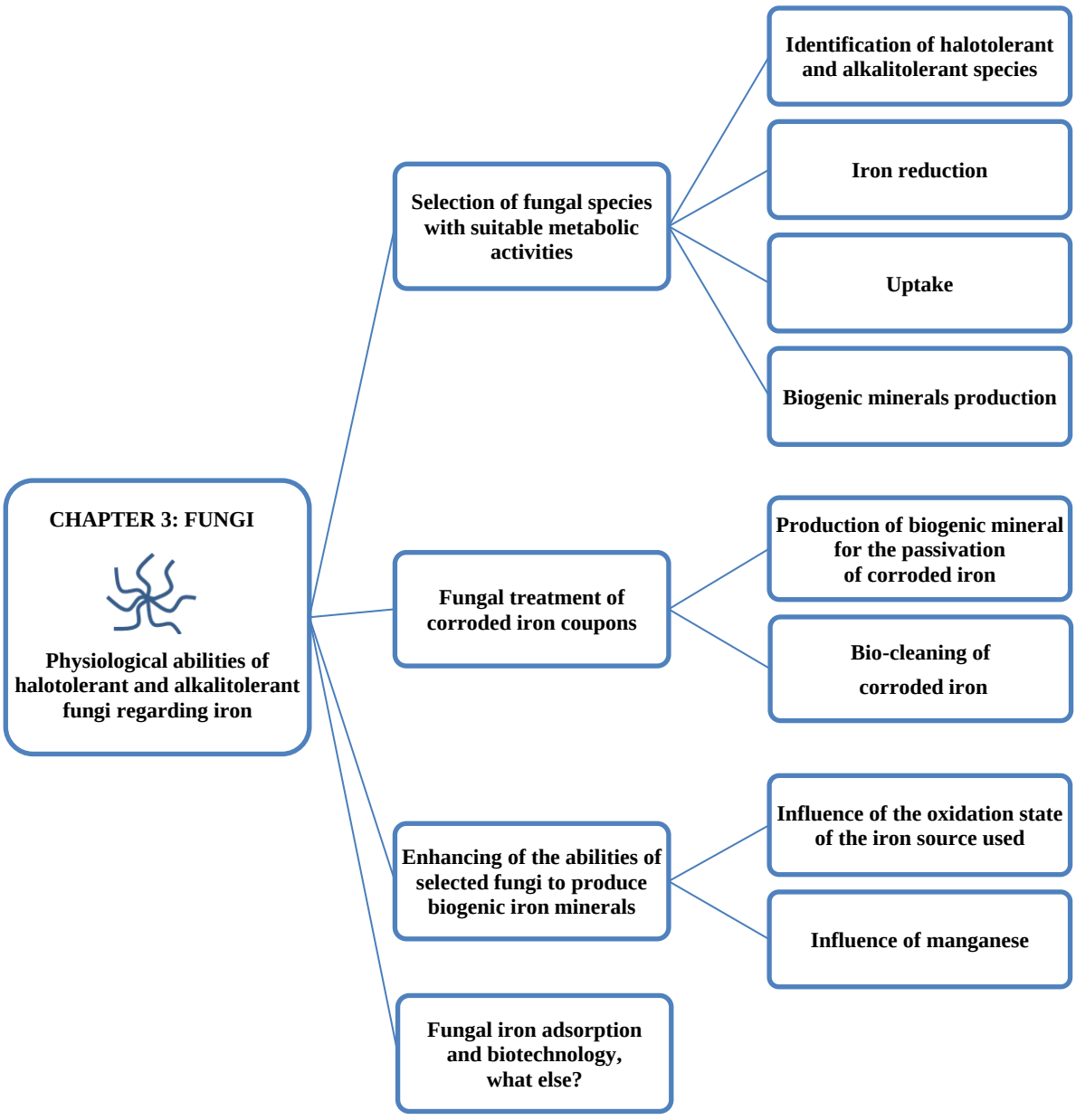


Figure 24: Graphic summary of the overall structure of the chapter 3: Physiological abilities of halotolerant and alkalitolerant fungi regarding iron.

3.2. Introduction

Iron oxidises readily in the presence of oxygen and water. In contrast, optimal fungal growth generally requires oxygen and high relative humidity. Thus, fungi could be seen as an unsuitable choice to develop a biotechnological approach to fight iron corrosion. However, iron corrosion can be avoided by using an alkaline solution (Selwyn 2004), and hence a new fungal treatment can be proposed for the stabilization of corroded iron considering an aqueous solution with an alkaline pH. In addition, the organisms used for such approach needs to be halotolerant, as the stabilization of iron leads to the release of chloride with a consequent increase in concentration in the treatment solution. To meet these criteria, a screening aiming to identify fungal strains able to grow under alkaline conditions and in presence of chlorinated salts was performed.

Even if iron is an essential element for fungal physiology and in soil dynamics, at present the general knowledge of possible fungal reactions involving iron is incomplete. For instance the role of filamentous fungi in the geochemical cycling of iron is still poorly documented (e.g. (Kosman 2003)). In order to develop new biotechnological approaches related to iron, the first step is to understand fungal abilities regarding this metal. Therefore, aiming to identify a suitable fungal metabolic activity for the development of a stabilisation method for corroded iron, the metabolic activities of selected fungal species regarding iron were also investigated.

It is well known that the precipitation of metal oxalates is a resistance mechanism allowing fungi to cope with environmental metal contamination (Fomina, Hillier et al. 2005, Gadd 2007). Nevertheless, the stability and the production of biogenic iron minerals by fungi are underexplored and little information is available *(Section 1.5. Metabolic activities of microbes regarding iron)*. In order to develop an ecologically friendly and sustainable method to stabilize the corrosion layer of archaeological iron objects, the ability of the fungus *B. bassiana* to produce biogenic iron minerals on corroded iron coupons was evaluated.

B. bassiana is an entomopathogenic fungus reported to be able to produce copper as well as iron oxalates as a consequence of oxalic acid production (Joseph, Cario et al. 2011). This compound plays multiple roles in fungal activity (Müller 1975). Indeed, oxalic acid is a pathogenicity factor that allows phytopatogenic fungi to degrade plant cell wall components (Dutton and Evans 1996, Ruijter, van de Vondervoort et al. 1999). A similar mechanism is used by entomopatogenic fungi that use oxalic acid for the degradation of insect cuticles (Kirkland, Eisa et al. 2005). In addition, due to its chelating properties, oxalic acid is reported

to act as a resistance mechanism against heavy metals, and, in particular against copper contamination (Gadd 1993, Gadd 1999, Gadd 2007). Experimental evidence suggests that the production of oxalic acid could also be involved in microbial competition (Shantha and Rati 1990, Ruijter, van de Vondervoort et al. 1999). Another important role of this organic acid is the solubilisation of minerals leading to an increase of the availability of important trace elements, such as calcium and iron (Dutton and Evans 1996, Ruijter, van de Vondervoort et al. 1999, Gadd 2010). Finally, oxalic acid is an important compound during wood degradation (Shimada, Ma et al. 1994, Dutton and Evans 1996). Several pathways have been described for the production of oxalic acid (Ruijter, van de Vondervoort et al. 1999), one of them is the conversion of oxaloacetate into oxalate and acetate with the Mn(II)-dependent enzyme oxaloacetate acetylhydrolase (Ruijter, van de Vondervoort et al. 1999). This reaction has been observed in the fungus *Aspergillus niger* (Hayaishi, Shimazono et al. 1956, Müller 1975, Lenz, Wunderwald et al. 1976), as well as in several other fungal species (Dutton and Evans 1996) and in some *Streptomyces* strains (Houck and Inamine 1987, Ruijter, van de Vondervoort et al. 1999). Since, manganese is a cofactor of the enzyme responsible for the conversion of oxaloacetate into oxalate in several fungi, in this section the potential enhancement of iron oxalate production by the addition of manganese, was tested.

It is widely recognised that the oxidation state of the metal involved in the mineralisation process is really important. Experimental evidence suggests that the stability and the kinetics of formation are highly influenced by the oxidation state of metals. For instance, it seemed that only iron oxalates produced from divalent cations are stable (Eckhardt (1985), Varadachari, Barman et al. (1994), Eick, Grossl, 1996). Therefore, in this section the influence of the oxidation state, as well as the pH of the medium were investigated.

Another interesting ability regarding iron for the development of a biotechnological approach to treat corroded iron is the uptake of this element. In fact, this ability could be exploited to develop a biological cleaning method that will remove the external part of the corrosion layer without damaging the original surface of an object. In fact, as previously described (*1.4. Currently used stabilisation methods*) cleaning procedures are performed mechanically, through sandblasting, or chemically, using acidic solutions. Both these techniques are highly invasive, and rely on the experience of the conservator-restorer to prevent the removal of engraving and decorations. In addition, electrolytic reduction is also used as a pre-treatment for heavily encrusted archaeological objects. However this technique leads to an uncontrolled removal of material (Scott and Eggert 2009). Fungal removal of part of the oxidation layer

could be then a suitable and smooth alternative cleaning method. For the development of a biocleaning method, *Alternaria* sp. was employed. This microorganism is an ubiquitous fungus known to be one of the most abundant rock inhabiting fungi (Sterflinger 2000). In fact, its melanised cell wall, make this fungus resistant to UV radiation as well as to heavy metal contamination. Indeed, experimental evidence demonstrated that melanin is able to chelate heavy metals like copper (Gadd 1993), avoiding the penetration inside the fungal cell.

Nevertheless, iron is an important trace element but often not bioavailable in nature for microorganisms. Then in this study the possible exploitation of the iron chelated in the dead biomass of *Alternaria* sp. as source of bioavailable iron by the *bacterium Pseudomonas fluorescens* was assessed.

3.3. Material and methods

3.3.1. Selection of fungal species with suitable metabolic activities

Identification of halotolerant and alkalitolerant fungal species

To select alkalitolerant fungi, a screening of different strains with diverse ecologies (Table 6) obtained from the fungal collection of the laboratory of microbiology of the University of Neuchâtel was performed on solid malt extract (1.2 %[wt/wt] in deionised water) agar (1.5 %[wt/wt] in deionised water) (MA) medium buffered at pH 9.2 with ($NaHCO_3$ 0.765 % and $NaCO_3$ 0.106 % [wt/wt] in deionised water). Additional experiments were carried out on solid MA medium amended with 50 mM of NaCl aiming to select halotolerant fungal strains. This concentration was selected according to literature suggesting that archaeological iron objects contain an average of 50 mM of chlorine (Rimmer, Watkinson et al. 2012). Each strain was inoculated in three replicates and incubated at room temperature.

Metabolic activities regarding iron

Iron reduction was studied in 1.2 % malt extract liquid cultures amended with 10 mM $FeCl_3$ and buffered at pH 9.2. Abiotic controls were also performed. In order to quantify iron reduction, 0.5 ml samples were collected from the liquid cultures during inoculation, and 2 and 4 days after incubation. Fe(II) concentration was measured with the Ferrozine® reagent as previously described (*2.3.4. Reduction of soluble iron phase in presence of chlorine*).

Spectophotometric analyses with the Ferrozine® were also conducted in order to assess iron uptake/adsorption by the selected fungal species.

These experiments were conducted as indicated above for the iron reduction experiments using as well 1.2 % malt liquid cultures amended with 10 mM $FeCl_3$ and buffered at pH 9.2.

Siderophore production was also studied for the selected fungal species using a CAS-amended media (chrome azurol S) as previously described (Daghino, Turci et al. 2006).

Table 6: List of the 30 species selected for the screening of alkalitolerance and halotolerance abilities.

Species tested	Ecology
Acremonium sp.	Saprophyte
Agaricus lignicola	Saprophyte
Alternaria alternata	Saprophyte, phytopathogen
Armillaria mellea	Saprophyte, phytopathogen
Aspergillus niger F75	Saprophyte
Aureobasidium sp.	Saprophyte, endophyte
Beauveria bassiana	Entomopathogen
Beauveria caledonica	Entomopathogen
Botritis cinerea	Phytopathogen
Alternaria sp.	Saprophyte, phytopathogen
Clitocybe geotropa	Saprophyte
Coprinus comatus	Saprophyte
Cordiceps sinensis	Entomopathogen
Fusarium culmorum NEU F376	Phytopathogen
Fusarium graminearum	Phytopathogen
Fusarium sp.	Phytopathogen
Ganoderma lucidum	Saprophyte
Laetiporus sulphureus	Saprophyte
Lepista nuda	Saprophyte
Morchella sp.	Saprophyte, ectomycorrhyze
Phanerochaete chrysosporium	Saprphyte
Pleurotus ostreatus	Saprophyte
Pycnoporus cinnabarinus 1	Saprophyte
Pycnoporus cinnabarinus 2	Saprophyte
Pycnoporus cinnabarinus 3	Saprophyte
Trametes versicolor	Saprophyte
Trichoderma sp.	Saprophyte, mycoparasite
Ulocladium sp.	Saprophyte
Verticillium dahlia	Phytopathogen

The production of biogenic iron minerals was assessed using solid MA medium amended with different iron sources (soluble iron citrate and insoluble FeO(OH)) at 10, 50,100 and 150 mM of iron. Sampling was carried out once per week during 1 month and samples were observed with a Leica DMR optical microscope coupled with a Leica DFC 7000 T camera.

The production of EPS and their abilities to chelate iron was also evaluated in *B. bassiana* incubating this fungus in 1.2% malt liquid medium amended with 10 mM of iron citrate. Scanning Electron Microscopy coupled with Energy Dispersive X-Ray Spectroscopy (SEM-EDX) observations were carried out in order to study the size, shape and composition of the particles newly formed in the presence of iron in liquid cultures. Samples were prepared as previously described (Bindschedler, Cailleau et al. 2014). Briefly, a primary fixation in 2.5 % glutaraldehyde in phosphate buffer solution (PBS) and a secondary fixation in 1 % osmium tetroxide in PBS were performed. After, a dehydratation in ethanol series of increasing concentrations with tetramethylsilane (TMS) ($Si(CH_4)_3$) was carried out. Fungal biomass was placed on a SEM stub and gold-coated with thicknesses of 18 nm. SEM analysis was performed using a Tescan Mira LMU operated at a distance of 10 mm and a voltage of 10 keV acceleration. Elemental microanalyses were performed with an EDAX energy-dispersive spectrometer (EDS) coupled to the SEM.

3.3.2. Fungal treatments of corroded iron coupons

For this experiment *B. bassiana* and *Alternaria* sp. were grown on liquid malt extract (1.2 % [wt/wt] in deionised water) medium using aqueous solutions with a pH > 9.2 allowing the passivation of iron (Selwyn 2004). Therefore, in order to avoid further corrosion of the coupons the medium was buffered at pH 9.2 with $NaHCO_3$ 0.765 % [wt/wt] in deionised water and $NaCO_3$ 0.106 % [wt/wt] in deionised water. The buffer solution was autoclaved separately in order to avoid darkening of the medium. Corroded iron coupons presenting an urban corrosion layer mainly composed of lepidocrocite and goethite (12.5 x 25 x 2-3 mm) were sterilized by spraying a solution of ethanol 70 % w/w in deionised water, and by UV exposure (20 minutes each side). A second sterilisation step was carried out through autoclaving (120°C for 20 minutes). Afterwards, each coupon was placed in a 50-mL cultivation flask containing 20 mL of the culture medium previously described. Cultures were prepared in triplicates. For biogenic mineral formation, 1 mL of a suspension of spores of *B. bassiana* (Tween 0.012 mM in deionised water) was added to the culture medium, whereas for the biocleaning test the mycelium of *Alternaria* sp. was taken out from a solid culture and

added to the culture medium. To evaluate the eventual interaction between the medium and the corroded coupons, abiotic controls were also performed. Samples were incubated under agitation (150 rpm) at room temperature, for one week, two weeks, and four weeks. After fungal treatments the coupons were sterilised and washed by spraying a solution of ethanol 70 % [wt/wt] in deionised water, and by exposure to UV radiation (20 minutes each side), and stored under dry conditions until colorimetric measurements and Fourier transformed infrared spectroscopy (FTIR) analyses were performed.

For the estimation of colour changes during fungal treatment coupons were analysed with a Minolta CM-508D spectrophotometer. The following measurement conditions were employed: Specular Component Included (SCI), Illuminant D65 (daylight containing UV component, colour T 6504K), d/8° geometry, 10° observer, measurement area diameter 8 mm, illumination with Xe flash light source 100% UV containing all UV components or 0% UV containing no UV components, CIELab 1976 colour space. Due to the small dimensions of the coupons, it was possible to perform only 2 measurements on each sample.

For Fourier transform infrared spectroscopy (FTIR) a iS5 Thermo Scientific spectrometer with a diamond Attenuated Total Reflectance (ATR) crystal plate (iD5™ ATR accessory) was used. Samples were analysed without any preparation. All spectra were acquired in the range 4000–650 cm^{-1}, at a spectral resolution of 4 cm^{-1}. A total of 32 scans were recorded and the resulting interferograms averaged. Data collection and post-run processing were carried out using Omnic™ software.

3.3.3. Enhancing of the abilities of selected fungi to produce biogenic iron minerals

Influence of the oxidation state

To test the influence of the oxidation state on the production of biogenic minerals, 4 media were prepared: a malt extract (1.2 %) agar (1.5 %) medium amended with iron(III)-citrate 10 mM, and a malt extract (1.2 %) agar (1.5 %) medium amended with iron(II)-chloride 10 mM. Each of these media was then prepared at neutral pH as well as buffered at pH 9.2 with $NaHCO_3$ 0.765 % and $NaCO_3$ 0.106 %. The 9 halotolerant and alkalitolerant strains enumerated in the table 3 (section 3.1) were inoculated by placing a cube of a pre-culture aged of 1 week in Petri dishes containing the four media in triplicates. To evaluate fungal growth the diameter of the colony was measured each 2-3 days for 14 days. After 1 moth of

incubation microscopic investigations were carried out on each sample, to detect crystals formation.

Influence of manganese

The study of the influence of manganese on the production of biogenic iron oxalates was carried out in solid malt extract (1.2 %) agar (1.5 %) media amended with two different sources of iron; iron(III)-citrate 10 mM and iron(III)-chloride 10 mM. Each medium was prepared with and without of a source of manganese ($MnCl_2 \cdot 4H_2O$ 10 mM). The same procedure described above was carried for the estimation of growth, as well as for the microscopic investigations.

3.3.4. Fungal iron adsorption and biotechnology; what else?

For this experiment each glass flask or utensil used was acid washed in order to avoid iron contamination. For the preparation of fungal biomass chelating iron, *Alternaria* sp. was cultivated in malt extract medium (1.2 %) amended with $FeCl_3$ 10 mM and buffered at pH 9.2 with $NaHCO_3$ 0.765 % and $NaCO_3$ 0.106 %. When not specified differently, all solutions were prepared in deionised water. Cultures without any iron source were also performed as a control. After 1 month of incubation, fungal biomass was separated from the medium by filtration. Several washing steps were performed in order to remove residual free iron from the culture. Briefly, after filtration fungal biomass was collected in 50-mL falcon tubes, and 20 mL of milliQ water were added. After, tubes were gently shaked and centrifuged at 4000 g for 5 minutes to collect biomass. In order to verify the absence of residual iron in the biomass, iron content was measured in the washing solution with the Ferrozine assay as previously described (*Chapter 2.2. Use of bacteria to stabilise archaeological iron*), with modifications. Since in this experiment total iron and not only Fe^{2+} had to be quantified, 15 µL of hydroxilamine 1.5 M were added to reduce all the iron before the addition of the Ferrozine reagent. Biomass was considered to be clean when the value of total iron was similar to the one of milliQ water. Hereafter, Biomass was homogenised with Ultra-Turax®, freeze-dried, homogenised, and sterilized through a dry autoclaving (20 minutes, 120°C).

Total iron content was measured also in the dead sterilised fungal biomass after acidic digestion with HCl 10 M. In order to verify that no free iron was present, samples of this dead fungal biomass were also immersed for 6 hours in milliQ water as well as in M9 medium, and the amount of iron in solution was measured with the Ferrozine assay.

After the preparation of the fungal dead biomass the second step was the preparation of bacterial cultures. In order to remove previous traces of iron, *P. fluorescens* was inoculated 3 times consecutively in iron free M9 media. The scheme in Figure 25 illustrates the different conditions tested. Briefly, 200 µL of an overnight culture of *P. fluorescens* were inoculated in 20 mL of M9 standard medium with the normal content of iron (0.5 mM), in M9 media free of iron, in M9 media free of iron but amended with fungal biomass without iron (2 different amounts of biomass: 4 and 18 mg), and finally in M9 media free of iron but amended with fungal biomass chelating iron (2 different amounts of biomass; 4 and 18 mg).

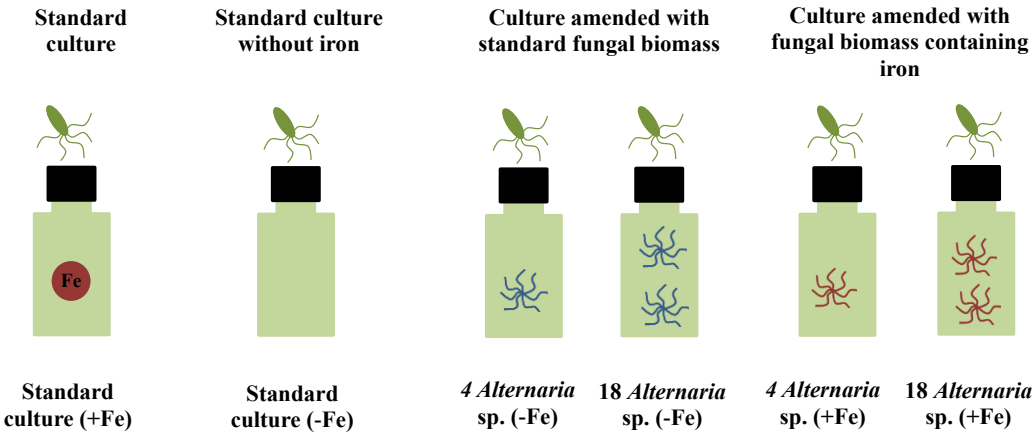


Figure 25: Scheme representing the different parameters tested during the experiment

The quantity of fungal biomass added to the culture (4 mg) was calculated in order to have nearly the same amount of iron than in the standard culture (0.5 mM), and a second culture was performed with an amount of fungal biomass (18 mg) corresponding to concentration of 2 mM of iron.

The ability of *P. fluorescens* to use iron chelated in the fungal biomass as an iron source was evaluated through the quantification of the fluorescent siderophore pyoverdine with spectrophotometric analysis. For the quantification of this compound a calibration curve was performed with known concentration of commercially available pyoverdine (Sigma Aldrich). After 1 and 2 days of incubation, 1 mL of each culture was sampled. 200 µL were used for the quantification of the cell density through spectrophotometric analysis at 600 nm, while for pyoverdine quantification, the rest were centrifuged at 10000g and the absorbance at 405 nm

was measured in the supernatant. Abiotic controls were also prepared, and each experiment was performed in triplicates.

3.4. Results and discussion

3.4.1. Selection of fungal species with suitable metabolic activities

Nine out of the 29 fungal species were able to grow under alkaline conditions and to tolerate a NaCl concentration of 50 mM (Table 7).

Concerning, the metabolic activities of this alkalinotolerant and halotolerant fungi regarding iron, Fe(II) quantification in the culture medium demonstrated that *Agaricus lignicola* had the ability to reduce iron (Table 7), which was probably due to the production of extracellular reductive enzymes or Fe^{3+} reductant compounds. Indeed, this fungus is able to degrade lignocellulose through the Fenton reaction mechanism, which involves such iron reducing molecules (Arantes, Milagres et al. 2011).

Once purified, these substances could be used for proposing a biological treatment that does not involve living microorganisms, but only the active molecules. This could avoid the sometimes fastidious supply of all the factors affecting fungal growth such as a carbon source, moisture and pH. Reduction of the chlorinated iron corrosion compound present in the archaeological object will then allow a decrease in the porosity of the corrosion layer and consequently dissolve out the chlorine from the object.

Table 7: Summary of the results obtained through the study of fungal metabolic activities regarding iron of selected alkalitolerant and halotolerant fungi.

Alkalitolerant and halotolerant fungal species	**Reduction**	**Uptake**	**Siderophore**	**Crystals**	**EPS**
Acremonium sp.	-	-	-	-	-
Agaricus lignicola	+	-	+	-	-
Alternaria sp.	-	+	-	-	-
Beauveria bassiana	-	-	+	+	+
Coprinus comatus	No growth	No growth	+	-	-
Cordiceps sinensis	-	-	-	-	-
Fusarium culmorum	-	+	+	-	-
Fusarium graminerarum	-	+	+	-	-
Morchella esculenta	No growth	No growth	+	-	-

Spectophotometric analyses with the Ferrozine® were conducted in order to assess iron uptake/adsorption by the selected fungal strains. Iron uptake/adsorption in *Alternaria* sp., *Fusarium culmorum* and *F. graminaerum* was demonstrated (Table 7). After two days of

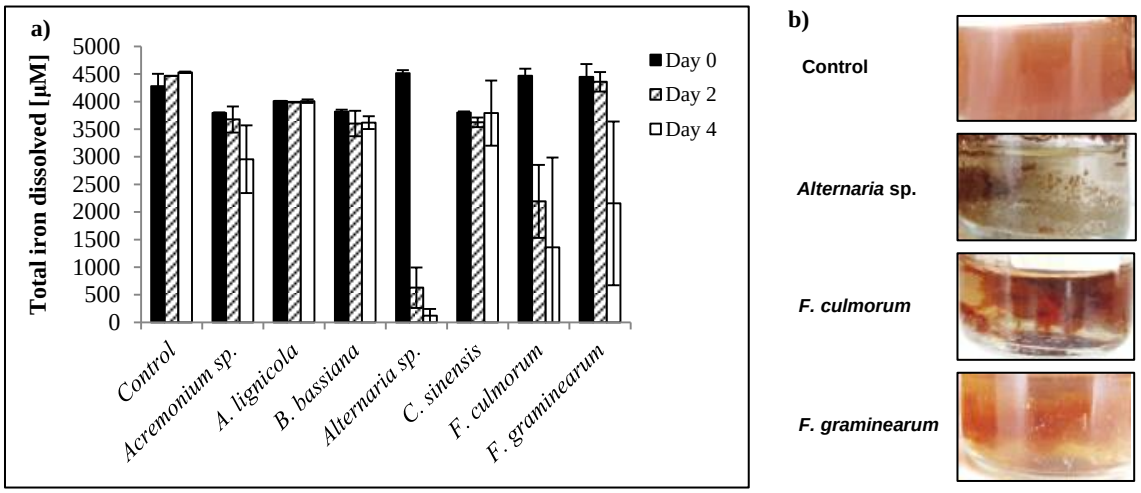


Figure 26: a) Total dissolved iron measured after 0, 2 and 4 days incubation of nine alkalitolerant and halotolerant fungal strains, in liquid malt extract medium amended with 10 mM $FeCl_3$. b) Control and cultures of *Alternaria* sp., *F. culmorum* and *F. graminearum* after 4 days incubation.

incubation the iron concentration decreased from 4514 µM to only 627 µM in the culture medium of *Alternaria* sp. (Figure 26). The two strains of *Fusarium* were less efficient, as iron concentration in *F. culmorum* cultures decreased from 4468 µM to 1356 µM, while in *F. oxysporum* cultures, it remained stable the first two days of incubation (4447 µM at day 1 and 4358 µM at day 2) and then decreased at day 4 (2156 µM). In addition the high standard deviations of total iron values measured in the culture of this two *Fusarium* strains after 4 days of incubation, revealed also the unstable behaviour of this fungi regarding iron uptake/adsorption.

Inoculation on CAS-amended media revealed that, *F. culmorum* and *F. graminearum* were able to produce siderophores, whereas this capacity was not observed in the *Alternaria* sp. (Table 7). This suggests that in the latter iron uptake is not directly related to siderophore production but to another mechanism. It can be hypothesised that different strategies are employed and that since *Alternaria* sp. is able to melanise its cell wall, this fungus could exploit cell wall adsorption for iron mobilization and rather than active uptake. This capacity could then be used to develop a biological cleaning of the object, removing the external part of the corrosion layer without damaging the original surface. A biological removal of a part of the oxidation layer could for example replace electrolytic reduction that has to be monitored carefully due to the production of H_2.

Optical microscopy investigations allowed observation of biogenic particles only for *B. bassiana*. Brown rosette-like aggregations similar to copper oxalate were detected after 1 month of incubation in cultures amended with 10 mM iron citrate (Figure 27 a and b), as well as 100 mM and 150 mM FeO(OH) (Figure 27 c-f). However, the low amount of crystals formed did not allow their identification with Raman Spectroscopy. Further investigation is needed in order to better understand the mechanism of crystal formation and to increase the amount of biogenic crystals produced by *B. bassiana.*

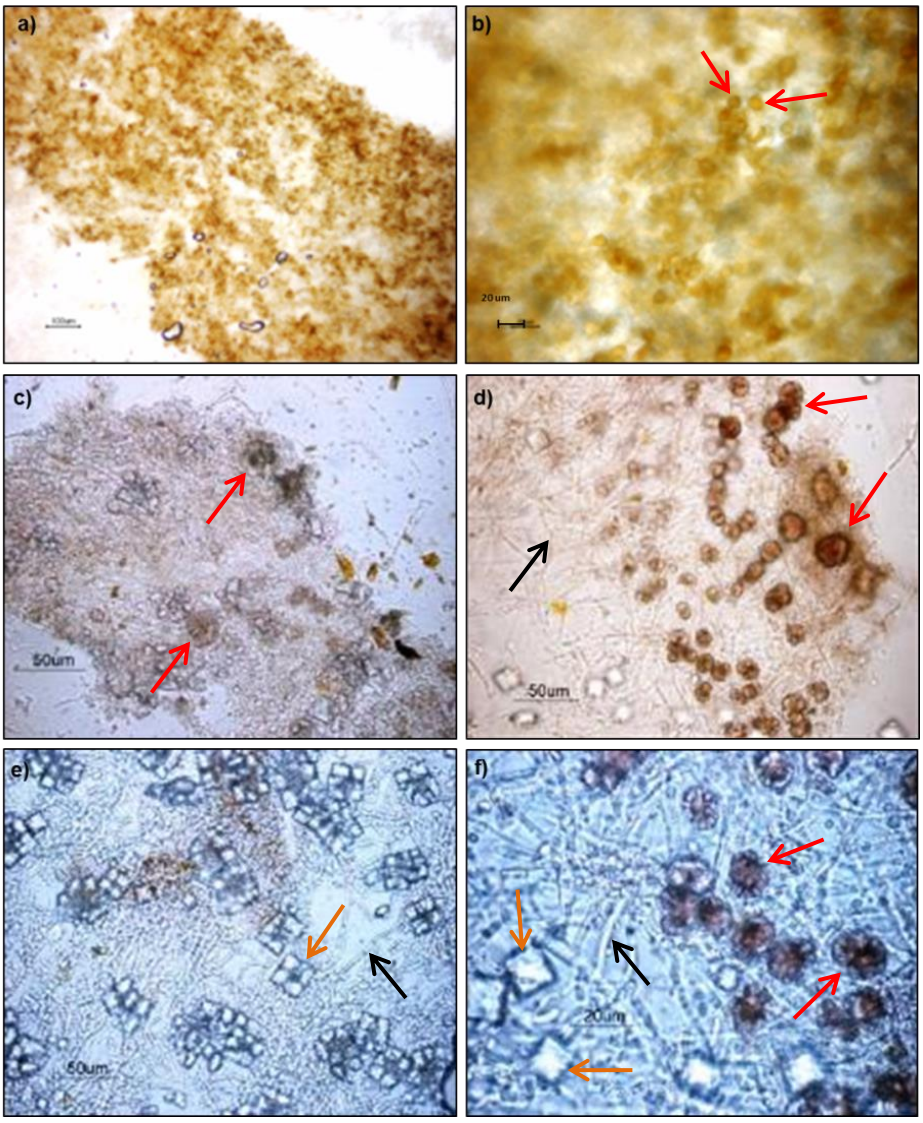


Figure 27: Biogenic minerals produced after 1 month of incubation of *Beauveria bassiana* with iron citrate a) at 100x original magnification and b) at 400x original magnification, or 10 mM FeO(OH) at 400x original magnification c)-f). In the figure two different types of crystals are presented, the first have a rosette-like habitus and brown colour (indicated by red arrows), while the second are transparent and seems to have a squared-bipiramidic habitus (indicated by orange). Fungal mycelium is also present and is indicated by black arrows.

The production of EPS and its ability to chelate iron was also evaluated in *B. bassiana* by incubating this fungus in 1.2% malt extract liquid medium amended with 10 mM iron citrate. Scanning electron microscopy coupled with Energy dispersive X-Ray spectroscopy (SEM-EDX) observations were carried out in order to study the size, shape and composition of the particles newly formed in the presence of iron in liquid cultures. SEM observations showed the presence of aggregates on the hyphae. Iron, carbon and oxygen were present in these particles, which were smaller than 1 μm (Figure 28).

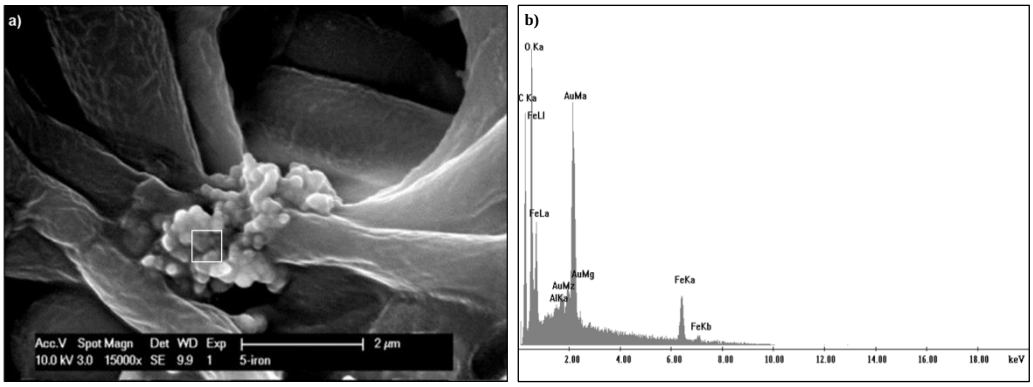


Figure 28: a) Secondary electron micrograph showing the aggregates on *Beauveria bassiana* hyphae and b) corresponding Energy Dispersive Spectroscopy spectrum.

3.4.2. Fungal treatments of corroded iron coupons

Observation of the abiotic control coupons, as well as the samples treated with *B. bassiana* had a similar colour to the untreated coupons (red-yellow) after incubation. In contrast, the colour of the plates changed drastically in the samples incubated with *Alternaria* sp. for the biocleaning assay. In fact, the corrosion layer of the coupons incubated 4 weeks with this fungus was partially removed, and the metallic surface was revealed (Figure 29). This visual observation was confirmed by the colorimetric measurements that revealed a shift of the colour coordinates measured on the surface of the coupons treated with *Alternaria* sp. towards green (less red), as well as higher lightness L* value (towards white) (Figure 29). The removal of a part of the corrosion layer was probably the consequence of the iron uptake abilities of *Alternaria* sp. In addition, colorimetric measurements allow to observe the same phenomenon, but less pronounced, on the coupons treated with *B. bassiana* for 4 weeks.

Indeed, the colour data revealed that this coupon was less red and had a higher lightness after the treatment. This could be explained by a leaching of the corrosion layer caused, for example, by organic acid production.

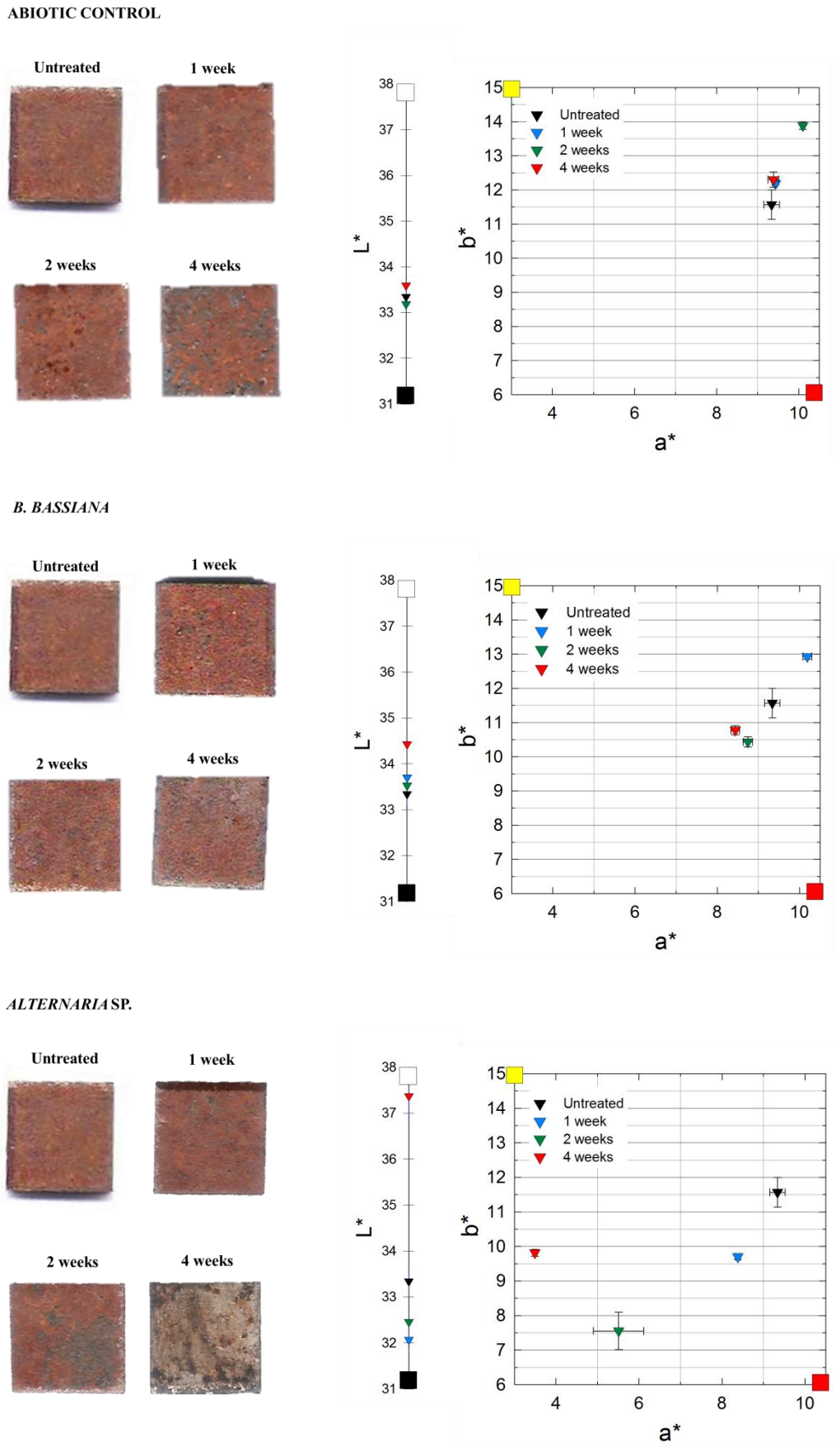


Figure 29: Colorimetry plots representing colour coordinates L^* (lightness, bright-dark) a^* (red/green opponent colours) and b^* (yellow/blue opponent colours) for all the samples, as well as the appearance of the coupons after the incubation with uninoculated medium (abiotic control) (in black), *B. bassiana*, and *Alternaria* sp., for 1 week (in blue), 2 weeks (in green), and 4 weeks (in red).

The molecular composition of the surface of the coupons before treatment allow the identification of lepidocrocite (1016 cm^{-1}) and a contamination of lipids (2917, 2849, 1470, and 1417 cm^{-1}) (Figure 30 a) (Jiao, Cody et al. 2010). Oxyhydroxides like lepidocrocite are currently found in the corrosion layer of iron objects, while lipids are probably the results of a contamination occurred during the cutting and marking procedure of coupons. FTIR investigations on the abiotic control samples revealed the presence of malt. In fact, the double peak of maltose at 2980 and 2900 cm^{-1}, and the one of carbohydrate at 1060 cm^{-1} were identified. This corresponds probably to rests of the culture medium that remained on the corrosion layer of the abiotic control coupons even after the washing step with ethanol 70 %. Additionally, due to the presence of a peak at 1394 cm^{-1} (*), the production of siderite is supposed. This phenomenon could be the result of the interaction between the iron compounds of the corrosion layer and the carbonates added in the medium as buffer solution. In this case no difference was observed between the samples incubated at different times. On the other hand, FTIR spectra recorded on coupons treated with the fungus *B. bassiana* differed depending on the time incubation (Figure 30 b). Indeed, measurements realised on the sample incubated for 1 week revealed the presence of malt (same absorbance peaks than in the abiotic control at 2980, 2900 and 1060 cm^{-1}), and lepidocrocite (principal vibrational band partially hidden by the carbohydrate absorbance peak at 1060 cm^{-1}, but presence of the absorbance peaks at 891 and 787 cm^{-1}). Whereas, spectra recorded on the sample incubated for 2 weeks had a similar pattern than the untreated samples. In fact, lipids (2918, 2850 cm^{-1}) and lepidocrocite (1008 cm^{-1}) were detected. This result could be attributed to a problem on fungal growth. Finally, the analysis carried out on the sample treated with the same fungus but incubated for 4 weeks, revealed the presence of residues of growth medium (2970, 2914 and 1057 cm^{-1}), as well as proteins (amide I peak at 1645 cm^{-1} and amide II at 1539 cm^{-1}), and lepidocrocite (principal absorbance peak hidden by the carbohydrate absorbance peak at 1060 cm^{-1}, but presence of the vibrational band at 892 cm^{-1}). The presence of proteins could be the results of the production of extracellular polymeric substances (EPS) in contact with the iron surfaces. In fact, FTIR spectra performed on fungal EPS displayed the same absorbance peaks for carbohydrates and proteins (Jiao, Cody et al. 2010). Moreover, experimental evidence demonstrated that this fungus exposed to iron produces small aggregated composed by EPS chelating iron ions (Comensoli, Bindschedler et al. 2017) (*3.1. Iron and fungal physiology: A review of biotechnological opportunities, Figure 28*). Finally, due to the possible overlap of the absorbance peak of amide I (at 1645 cm^{-1}) with the one of iron oxalates (at 1627 cm^{-1}) the identification of this latter was not possible with certitude, despite the fact that the production

of iron oxalates by *B. bassiana* has been already reported (Joseph, Cario et al. 2011). In regards to the biocleaning assay with *Alternaria* sp., FTIR measurements did not detect either carbohydrates or proteins (Figure 30 c). The spectra recorded on these coupons were similar to the abiotic controls and only lipids (2917 and 2848 cm^{-1}) and lepidocrocite (1009, 889, and 798 cm^{-1}) were detected.

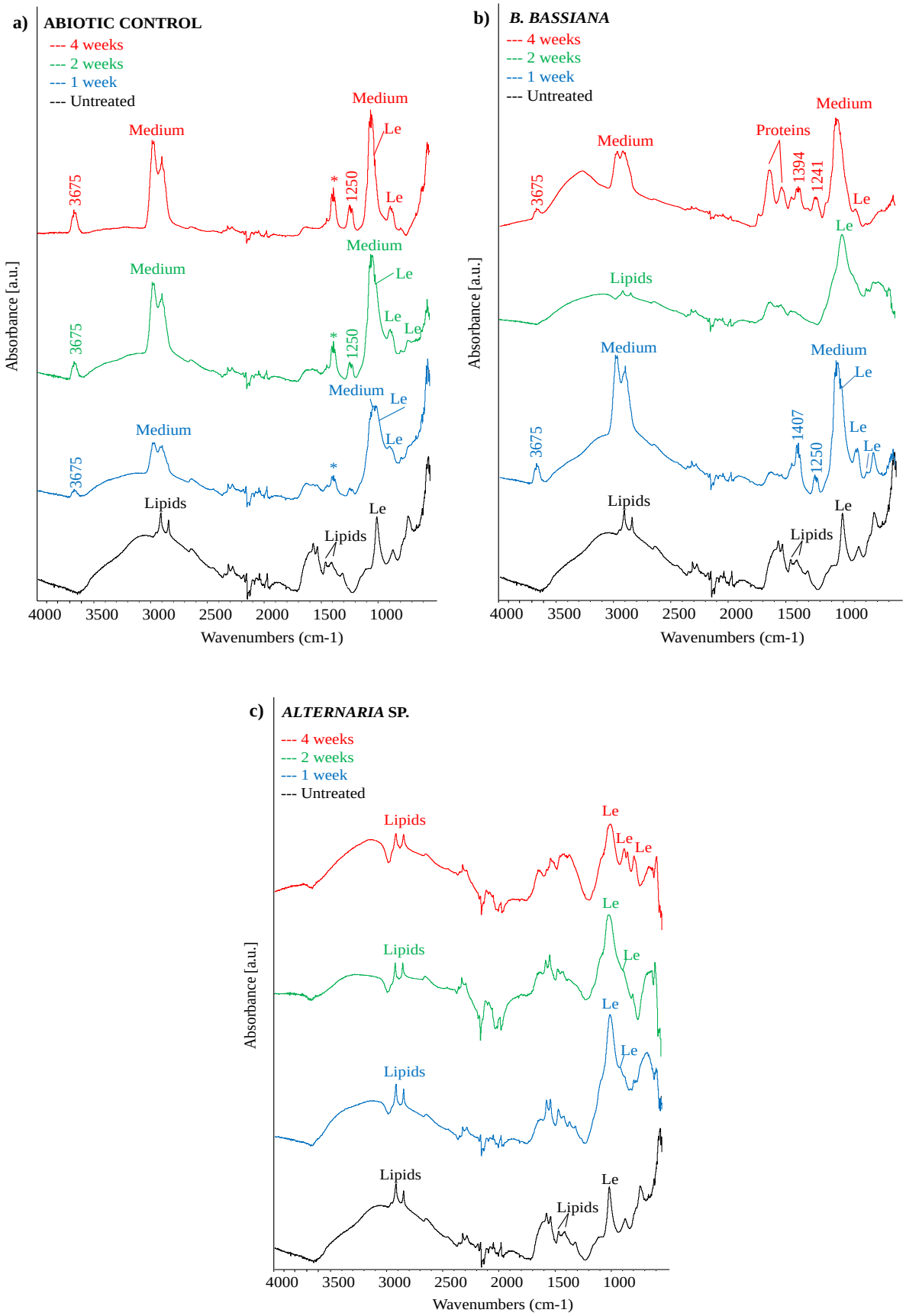


Figure 30: ATR-FTIR spectra recorded on the untreated coupons as well as on the coupons incubated with; a) uninoculated medium (abiotic control), b) *B. bassiana*, and c) *Alternaria* sp. for 1 week (in blue), 2 weeks (in green), and 4 weeks (in red). Principal compounds were identified lipids, lepidocrocite (Le), carbohydrates from the culture medium, and proteins. The asterisk indicates the absorbance peak that can be attributed to siderite.

3.4.3. Enhancing of the abilities of selected fungi to produce biogenic iron minerals

Influence of the oxidation state

The inoculation of halotolerant and alkalitolerant fungi on media amended with different iron sources, as well as at neutral and alkaline pH, allows studying biogenic minerals production depending on the iron oxidation state. Except for *C. comatus, F. colmorum,* and *M. esculenta,* only small differences were observed on the growth rate of the fungi inoculated in the medium amended with iron(III) citrate at neutral pH compared with the same medium buffered at pH 9.2 (Figure 31). In the medium amended with iron(II) chloride pH seemed to have a greater influence on the fungal growth rate.

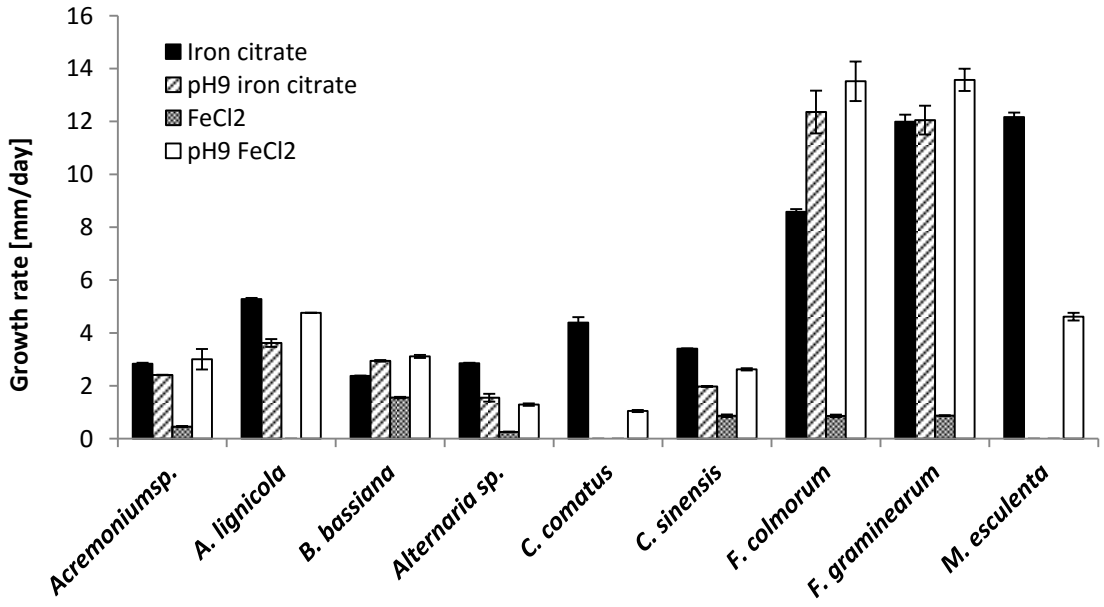


Figure 31: Growth rate of the 9 halotolerant and alkalitolerant fungi inoculated on media amended with Iron(III)-citrate and iron(II)chloride at neutral as well as 9.2 pH.

In fact, *A. lignicola, C. comatus*, and *M. esculenta* did not grow in the medium at neutral pH, whereas fungal development was observed in the same medium buffered at pH 9.2. Even though growth was detected for all the other species in the medium amended with iron(II) chloride at neutral pH, the same trend than for the previous species was noticed. Indeed, growth rate values of these fungi were at least 5 times lower in the medium with neutral pH than in those buffered at pH 9.2.

The fungal species less influenced by this culturing condition (medium containing iron(II) chloride at neutral pH) were *B. bassiana* and *C. sinensis*. The growth rate of these two species dropped from 3.1 to 1.5 mm/day, and from 2.6 to 0.9 mm/day for *B. bassiana* and *C. sinensis*, respectively (Figure 31).

Regarding biogenic mineral production, none of the conditions stimulated this ability. Crystals were detected only in the medium inoculated with the fungus *B. bassiana* amended with iron(III) citrate (Figure 32). These minerals were already reported to be produced in these conditions (*3.4.1. Selection of fungal species with suitable metabolic activities*) and even if no molecular characterisation were performed, due to rosette-like habitus similar to the one of copper oxalates and the ability of this fungus to produce large amount of oxalic acid, the production of iron oxalates is highly suspected. Interestingly, crystal formation was not observed in the same medium but buffered at pH 9.2. This could be attributed to the presence of sodium ions present in the medium, added with the buffer solution ($NaHCO_3$ and Na_2CO_3) that could interact with oxalic acid. Since, even *B. bassiana* was not able to produce biogenic minerals in medium containing iron(II) -chloride, it can be assumed that for the production of biogenic iron minerals an iron(III) source is needed.

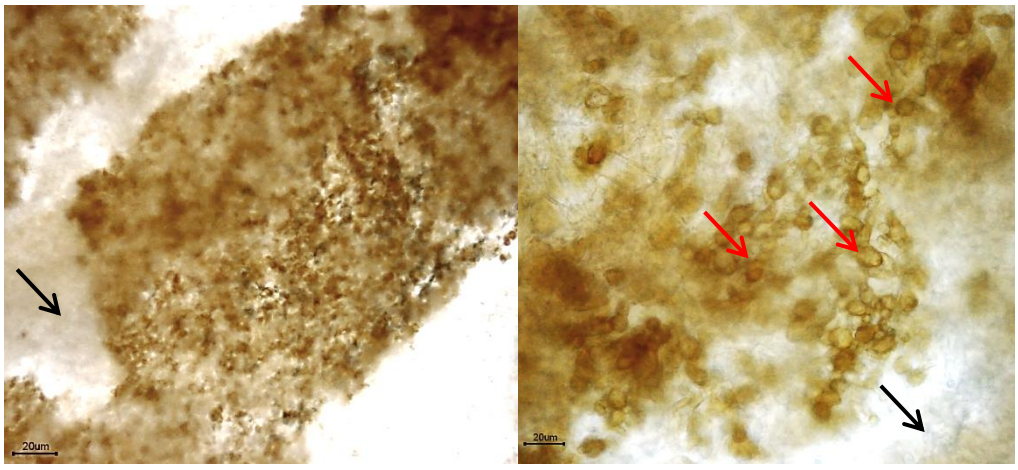


Figure 32: Biogenic minerals produced by *B. bassiana* after 1 month of incubation in solid malt extract (1.2 %) agar (1.5 %) media, amended with iron(III)-citrate at neutral pH. In the figure with crystals rosette-like habitus and brown colour are indicated by red arrows, while fungal mycelium is indicated by black arrows.

Influence of manganese

The second experiment aimed to verify the influence of manganese on the production of biogenic minerals in two oxalic acid-producing fungi (*B. bassiana and B. caledonica*). Overall the growth rate of the fungi inoculated in the medium with and without manganese did not vary (Figure 33). Even though this experiment did not allow to conclude that manganese stimulates oxalic acid production, microscopic investigations allowed to observe that manganese had an inhibitory effect on the production of biogenic minerals. Indeed crystals were produced (by both strains) only in the medium amended with iron(III) citrate and no manganese.

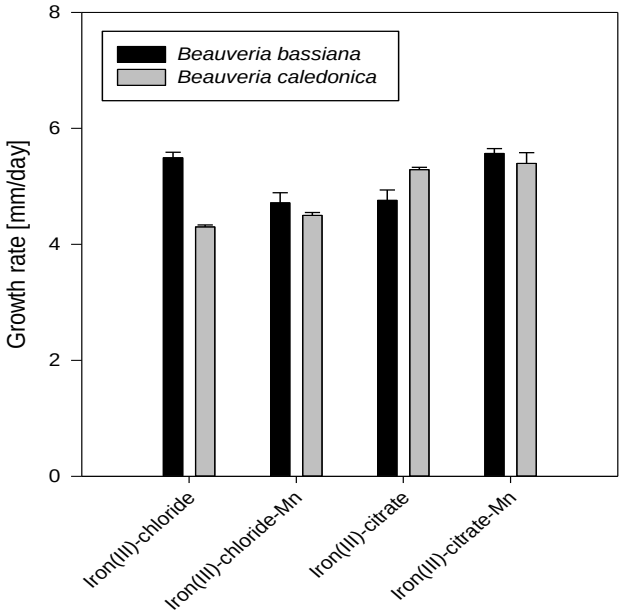


Figure 33: Growth rate of *B. bassiana* and *B. caledonica* inoculated on iron(II) chloride and iron(III) citrate with and without manganese 10 mM amendment.

3.4.4. Fungal iron adsorption and biotechnology; what else?

Fungal biomass exposed to iron showed a black colouration after 1 month of incubation with iron (Figure 34), and spherical growing plugs of different dimensions were observed. When cut these aggregates revealed a rusted pigmentation probably due to iron content (Figure 34 b). In addition, at the end of the biomass preparation a clear colour difference was observed between fungal biomass grown without and with iron; standard fungal biomass had a beige colour, while brown-dark pigmentation was observed for the biomass incubated with iron (Figure 34 c).

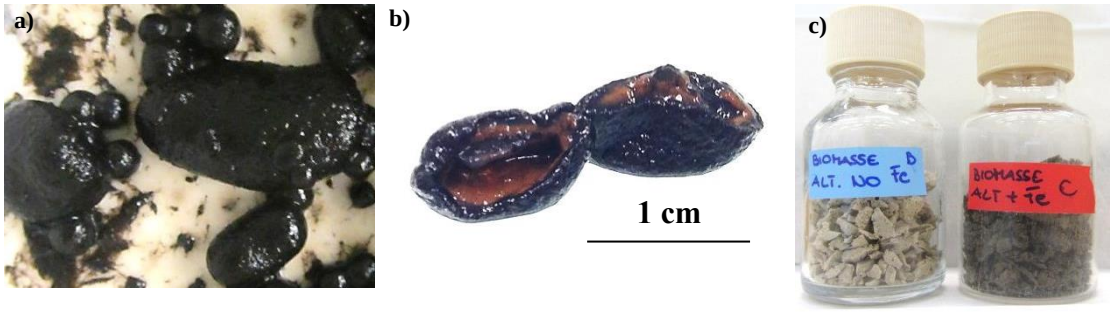


Figure 34: Appearance of the fungal biomass containing iron a) after filtration, and b) cut. c) Appearance of the biomass after freeze-drying and with or without iron.

This difference was also confirmed by iron quantification in the fungal dead biomass. In fact 2.159 µmol of iron per mg of dry biomass were measured after acidic digestion of the fungal dead biomass incubated with iron 10 mM, while only 0.004 µmol of iron per mg of dry biomass were released after the same procedure was applied to the dead biomass of the fungus incubated in the absence of iron (Table 8).

Spectrophotometric quantification of iron released in water as well in M9 medium, allowed to confirm that iron was chelated on the biomass and almost no iron was free (Table 8). Indeed, only 0.006 and 0.007 µmol of iron per mg of dry biomass were released in water and M9 medium respectively after immersion of fungal dead biomass chelating iron for 6 hours.

Table 8: Quantification of iron released from the fungal biomass incubated for 6 hours in water, M9 medium, and HCl 10 M.

	Iron released [µmol/mg of dry weight]	
Treatment	**Biomass cultivated without iron**	**Biomass cultivated with iron**
Water	0.003 ± 0.001	0.006 ± 0.001
M9 medium	0.002 ± 0.001	0.007 ± 0.001
HCl 10 M	0.004 ± 0.001	2.159 ± 0.9

Spectrophotometric analysis of the amount of pyoverdine produced confirmed the hypothesis that *P. fluorescens* is able to use iron chelated in the fungal dead biomass as a source of bioavailable iron. In fact, in the culture amended with fungal dead biomass chelating iron, the amount of pyoverdine produced was 6.5 mg/L/DO_{600} for the culture incubated with 4 mg of dead biomass and 7.6 mg/L/DO600 in the culture incubated with 18 mg of fungal dead biomass (Figure 35). These values were similar to the one measured in the standard culture containing bioavailable iron (7.5 mg/L/DO600 of pyoverdine). Whereas in the culture without iron the amount of pyoverdine produced after 1 day of incubation were higher. Indeed, in the standard culture free of iron 13.6 mg/L/DO600 of pyoverdine were measured, and similar values were detected in the cultures amended with fungal biomass free of iron; 14.6 mg/L/DO600 for the culture containing 4 mg, and 12.7 mg/L/DO600 in the culture containing 18 mg of dead biomass free of iron. The same trend was observed after 2 days of incubation (Figure 35).

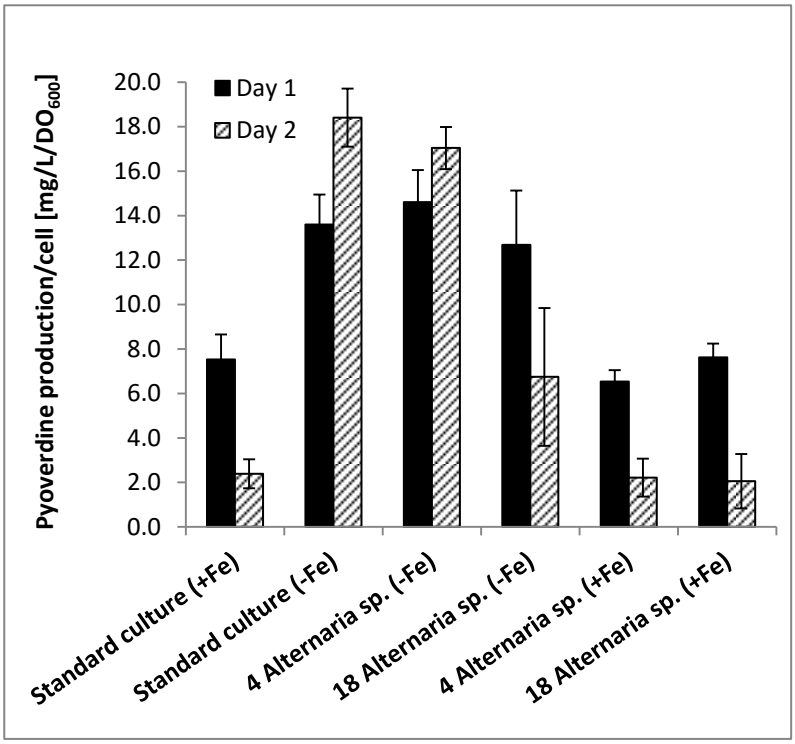


Figure 35: Quantification of pyoverdine normalized by the cell optical density measured at 600 nm. Measurements were effectuated after 1 and 2 days of incubation, in the culture of *P. fluorescens* incubated with and without an iron source.

3.5. Conclusions

The screening of selected fungal species allowed to obtain 9 halotolerant and alkalitolerant fungi. However, only 3 species were found to have metabolic activities potentially useful for the development of stabilisation methods for corroded iron. First, *A. lignicola*, was able to reduce soluble iron. Further investigation is still required, but the isolation of one or several iron reductive molecules could permit developing a stabilisation method based on the reduction of the iron compounds present in the corrosion layer. Second, iron adsorption abilities were shown for *Alternaria* sp. This capacity could be exploited to develop a biological treatment aiming to remove the corrosion layers in a selective way, without damaging the original surface, replacing for example electrolytic reduction methods. Third, *Beauveria bassiana* displayed the ability to produce biogenic iron minerals in solid media amended with several iron sources, but also to produce EPS chelating iron in liquid cultures. These metabolic abilities could be exploited to convert a part of an unstable corrosion layer into more stable iron compounds, such as iron oxalates and EPS chelating metals. This conversion could stabilize the iron object by two mechanisms, first by the production of a passivation layer on their surface and second by an indirect chlorine extraction from the corrosion layer.

The experiments performed on corroded iron coupons allowed to reveal that *B. bassiana* is not the ideal candidate for the development of a biotechnological approach for the stabilisation of corroded iron through the production of iron biogenic minerals. In fact, it appeared that even if *B. bassiana* has the ability to produce iron(II) oxalates (Joseph, Cario et al. 2011), when exposed to iron it produce preferentially EPS, compounds able to chelate this metal. In addition, experimental evidence suggests that only metal oxalates produced with divalent cations are insoluble salts (Eckhardt 1985, Varadachari, Barman et al. 1994) but Fe^{2+} is not reported to produce iron oxalates (Eick, Grossl, 1996). Therefore it can be questioned if fungal iron oxalates are stable minerals or not. As a consequence, fungi exposed to high concentrations of iron probably deploy a different detoxification mechanism (for example the immobilisation of iron ions in the EPS matrix) and not the formation of iron oxalates. However, to verify that the chemical signature of iron oxalate in FTIR was not hidden by organic residues (such as malt coming from the culture medium or proteins produced by the fungus), SEM-EDS analysis should be carried out on the surface of the samples.

On the other hand, the incubation of corroded iron coupons during 4 weeks with the fungus *Alternaria* sp. allowed the removal of the main part of the corrosion layer. This encouraging result revealed the potential of fungi for the development of biocleaning methods for corroded iron surfaces. The currently used cleaning methods are sandblasting or electrolytic reduction. These techniques, remove mechanically part of the corrosion layer, but can also alter engravings present on the metallic surface. In contrast, fungal melanin will smoothly capture iron from the corrosion layer (oxidised forms of iron). Then, fungal biocleaning could be useful also with fragile objects having a thin corrosion layer and/or fragile metallic parts presenting a non-cohesive structure (when little of the metal is still present).

Experiments carried out to enhance the ability of two *Beauveria* sp. allowed to define that an iron(III) source is required for biogenic iron minerals formation. Indeed, while some crystals were produced by *B. bassiana* in the medium containing iron(III)-citrate at neutral pH, the same fungus was not able to produce biogenic minerals when exposed to iron(II)-chloride. Even if no molecular characterisation was performed, this could represent an additional confirmation that iron oxalates were not produced with iron(II) cations. In addition, the inhibitory effect of alkaline pH was demonstrated. Finally, manganese displayed also an inhibitory effect on the production of biogenic iron minerals. These results lead to the conclusion that in the case of fungi, biogenic iron minerals production is not the most important metabolic activity regarding iron. Furthermore, since alkaline pH inhibit biogenic minerals production but during the treatment of a corroded iron object alkaline conditions are required to avoid further corrosion, it can be concluded that biogenic mineral formations by fungi is not a microbial activity useful for the development of a biotechnological treatment of corroded iron.

The study of the interaction between dead fungal biomass chelating iron and the bacterium *P. fluorescens* allowed discovering an interesting bacterial-fungal interaction. Even if results are preliminary and other microbes have to be tested this experiment show that bacteria could benefit from iron chelated in fungal dead biomass. This phenomenon could be exploited to develop new fertilizers to amend iron to soils and to improve plant growth. This type of amendment could be interesting because as iron is chelated onto the biomass, it will not leach through soil, and at the same time dead biomass will provide an additional organic matter source. Through this experiment we showed that fungi could actively participate to iron dynamics in soil, not only via the solubilisation, and the production of oxyhydroxides, but

also via the uptake of this metal in their biomass, that could be an important source of bioavailable iron in soil for other microbes, as well as for plants.

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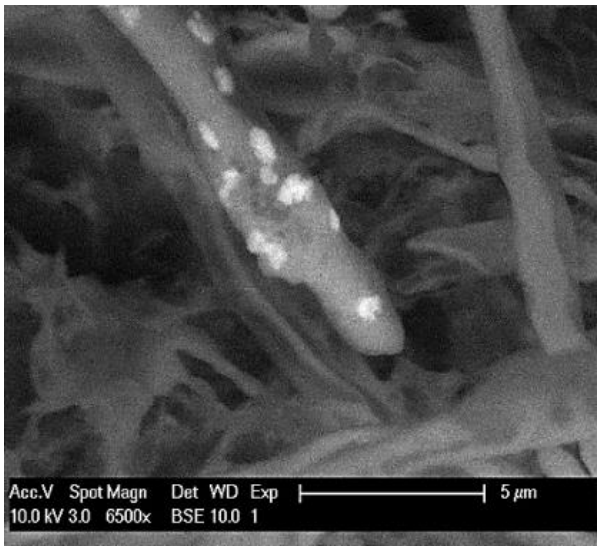
CHAPTER 4

Physiological abilities of alkalitolerant and halotolerant fungi



Hyphae of *B. bassiana* with chlorine aggregates

Part of this chapter is based on the following published articles:

Albini M., Comensoli L., Brambilla L., Domon Beuret E., Kooli, W., Mathys L., ... & Joseph E. (2016). Innovative biological approaches for metal conservation. *Materials and Corrosion*, *67*(2), 200-206.

Joseph E., Bindschedler S., Albini M., Comensoli L., Kooli W., & Mathys L. (2017). Chapter 35 Microorganisms for Safeguarding Cultural Heritage. In *The Fungal Community: Its Organization and Role in the Ecosystem, Fourth Edition* (pp. 509-518). CRC Press.

4.1. Abstract

Fungi are microorganisms able to deal with high levels of environmental stress and their resistance mechanisms could provide valuable solutions in biotechnology. In this chapter the potential of halotolerant fungi to develop innovative methods for the removal of chlorine from corroded iron was studied. To this purpose chlorine uptake was investigated in the fungus *Beauveria bassiana*. Even though this fungus was able to precipitate aggregates composed of potassium and chlorine, no evidence of chlorine uptake was obtained. In addition, accumulation of glycerol and K^+ was studied in media amended with different concentration of NaCl as well as $FeCl_3$ in three fungi belonging to the principal ecological categories (phytopathogenic, melanised saprophytic, and entomopathogenic) found in hypersaline environments. Glycerol accumulation in fungi exposed to NaCl was demonstrated for *F. culmorum* and *Alternaria* sp. but not for *B. bassiana*. In order to obtain additional halotolerant fungal strains, isolation from hypersaline environments was performed, and 12 halotolerant fungal strains were obtained. Finally, a first screening of the production of chlorinated volatiles organic compounds was assessed in all the halotolerant strains (selected and isolated). The aim of this last part was to identify one or several strains able to volatilize chlorine, and exploit this ability to remove this ion from the corrosion layer of corroded iron. Unfortunately the analysis of the results is not yet completed. Therefore only a preliminary overview of the influence of NaCl on the production of fungal volatiles organic compounds was presented at the end of this chapter. In fact, two fungi produced different volatile organic compounds when exposed to 3 % NaCl. Overall, the results obtained in this chapter did not allow assessing if direct chlorine extraction by fungi is a valuable approach to develop stabilisation methods for corroded iron. Nevertheless, some interesting results concerning the interaction between fungi and chlorine were obtained.

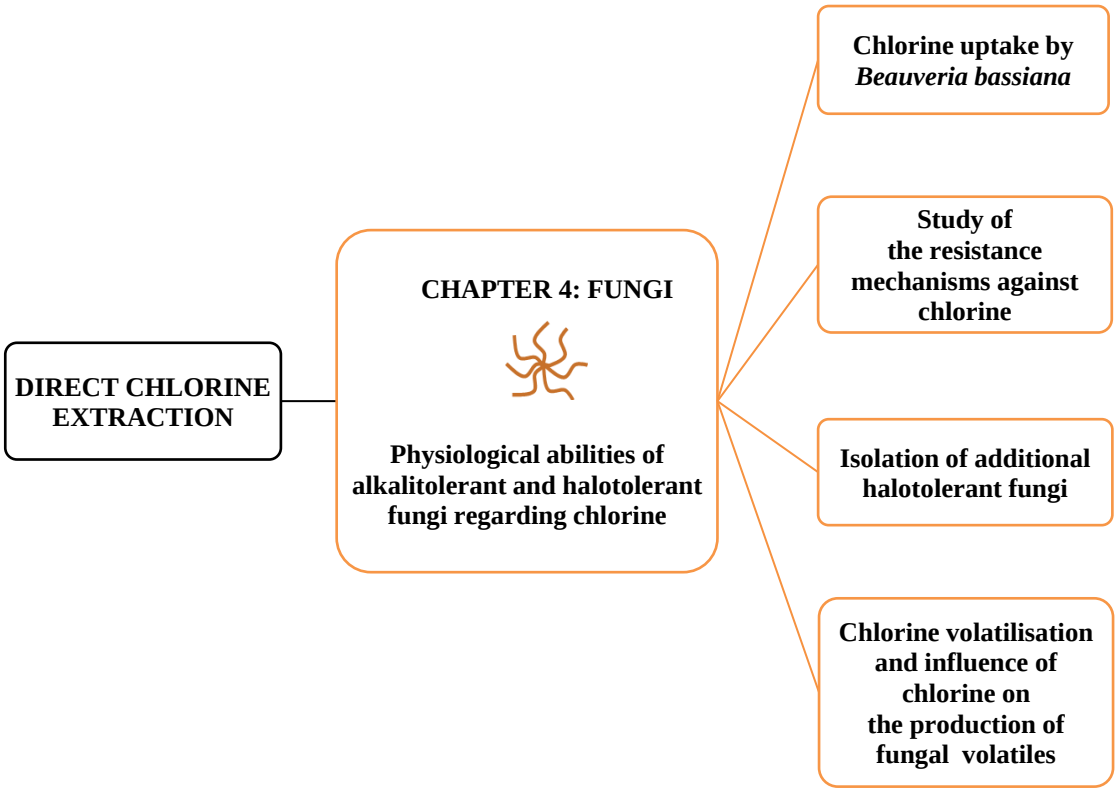


Figure 36: Graphic summary representing the overall structure of the chapter 4: physiological abilities of halotolerant and alkalitolerant fungi regarding chlorine.

4.2. Introduction

The field that provides more information about fungal metabolic activities regarding chlorine is the study of hypersaline environments, in particular halotolerant and halophilic fungi and their resistance mechanisms against chlorine. It is widely recognized that high salt concentrations are toxic for microbes. In hypersaline environments microorganisms will lose internal water as a consequence of osmosis (Yancey 2005, Kogej, Stein et al. 2007). Microorganisms have developed several strategies to deal with this issue. The first one is to maintain intracellular ion homeostasis via accumulation of KCl. This strategy is reported to be mainly found in extremely halophilic *Archea* and some bacteria (Oren 1999, Yancey 2005, Zajc, Kogej et al. 2014). The second is the accumulation or synthesis of compatible solutes helping to maintain turgor pressure, cell volume and concentration of electrolytes (Roberts 2005, Zajc, Kogej et al. 2014). Several types of molecules could be accumulated for this purpose. This includes, for instance, polyols, sugar, aminoacids, betaines, ectoines, and

uncharged peptides (Roberts 2005). Accumulation of compatible solutes is the strategy reported to be the predominantly used by fungi to achieved osmotic balance (Zajc, Kogej et al. 2014). In fungi the major compatible solutes observed are amino acids and polyols, mainly, glycerol, erythritol, ribitol, arabinitol, xylitol, sorbitol, mannitol, and galacticol (Hohmann 2002, Gunde-Cimerman, Ramos et al. 2009). Finally, experimental evidence suggest that melanin plays an important role, not only in protection against UV radiation, but also preventing desiccation and allowing glycerol retention (Kogej, Stein et al. 2007, Gessler, Egorova et al. 2014).

Several fungal species are reported to colonize hypersaline environments. Some of them are ubiquitous fungi that can be easily found in outdoor and indoor atmospheres. Those include species belonging to genera such as *Cladosporum*, *Alternaria*, *Aspergillus*, or *Penicillium*. Others are more specific to these extreme environments. These include the black yeast *Hortaea werneckii*, reported to be one of the most salt-tolerant organisms described up to now (Gunde-Cimerman, Zalar et al. 2000). This fungal species is able to growth in a nearly saturated salt solution (Gunde-Cimerman, Zalar et al. 2000, Zajc, Kogej et al. 2014).

Another interesting ability of fungi is the production of volatile organic compounds (VOCs), gas-phase and carbon-based compounds able to diffuse in air due to their small size (< 300 Da) (Morath, Hung et al. 2012, Weisskopf, Ryu et al. 2016). They are reported to play important roles in fungal ecology, such as mediation of the interaction with plants, arthropods, bacteria, and other fungi. VOCs can have plant growth promoting effect, as well as antifungal and antibacterial activities (Morath, Hung et al. 2012). Chloromethane (CH_3Cl) is a particular type of chlorinated organic volatile compound produced by white rot fungi (Anke and Weber 2006). This compound is considered to be the most abundant halogenated organic greenhouse gas, responsible for the destruction of the layer of ozone (Anke and Weber 2006). Its production is coupled to wood degradation. Scientists hypothesised that this compound acts as a methyl donor allowing the formation of veratryl alcohol, which is a cofactor in lignin degradation (Harper, Buswell et al. 1990, Anke and Weber 2006). White rot fungi are reported to be able to produce also non-gaseous chlorinated compounds, such as anysil alcohol. This compound is also involved in wood degradation. Indeed, it is required to produce H_2O_2 that activates lignin peroxidase (LiP) and manganese peroxidase (MnP) responsible for lignin degradation (Anke and Weber 2006). Experimental evidence demonstrated that the fungal production of chlorinated organic compounds can result in a substantial accumulation of halogenated compounds in soils (Anke and Weber 2006). For

instance, wood colonized by *Hypholoma* spp. contained 180 mg/Kg of halogenated compounds (Asplund and Grimvall 1991). Even though, the role of these compounds is still under investigation, in addition to lignin degradation their production seems to inhibit fungal chitin synthase activity (Pfefferle, Anke et al. 1990). Then, an additional proposed function of these molecules is chemical warfare for the protection against the colonization of antagonist microbes of the wood substrate (Anke and Weber 2006). Fungi are reported also to be able to degrade chloroaromatics (Harper 2000, Castro 2003). Research from Moore, Gut et al. (2005) demonstrated that even though fungi participate to the production of chloromethane, some fungal species are able to degrade this compound. Indeed, part of the chloromethane produced during wood degradation is immediately degraded by other fungi, and it does not reach the atmosphere (Moore, Gut et al. 2005).

From a biotechnological point of view, only a handful of studies have investigated the exploitation of fungal metabolism involving chlorine. One of the fields under study is the use of fungi to degrade xenobiotic halogenated organic compounds. In fact, white rot fungi are reported to be highly efficient on the degradation of anthropogenic chloroaromatic pollutants, such as the highly toxic insecticide DDT (dichloro-diphenyl-trichloromethane) or the wood preservative PCP (pentachlorophenol) (Rabinovich, Bolobova et al. 2004, Tortella, Diez et al. 2005). Another biotechnological approach exploiting fungal chlorine interaction was described by Bois, Bertrand et al. (2006). Their approach is the identification of halotolerant mycorrhizal fungi able to support plants growth on soils contaminated by salts. However, in the latter study only data on the salt tolerance of fungi was provided, but the effect on plant growth was not investigated.

This chapter aims at elucidating the interaction between fungi and chlorine and to identify a metabolic activity useful for the removal of chlorine from the corrosion layer of iron objects. To this purpose, the ability of *Beauveria bassiana* to uptake chlorine was first evaluated. Afterwards, to better elucidate fungal resistance mechanisms against chlorine, tolerance to this anion was studied in nine halotolerant and alkalitolerant fungi (Table 9). Furthermore, glycerol and K^+ accumulation was evaluated after exposure to different NaCl concentrations (0, 3, 10, and 15 %) in three selected strains. The selection was performed according to their different ecology: *Fusarium culmorum* a phytopathogen, *Alternaria* sp. a saprophyte that is melanised, and *Beauveria bassiana* an entomopathogen. Glycerol accumulation was also studied on the same fungal strains in medium amended with 10 mM of $FeCl_3$ to validate the hypothesis that this compound is involved in iron storage inside the fungal biomass. In

parallel, in order to obtain additional fungal strains that are adapted to high salt content, isolation from salt flats was performed. Finally, aiming to actively remove chlorine from iron objects, chlorine volatilization was investigated in all the halotolerant fungi (isolated strains and selected strains (Table 9), and the influence of chlorine on the production of fungal VOCs was assessed.

4.3. Material and methods

4.3.1. Chlorine uptake by *B. bassiana*

The ability of *B. bassiana* to uptake chlorine was investigated in two different culture media. First, the fungus was inoculated in solid malt extract (1.2 % w/w in deionised water) agar (1.5 % w/w in deionised water) medium amended with 50 mM $FeCl_2 \cdot 4H_2O$ in triplicates. After 15 days of incubation samples were collected and prepared for SEM investigation as previously described (Pearson, Kearsley et al. 2004). A Philips ESEM XL30 FEG environmental scanning electron microscope equipped with an energy-dispersive X-ray analyser was used. The samples were observed in secondary electrons and backscattered electrons modes at an acceleration potential of 10–25 keV and a working distance of 10 mm. Elemental mapping was carried out to ascertain the elemental composition of the aggregates produced by the fungus. Second, to quantify chlorine uptake, 50-mL liquid cultures of the fungus were carried out in liquid malt extract (1.2 % w/w in deionised water) medium amended with NaCl concentration of 0, 0.3, and 3.5 % (w/w in deionised water) in triplicates. After 28 days of incubation, 10 mL were sampled for dry weight measurements, and 5 mL were filtered at 0.2 µm pore-size for the quantification of chloride ions with ionic chromatography. Dry weight was assessed by drying the fungal biomass overnight at 70°C. Chloride concentration was measured by ion chromatography (Dionex ICS-1600) equipped with a conductivity detector and a Dionex Ion Pac™ AS14A (4 x 250 mm) anion exchange column and a Dionex Ion Pac™ RFIC™ (4 x 50 mm) guard column, using a 8 mM Na_2CO_3 and 1 mM $NaHCO_3$ solution at a flow rate of 1.3 mL min^{-1} as mobile phase. The detection limit was 0.05 mg L^{-1}.

4.3.2. Study of the resistance mechanisms against chlorine

To investigate in more detail the amount of chlorine tolerated by nine halotolerant and alkalitolerant fungi (Table 9), these strains were inoculated in triplicate in Petri dishes on malt extract (1.2 % w/w in deionised water) agar (1.5 % w/w in deionised water) amended with the following NaCl concentrations: 0, 0.2, 0.5, 1, 1.5 and 3 % (w/w in deionised water). Inoculation was performed by placing in the centre of each Petri dish a cube (2x2x2 mm^3) collected from a 10 day-aged solid pre-culture of each strain. To evaluate the influence of NaCl concentration on the growth of the selected fungal strains, the diameter of the colony was measured each 2-3 days.

Table 9: Selected halotolerant and alkalitolerant fungal strains

Alkalitolerant and halotolerant fungal species
Acremonium sp.
Agaricus lignicola
Alternaria sp.
Beauveria bassiana
Coprinus comatus
Cordiceps sinensis
Fusarium culmorum
Fusarium graminerarum
Morchella esculenta

Influence of NaCl on the accumulation of glycerol and K^+

To study the influence of NaCl on growth, glycerol and potassium accumulations, three fungi were selected from the nine halotolerant and alkalitolerant strains tested above: *F. oxysporum* (phytopathogen) *Alternaria* sp. (saprophyte and melanised fungus), and *B. bassiana* (entomopathogen). To investigate the influence of NaCl content on fungal growth, the three strains were inoculated in triplicate in malt extract (1.2 % w/w in deionised water) agar amended with 0, 3, 10, and 15 % w/w NaCl. The diameter of the colony was measured each 2-3 days during 10 days.

Regarding the quantification of glycerol and potassium ions, 50-mL cultures in liquid malt extract (1.2 % w/w in deionised water) medium were prepared with the same NaCl concentrations described previously. One mL of spore suspension was added to the media as inoculum. After 9 days of incubation at room temperature the biomass was collected though filtration at 0.45 µm pore-size. Afterwards, the biomass was washed and frozen with liquid nitrogen and freeze-dried. To extract polyols from the freeze-dried fungal biomass, the protocol described by Bligh and Dyer (1959) was employed with a few modifications (Kogej, Stein et al. 2007). Briefly, nearly 30 mg of freeze-dried biomass were suspended in 500 µL of Bligh & Dyer solution (methanol/chloroform/deionised water, 10/5/4) in a 2-mL tube. After, the samples were shaken for 30 minutes (327 t/min). 230 µL of chloroform and 230 µL of deionised water were added, and the samples were shaken for 30 minutes. To allow phase separation samples were centrifuged at 5510 g for 10 minutes (Eppendorf miniSpin). After centrifugation the aqueous phase (polar) containing polyols was transferred to a fresh 2-mL tube. This solution was then used for the quantification of glycerol as well as of K^+ ions. Glycerol and K^+ ions were quantified spectrophotometrically with a glycerol kit assay (Sigma Aldrich) and a potassium assay kit (Diazyme Laboratory), respectively. Given the standard deviation on the measurements, the difference between values was verified with an ANOVA test (n=3), followed by a Tukey's test with R® software.

Influence of $FeCl_3$on the accumulation of glycerol

To assess the influence of $FeCl_3$ on the accumulation/production of glycerol and melanin, liquid cultures of *F. culmorum*, *Alternaria* sp, and *B. bassiana* were performed in malt extract (1.2 % w/w in deionised water) medium amended with 10 mM $FeCl_3$ and buffered at 9.2 pH with $NaHCO_3$ 0.765 % w/w in deionised water and $NaCO_3$ 0.106 % w/w in deionised water.

This pH was chosen as it was previously observed that iron uptake was more pronounced under alkaline conditions for *Alternaria* sp. (data not shown). After 15 days of incubation, the fungal biomass was collected through filtration at 0.2 μm pore-size, and washed several time with deionised water. Part of the biomass was freeze-dried for glycerol extraction and part was used for the quantification of total iron after acidic digestion as previously described (Daghino, Martino et al. 2008). Glycerol extraction and quantification, as well as total iron measurements were performed as described above.

4.3.3. Isolation of halotolerant and halophilic fungi

To isolate halotolerant and halophilic fungi living in hypersaline environments enrichments were performed with samples obtained from the salt lake of Alikis in the island of Kos, Greece (Figure 37). In this location two different sites were identified according to anthropogenic impact. Site 1 was exploited until 5 years ago for the commercial production of salt. In this site water was present and salts crystals had a light-pink colour (Figure 37). Here, three types of samples were collected: superficial salt crystals, salt crystals from the bottom of the pond, and salty water. Site 2 corresponded to a part of the salt lake completely dried where no exploitation was recorded. In this site only superficial salts crystals were sampled since the sediments under the salt crust were completely anoxic. Even if no human exploitation was performed in this location, organic matter coming from birds was observed. In fact, excrements as well as feathers and dead birds were present. Samples were stored at 4 °C, in sterile bags for 2 weeks. Inoculation was performed in the laboratory under sterile conditions in media amended with malt extract (0.12 %) agar (1.5 %) 3 % of NaCl, and chloramphenicol and streptomycin (final concentration 10 mM for both the antibiotics) were added to avoid bacterial growth. Except for the filter obtained after water filtration, which was cut in 4 pieces and placed in the middle of a Petri dishes, for the other samples two different inoculation procedures were carried out to increase the number of isolates. First, 3 particles of each sample were posed in the centre the culture media. The second procedure used dilution to facilitate obtaining pure cultures. Briefly, around 3 g of sample were added to 10 ml of sterile saline water (3 % NaCl) containing Tween80® (0.06 mM) in order to reduce hydrophobicity of particles. After vortex, 1 mL of each dilution (10^0) was used to perform the following serial dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. Five hundred μL of each dilution were inoculated in triplicates on Petri dishes. Cultures were incubated at 30°C for 1 month, and each 2-3 days the Petri dishes were observed to verify fungal growth. Each fungal strain grown was purified through

7 successive inoculations in new culture media. It has to be mentioned, that several strains did not grow after a few successive passages.

Fungal strains isolated from the salt flat sediments were identified by DNA sequencing. DNA extraction was performed according to the instructions of the PowerSoil R _ DNA Isolation Kit (MoBio, Carlsbad, CA, USA) with a bead-beating step of 5 min at 50 beats·s^{-1} (Qiagen, Hilden, Germany). Quantification of DNA extracts was performed using a Qubit fluorometer. DNA concentration ranged from 1.32 to 54.8 ng/μL. For performing PCR amplification of the internal transcribed spacer (ITS) region of the 5.8S rRNA gene, the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') were used; (Anderson, Campbell et al. 2003). PCR master mix contained (in a final volume of 50 μL): 5 μL buffer, 0.25 mM dNTPs mix, 0.4 μM of each primer and 1 U Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA). One microliter of DNA template (concentration ranged from 1.14 to 2 ng/μL of DNA) was added. The PCR program consisted of an initial denaturation at 94◦C for 5 min followed by 30 cycles of denaturation at 94◦C for 30 s, annealing at 55◦C for 30 s, and elongation at 68◦C for 30 s. Final extension was performed at 68◦C for 5 min. The PCR products were purified with a MultiScreen PCRμ96 Filter Plate. Amplicons were quantified using a Qubit fluorometer (DNA concentrations ranged from 22.5 to 43.1 ng/μL) and sent for Sanger sequencing to GATC Biotech AG. The search for similarity against sequences from the ITS region on 5.8S rRNA gene was performed using BLAST.

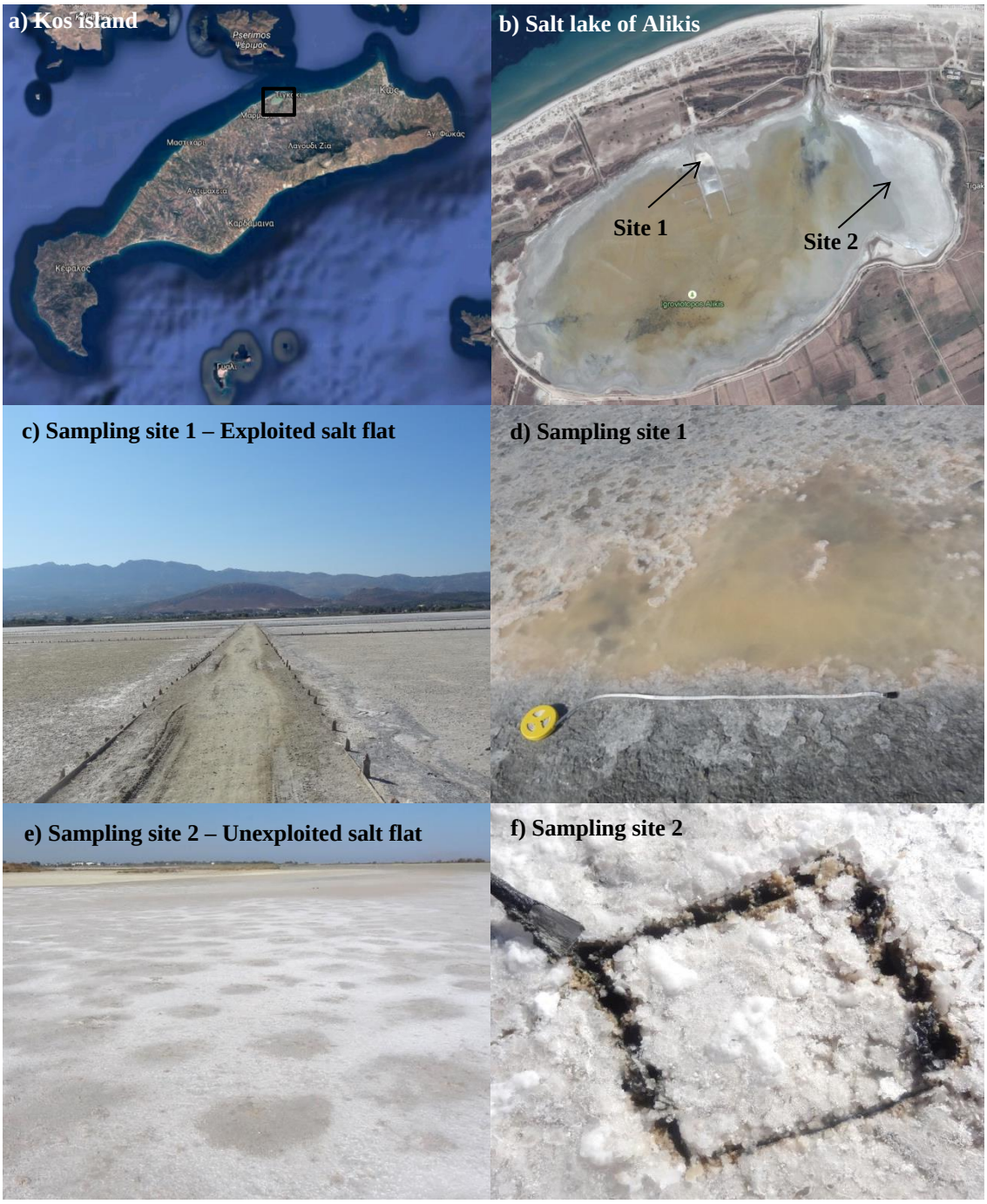


Figure 37: Localization of sampling sites. a) Map showing an overview of the island of Kos, b) Appearance of the salt lake of Alikis, c) Exploited salt flats, d) appearance of the chosen sampling site on the exploited areas (site 1), e) unexploited salt flats, f) appearance of the chosen sampling site on the unexploited areas (site 2).

4.3.4. Influence of chlorine on the production of volatiles compounds

Various fungal species were grown during 15 days on malt extract (1.2 % w/w in deionised water) media amended with NaCl at 0 and 3 % w/w in deionised water. All fungi were incubated in individual 20-mL glass vials hermetically closed by a metallic cap with a PFTE-silicone septum. Volatile compounds released by fungi were analysed using a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer detector (Agilent 5975C). All components were passively trapped with the Stir Bbar Sorptive Extraction (SBSE) technique. One individual bar coated with polydimethylsiloxane polymer (Twister® Gerstel GmbH, 10 mm length, 0.5 mm film thickness) was directly placed under the cap of the sampling vial during 24 hours. Each bar was then dried for 2 min in 2-mL glass vials under technical N_2 flow (20 mL/min) at room temperature before placing them in a thermal desorption unit (TDU) using a robotic sampler (MPS, Gerstel GmbH). For desorption, TDU was set in split less mode, initial temperature was 40°C for 0.5 min and then the temperature was increased at a rate of 80°C/min to up to 250°C (hold time 3 min). A cryo injection system (CIS) at -80°C was used to first focus on the compounds that were released during desorption before releasing them simultaneously (12°C/sec to 280°C, hold time 5 min). The PTV inlet was operated in the solvent vent mode, a vent pressure of 14 PSI, a vent flow of 50 mL/min, and a purge flow of 50 mL/min. The helium carrier gas pressure was 13.3 PSI (flow rate 1.65 mL/min) at constant flow mode and pushed the sample onto an Agilent HP-5MS column (30 m length x 0.25 mm i.d., and 0.25 µm film thickness). The GC oven program started at 40°C for 5 min, then ramped to 200°C at a rate of 6°C/min. A 2 min post run at 260°C was carried out. The detector transfer line temperature was set at 280°C and the ion source and quadrupole temperatures were set at 230°C and 150°C respectively. Electron impact (EI) mode and ionization potential of 70 eV were used with a scanning over the mass range of 10-200. Two substrate controls and one blank analysis were run in order to distinguish the volatiles only released by the studied organisms. Preliminary identification of the compounds was based on comparisons to the NIST11 mass spectral library as well as PBM Library Search (Agilent Technologies, Inc.). No internal standard was added to the initial samples as we did attempt to quantify the VOCs released.

4.4. Results and discussion

4.4.1. Chlorine uptake by *B. bassiana*

To evaluate the ability of *B. bassiana* to uptake chlorine, this fungus was inoculated on solid malt extract medium amended with 10 mM $FeCl_2$. Areas with different elemental composition were observed by back-scattered electrons observations of hyphae of *B. bassiana*. EDS spectra recorded on these aggregates (localized inside the fungal mycelium) revealed the presence of carbon (probably originated from the coating), nitrogen, oxygen, sodium, phosphorus, sulphur, chlorine and potassium (Figure 38). Since some of these elements are constitutive of fungal biomass, to better investigate the composition of these aggregates, elemental mapping was performed. This analysis revealed that aggregates found on fungal hyphae were mainly composed of potassium and chlorine, while hyphae were composed mainly of phosphorous, oxygen, sodium, nitrogen, and traces of manganese (Figure 39). These results suggest that *B. bassiana* incorporated chlorine inside its mycelium in order to decrease the chlorine content in the medium. Indeed as high chlorine concentration could produce an excessive osmotic pressure for the fungus, immobilization in the mycelium could be a detoxification mechanism. This ability is particularly interesting for our purpose to actively remove chlorine from archaeological iron artefacts.

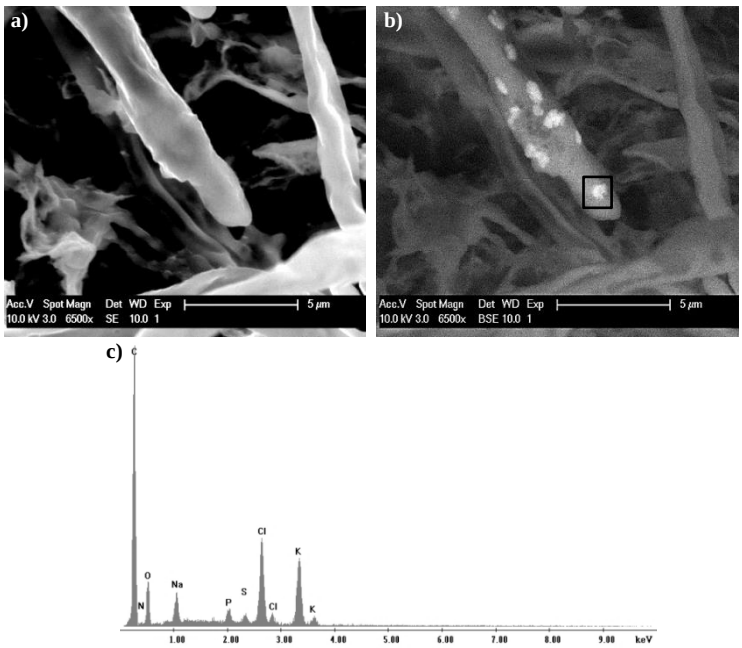


Figure 38: Study of chlorine uptake ability of *Beauveria bassiana*. a) Secondary electrons image of a fungal hypha, b) corresponding back-scattered electrons image showing the presence of aggregates with a different elemental composition compared to the fungal biomass, and a black box indicating the area submitted to EDS analysis. c) EDS spectrum revealing the elemental composition of the aggregates.

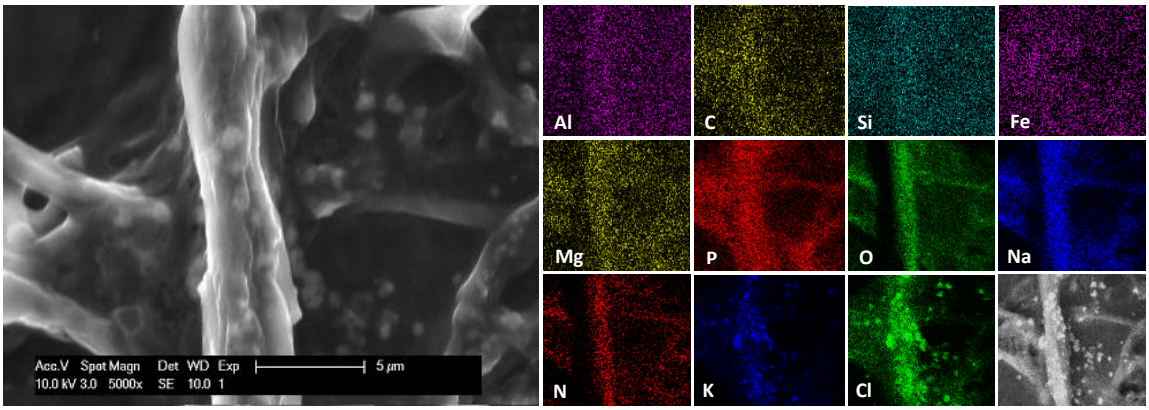


Figure 39: Investigation of the composition of the aggregates produced by *Beauveria bassiana* in presence of 10 mM $FeCl_2 \cdot 4H_2O$. On the left, secondary electrons image on a hypha presenting aggregates, and on the right back-scattered electrons image of the area submitted to elemental mapping with elemental distribution of aluminum, carbon, silicon, iron, manganese, phosphorous, oxygen, sodium, nitrogen, potassium, and chlorine.

To quantify the ability of *B. bassiana* to uptake chlorine, liquid cultures amended with NaCl at 0, 0.3, and 3.5 % w/w in deionised water were performed. Dry weight measurements revealed that this fungus produced more biomass in presence of 3.5 % of NaCl. In fact, dry weight values increased from nearly 60 and 52 g/L, in the cultures with 0 and 0.3 % of NaCl respectively, to 112 g/L in the ones amended with 3.5 % of NaCl (Figure 40). These values suggested that *B. bassiana* grows better in an environment having an elevated osmotic pressure. This could be explained by the fact that as an entomopathogenic fungus, *B. bassiana* should have an efficient mechanism allowing growth under the high osmotic pressure of insect haemolymph. It could be also hypothesised, that a higher amount of biomass allowed this fungus to accumulate more potassium and chlorine inside its mycelium, decreasing the external osmotic pressure. However, ionic chromatography measurements did not reveal a substantial decrease in the chlorine content of the culture medium (Table 10). These results suggested that chlorine and potassium uptake is probably not the only resistance mechanisms employed by *B. bassiana* to overcome osmotic stress. Therefore, even if chlorine uptake is probably used by *B. bassiana* to deal with problematic osmotic pressure, since we do not understand the factors regulating this ability, at this stage it would be difficult to exploit this ability for the development of a biotechnological method to remove chlorine from corroded iron objects.

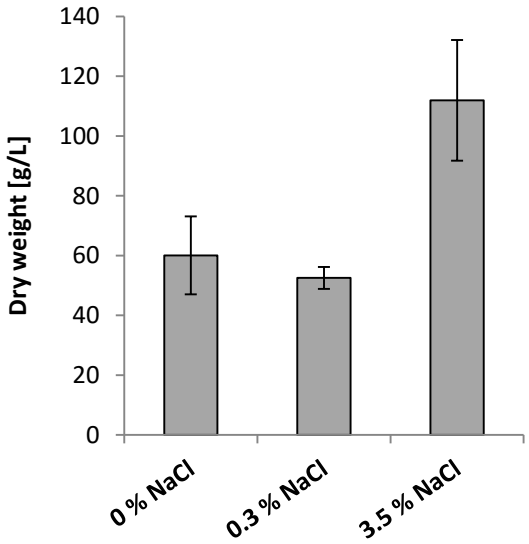


Figure 40: Dry weight quantification of *Beauveria bassiana* exposed to NaCl concentration of 0, 0.3, and 3.5 % w/w in deionised water after 28 of incubation in liquid malt extract (1.2 % w/w in deionised water).

Table 10: Quantification of chlorine by ionic chromatography, after 28-days incubation of the fungus *B. bassiana* in liquid media amended with NaCl at 0 and 3.5 % w/w in deionised water.

	Chlorides ions concentration [mg/L]	
	Day 0	**Day 10**
Abiotic control	16.1 ± 0.3	16.3 ± 0.2
B. bassiana	16.3 ± 1.1	15.8 ± 0.5
Abiotic control NaCl	11316 ± 396.3	11224 ± 473.1
***B. bassiana* NaCl**	11270 ± 121.3	11206 ± 236.4

4.4.2. Study of the resistance mechanisms against chlorine

Chlorine tolerance in selected alkalitolerant and halotolerant fungi

Investigation on the amount of NaCl tolerance revealed that all the strains tested tolerated up to 2 % of NaCl. The growth of *Alternaria* sp., *Acremonium* sp., *B. bassiana*, the two strains of *Fusarium*, and *C. sinensis* was not affected by the chlorine concentrations tested (Figure 41). For some of the strains, a higher growth rate was observed when chlorine is present, especially at 1, 1.5, and 2 % of NaCl. For *A. lignocola* and *C. comatus* a decrease on the growth rate was observe with increasing NaCl concentrations. Regarding *A. lignocola* the maximum NaCl tolerated was 2 %, while in the culture amended with 3 % not growth was observed. *C. comatus* was able to grow at 3 % but the growth rate was 9 times lower than the value measured in the culture without chlorine.

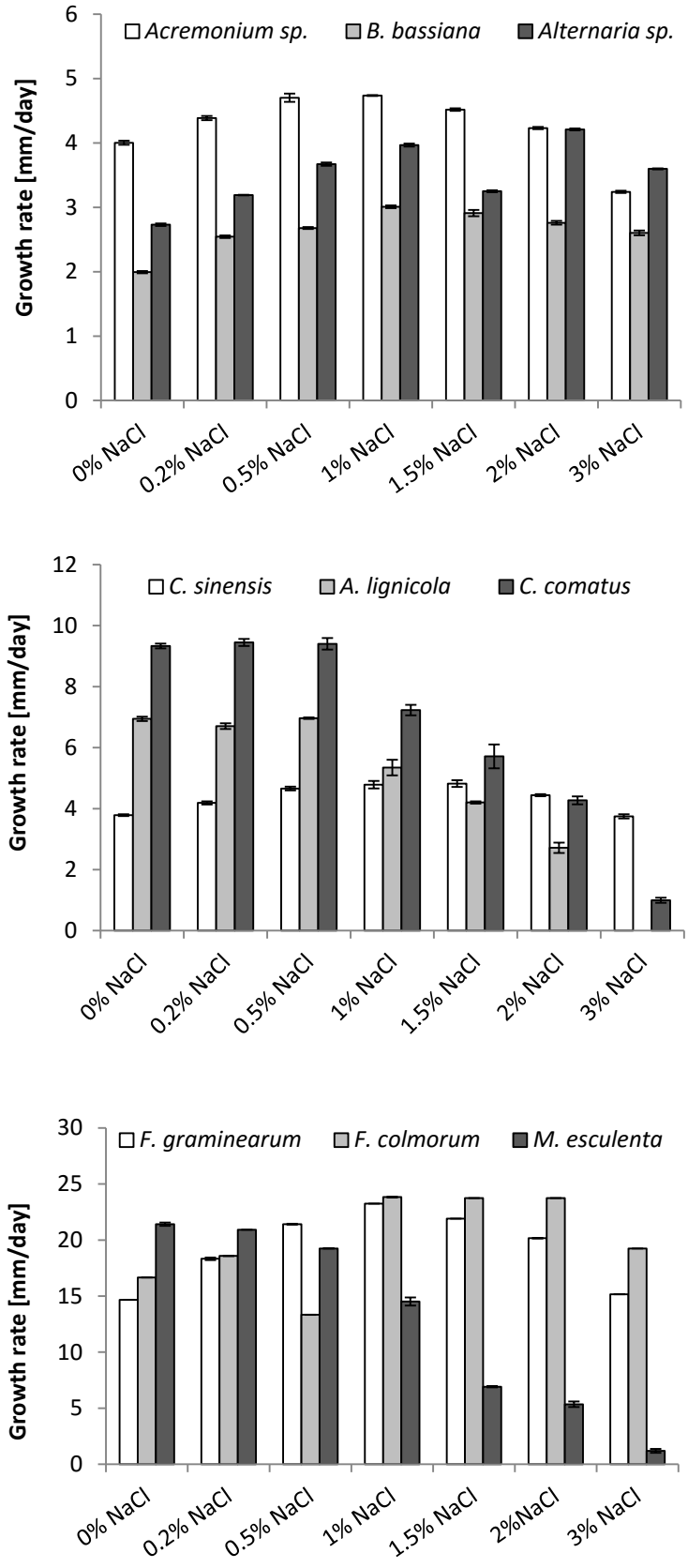


Figure 41: Growth rate of the selected halotolerant and alkalitolerant fungi a) *Acremonium* sp., *Beauveria bassiana*, and *Alternaria* sp., c) *Cordiceps sinensis*, *Agaricus lignicola*, and *Coprinus comatus*, and c) *Fusarium graminearum*, *F. colmorum*, and *Morchella esculenta*.

Influence of NaCl on the accumulation of glycerol and K^+

To study the influence of NaCl on the fungal accumulation of glycerol and K^+, spectrophotometric analysis were carried. Considering that most of the strains grew up to 3 % NaCl, to investigate resistance mechanisms against chlorine, the influence of higher concentrations of NaCl (10, and 15 %) was measured using *F. culmorum*, *Alternaria* sp. and *B. bassiana*. *F. culmorum* and *Alternaria* sp. changed their pigmentation pattern depending on the concentration of NaCl present in the medium (Figure 42). *F. culmorum* had a yellow colour at 0 %, orange-brown at 3 %, and white at 10 % of NaCl. Regarding *Alternaria* sp., a depigmentation of the cultures was observed starting from a NaCl concentration of 10 %. Colonies of this species were black in the culture amended with 0 and 3 % of NaCl, while at 10 % of NaCl the colony had a white pigmentation. In addition, a serious growth defect occurred for this species at 10 % of NaCl, which indeed could affect the melanisation process. On the other hand, observation of *B. bassiana* cultures demonstrated that this fungus was able to growth only until a concentration of 3 % of NaCl, and no change in the pigmentation was noticed. Depigmentation with exposition to increasing amounts of chlorine is a surprising result, especially for species naturally melanised such as *Alternaria* sp., as melanisation is a

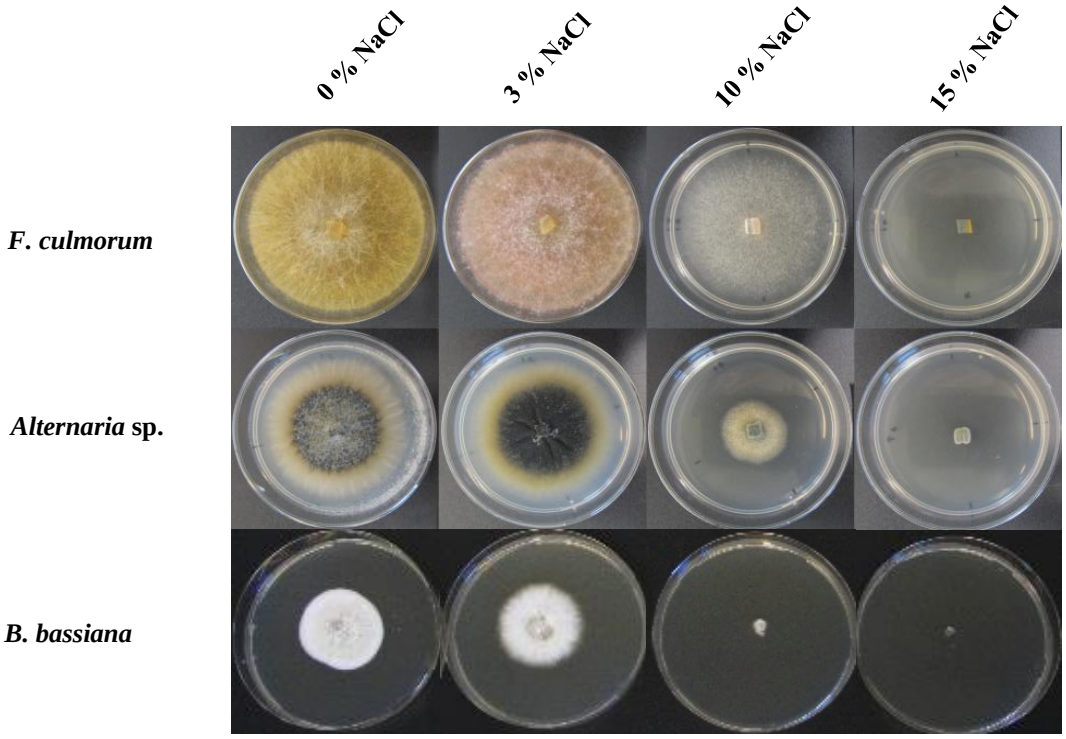


Figure 42: Macroscopic appearance of the fungal colonies of *Fusarium culmorum*, *Alternaria* sp., and *B. bassiana*, inoculated on malt extract (1.2 %) agar (1.5 %) amended with the following NaCl concentrations: 0, 3, 10, and 15 %.

resistance mechanism employed by several fungi to deal with high chlorine concentrations (Kogej, Stein et al. 2007).

Growth rate measurements allowed observing that for the three fungi tested concentrations above 3% have a drastic effect on growth. For instance, *F. culmorum* had similar growth rates at 0 and 3 % NaCl (16.9 and 16.4 mm/day, respectively), while a drastic decrease on this value was observed on the culture amended with 10 % of NaCl (5.8 mm/day), and no growth was measured in the culture containing 15 % of NaCl (**Figure 43** a). A similar behaviour was observed for *Alternaria* sp: no difference was observed between 0 and 3 % of NaCl (5.0 and 5.1 mm/day, respectively; **Figure 43** b), while a significant decrease at 10 and 15 % was noticed. Regarding *B. bassiana*, optimal growth was observed in media amended with 0 and 3 % NaCl (2.85 and 3.3 mm/day, respectively), whereas almost no growth was measured in the medium containing 10 % NaCl (0.27 mm/day) (Figure 43 c).

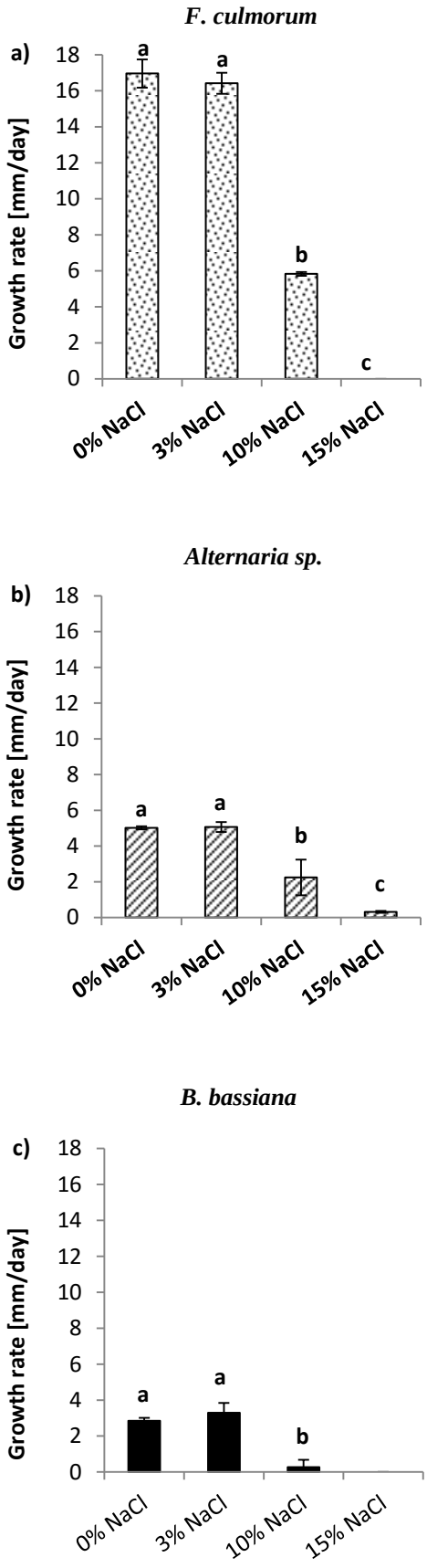


Figure 43: Growth rates of *Fusarium culmorum*, *Alternaria* sp., and *Beauveria bassiana*, on media amended with the following NaCl concentration: 0, 3, 10, and 15 %. Difference between the growth rates of fungi inoculated in media containing divers amount of NaCl were compared with an ANOVA and Tukey as post hoc-test. Statistically significant differences in fungal growth rates are indicated by different letters ($P \leq 0.05$).

Regarding the quantification of glycerol and K^+ ions on liquid cultures amended with several NaCl concentrations, it has to be indicated that all three fungal species did not grown in the liquid cultures amended with 15 % of NaCl. Glycerol analysis on the fungal biomass demonstrated that this polyol plays an important role in the resistance of *F. culmorum* and *Alternaria* sp. against chlorine, with a significant increase on the amount of glycerol detected when exposed to chlorine (Figure 44 a). This phenomenon was more pronounced for *F. culmorum*, which produced 2.6 µmol/g of fungal dry biomass of glycerol on the culture without NaCl, while glycerol concentration rose to 48.3 µmol/g and 700.8 µmol/g in the cultures amended with 3 and 10 % of NaCl, respectively. Glycerol content increased also in the culture of *Alternaria* sp. exposed to increasing concentration of NaCl. Values increased from 7.6 µmol/g in the culture without NaCl, to 182.1 µmol/g in the one amended with 3 % of NaCl. No significant difference in glycerol content was observed between the culture exposed to 3 % and 10 % of NaCl. Regarding *B. bassiana*, no significant difference between the culture inoculated on media with 0 and 3% of NaCl were detected and glycerol content was 3 and 9 µmol/g, respectively, in these cultures (Figure 44 a). This result suggested that *B. bassiana* possesses other resistance mechanisms against osmotic pressure than glycerol accumulation. It could be hypothesised that since other polyols are reported to be produced by this fungus (Hallsworth and Magan 1997), *B. bassiana* could accumulate other compatible osmolytes, such as erythriol, arabitol, mannitol, and trehalose, which were not measured here.

K^+ ions analysis revealed a change in the amount of this ion in the biomass of *F. culmorum* and *B. bassiana* exposed to different NaCl concentrations, while no difference was noticed for *Alternaria* sp.. Indeed, cultures of *F. culmorum* exposed to 10 % of NaCl had lesser amounts of K^+ ions in their biomass (320.0 µmol/g), compared to the culture without NaCl (511.1 µmol/g) and 3% NaCl (474.3 µmol/g). *B. bassiana* had a similar behaviour concerning K^+ accumulation. Indeed chlorine amendment caused a decrease in K^+ concentration in the fungal biomass from 201.5 µmol/g (culture without chlorine) to 62 µmol/g (at 3 % NaCl). In the case of *Alternaria* sp., the concentration of K^+ measured in the biomass did not vary significantly for the NaCl concentrations tested (Figure 44 b).

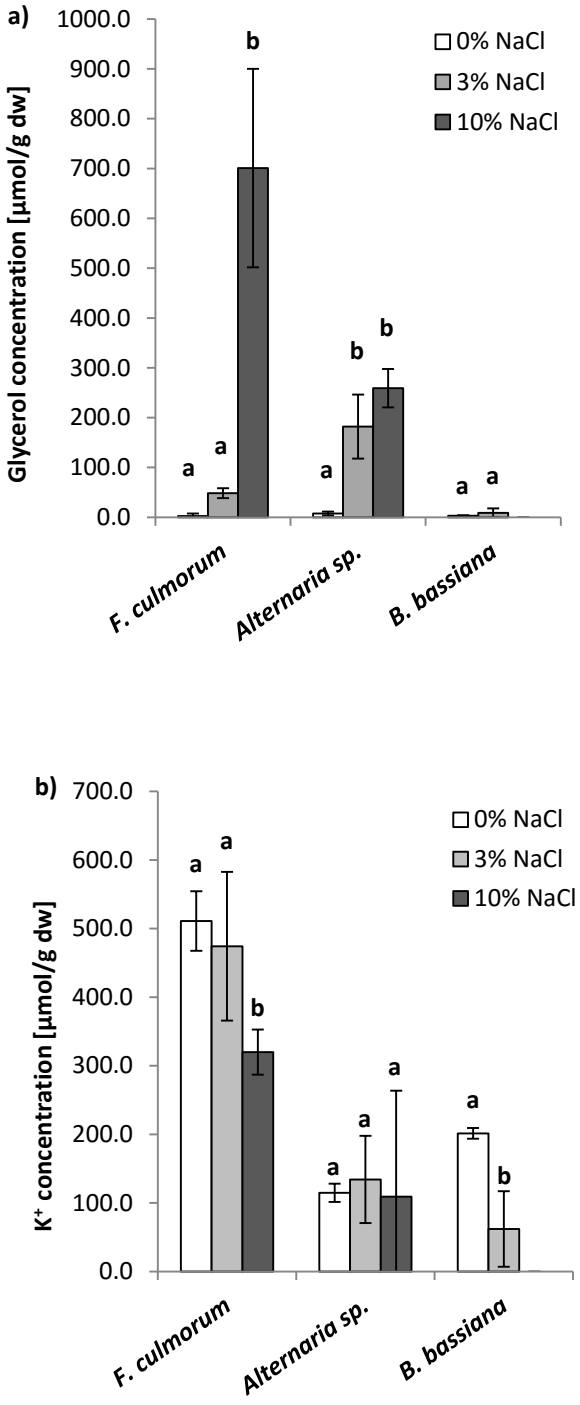


Figure 44: Study of the resistance mechanisms in *Fusarium culmorum*, *Alternaria* sp, and *Beauveria bassiana*. a) Glycerol quantification, and b) K^+ concentration in fungal dry biomass inoculated in malt extract (1.2 %) agar (1.5 %), amended with 0, 3, and 10 % of NaCl. Difference between the amount of measured compounds (glycerol or K^+) produced by fungi inoculated in media containing divers amount of NaCl were compared with an ANOVA and Tukey as post hoc-test. Statistically significant differences in fungal growth rates are indicated by different letters ($P \leq 0.05$).

Influence of $FeCl_3$ on the accumulation of glycerol

Iron uptake ability of fungi has been already evaluated in chapter 3. Nevertheless, during the study of resistance mechanisms of *F. culmorum*, *Alternaria* sp. and *B. bassiana* against chlorine, some interesting results suggesting a connection between iron uptake and glycerol were obtained. In order to ascertain iron uptake, total iron was measured in the fungal biomass after acidic digestion. Results showed that for *F. culmorum* the amount of iron contained in the biomass after 15 days of incubation was 7.25 mM in the culture without iron, and 5291 mM in the one amended with $FeCl_3$ (Figure 45). The same behaviour was observed for *Alternaria* sp. that accumulated 5360 mM of iron in the biomass, which correspond nearly to half the amount of iron contained in the culture medium. Regarding *B. bassiana* an uptake of iron was also noticed but was less pronounced than for the other two strains. In fact, only 555.8 mM of iron were measure in its biomass (Figure 45).

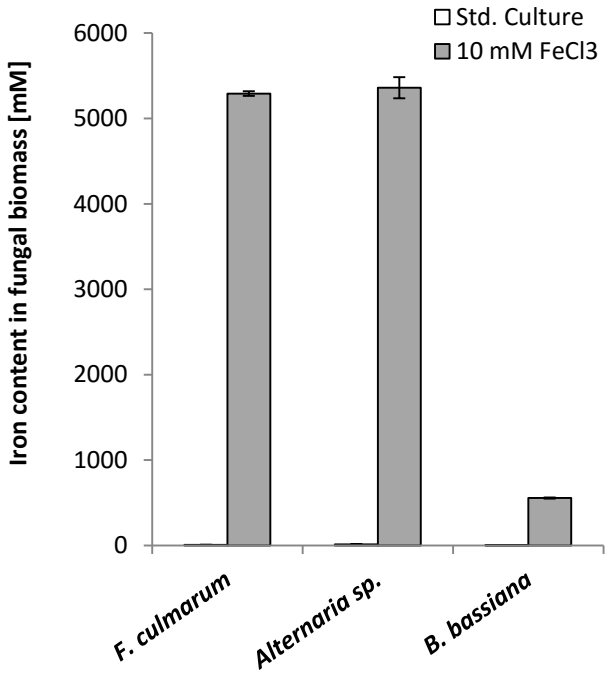


Figure 45: Total iron quantification in the fungal biomass of *Fusarium culmorum*, *Alternaria* sp., and *B. bassiana* after 15 days of incubation in standard malt extract (1.2 % w/w in deionised water) medium without and with 10 mM $FeCl_3$.

Biomass from the same cultures was analysed for glycerol content, and for *F. culmorum* and *Alternaria* sp. an interesting results was observed (Figure 46). In fact, even though an increase in the amount of glycerol was expected considering the results obtained for the cultures without iron, when the medium was amended with 10 mM $FeCl_3$ the results obtained revealed the opposite. For instance, 11.9 μmol/g of dry weight of glycerol were measured in the standard culture of *F. culmorum* incubated without iron, while only 3.8 μg/g were measured in the culture of the same strain amended with 10 mM $FeCl_3$. The same trend was observed in the biomass of *Alternaria* sp. containing 7.5 μg/g of dry weight in absence of iron, while glycerol concentration decreased to 1.2 μg/g in the culture amended with an iron source. On the other hand, no difference on the amount of glycerol was observed in the culture of *B. bassiana*. This is probably due to the fact that iron might inhibit the production of glycerol.

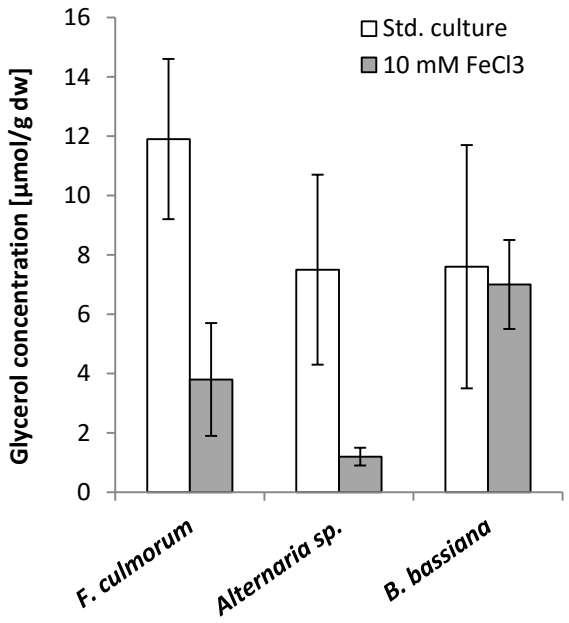


Figure 46: Glycerol quantification in the culture of *Fusarium culmorum*, *Alternaria* sp., and *Beauveria bassiana* after 15 days of incubation in standard malt extract (1.2 % w/w in deionised water) medium without and with 10 mM of $FeCl_3$.

Isolation of halotolerant and halophilic fungi

Isolation of fungi from salt flats was performed to obtain strains tolerating high amounts of chlorine and having a resistance mechanism potentially useful for the development of a fungal method to remove chlorine from corroded iron objects. 27 strains of fungi were originally isolated from salt flat samples, but only 12 successfully grew after the purification procedure. The isolated fungal strains had three different ecologies. Six isolates were identified as phytopathogenic: *Tranzscheliella hypodytes*, three strains of *Stemphilium solani, Stemphilium vecicarium, and Gibellulopsis nigrescens*; one as animal as well as plants pathogen: *Purpureocillium lilacinum;* one was recognised as entomopathogenic *Ophiocordyceps heteropoda*; and the remaining four strains were characterised as saprophytic: *Cladosporium ramotenellum, Alternaria abundans,* and 2 strains of *Penicillium citrinum.* Phytopathogens have naturally resistance mechanisms against osmotic pressure since they are able to colonise plant tissues. For instance, species belonging to *Stemphylium* spp. are reported to have the capacity to tolerate several stress factors (Buchalo, Wasser et al. 2009), and strains of *G. nigrescens* have already been isolated from hypersaline environments (Hujslová, Kubátová et al. 2010). In addition, strains of *P. lilacinum* are reported to also be able to grow under several stress conditions and to be present also in soda soils (Grum-Grzhimaylo, Georgieva et al. 2016).

Table 11: List of the fungal strain isolated from the salt flats in Kos island (Greece), as well as their ecology.

Code strain	Identification	Ecology
S-1	*Tranzscheliella hypodytes*	Phythopatogen
S-2	*Cladosporium ramotenellum*	Saprophyte
S-4	*Stemphylium solani*	Phytopathogen
S-5	*Stemphylium solani*	Phytopathogen
S-7	*Stemphylium vecicarium*	Phytopathogen
n°A	*Alternaria abundans*	Saprophyte
n°E	*Penicillium citrinum*	Saprophyte
n°I	*Penicillium citrinum*	Saprophyte
n°J	*Ophiocordyceps heteropoda*	Entomopathogen
n°K	*Stemphylium solani*	Phytopathogen
n°L	*Purpureocillium lilacinum*	Pathogen
n°N	*Gibellulopsis nigrescens*	Phtopathogen

The entomopathogen fungus *Tranzscheliella hypodytes* is not reported to colonised hypersaline environments, however, in order to colonise insect haemolymph it has to tolerate important osmotic pressure and thus it could be hypothesised that this ability make this fungus able to grow in hypersaline environments. The ubiquitous fungus *P. citrinus* could be an air contaminant. Nevertheless, several studies report the isolation of this species from hypersaline environments (Cantrell, Casillas-Martínez et al. 2006, Butinar, Frisvad et al. 2011, Ismail, Moubasher et al. 2016). Melanised saprophytic fungi such as those related to *Alternaria* spp. are frequently isolated from hypersaline environments as suggested by Grishkan, Nevo et al. (2003). Finally, species related to *Cladosporium* spp, and in particular *C. ramotenellum* are one of the most representative fungal species of hypersaline environments (Zalar, De Hoog et al. 2007). From a biotechnological point of view, it is clear that pathogenic fungi such as *P. lilacinum* cannot be considered for further investigation.

Influence of chlorine on the production of volatiles compounds

All the samples were analysed for volatiles organic compounds production in media containing 0 and 3 % of NaCl. However, due to technical problems occurred during this collaboration, it was not possible to analyse all the results obtained. Nevertheless, interesting preliminary results on the effect of NaCl content on the production of the fungal volatiles was observed. In fact, it appeared that the presence of NaCl induced the production of different type of volatile compounds in fungi. For instance, the fungus *Phellinus tuberculosus*, used as positive control for production of halogenated volatile organic compounds, had a different profile when exposed to 3 % NaCl (Figure 47). In fact, the production of four different substances, not present in the culture without NaCl amendments was detected: 1-butanol, 3-methyl-, 1-butanol, 2-methyl, benzoic acid, ethylester, and Benzenepropanol (Figure 49 and Figure 48). A similar behavior was noticed for *G. nigrescens*, a strain isolated from hypersaline environments. Indeed, this strain produced 1R-Ethoxy-3-trans-methoxy-2-cis-methylcyclohexane and 2H-Pyran-2-methanol, 6-ethoxy-3,6-dihydro-3-hydroxy- only when exposed to 3 % of NaCl (Figure 50).

P. tuberculosus

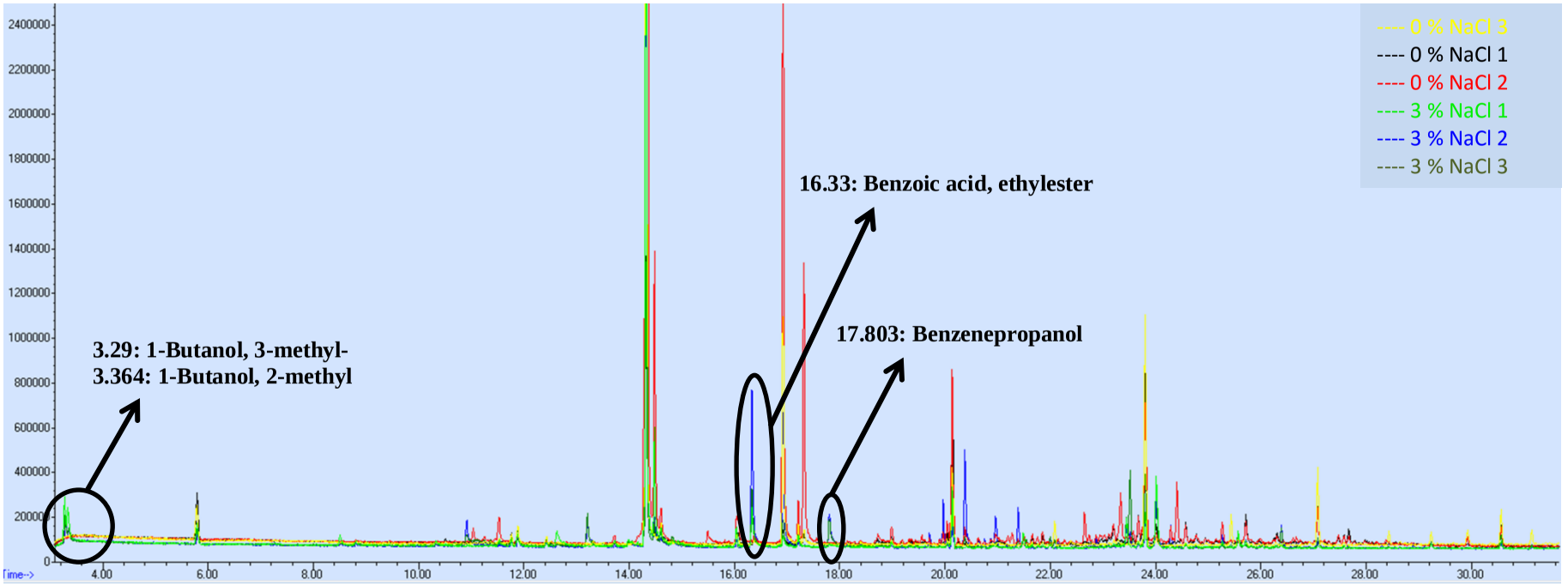


Figure 47: Volatiles organic compounds profile of *P. tuberculosus*, after 15 days of incubation on media amended with 0 and 3 % of NaCl.

P. tuberculosus – Detail 1

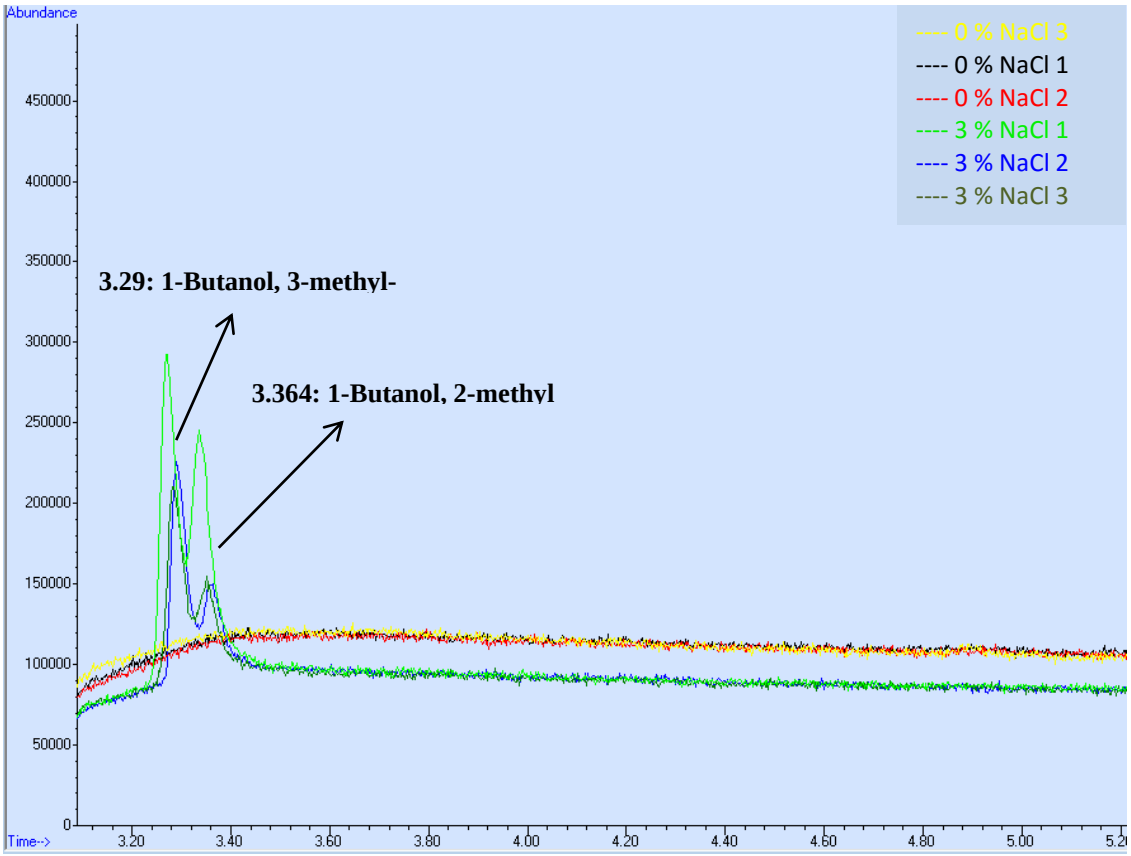


Figure 48: Detail of a part of the volatile organic compounds of *P. tuberculosus* showing the picks of 1-Butanol, 3-methyl and 1- Butanol, 2-methyl, produced only in the culture exposed to 3 % of NaCl.

P. tuberculosus – Detail 2

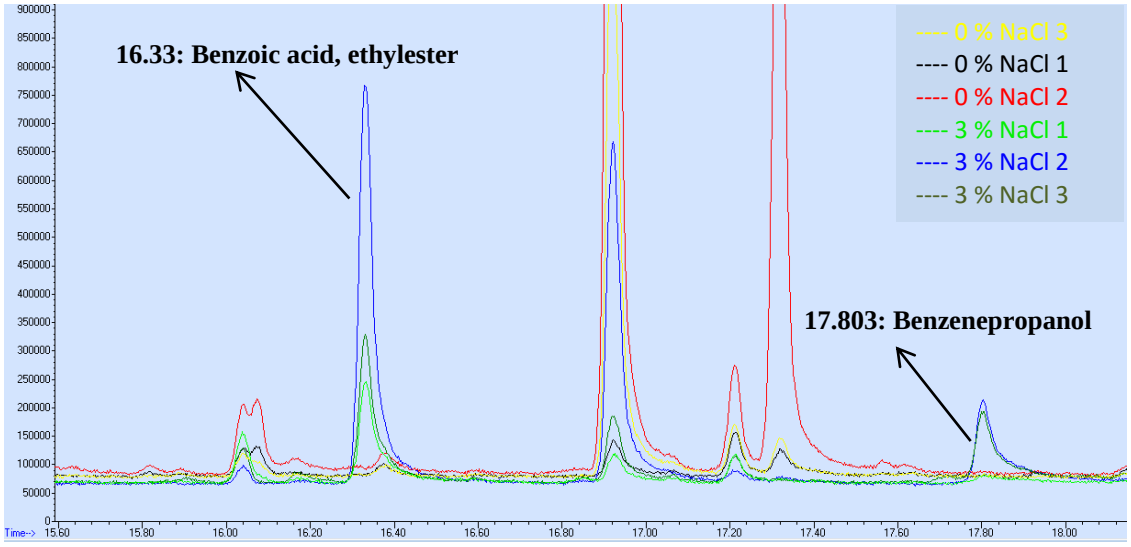


Figure 49: Detail of a part of the volatile organic compounds of *P. tuberculosus* showing the picks of benzoic acid, ethylester and Benzenepropanol, produced only in the culture exposed to 3 % of NaCl.

G. nigrescens

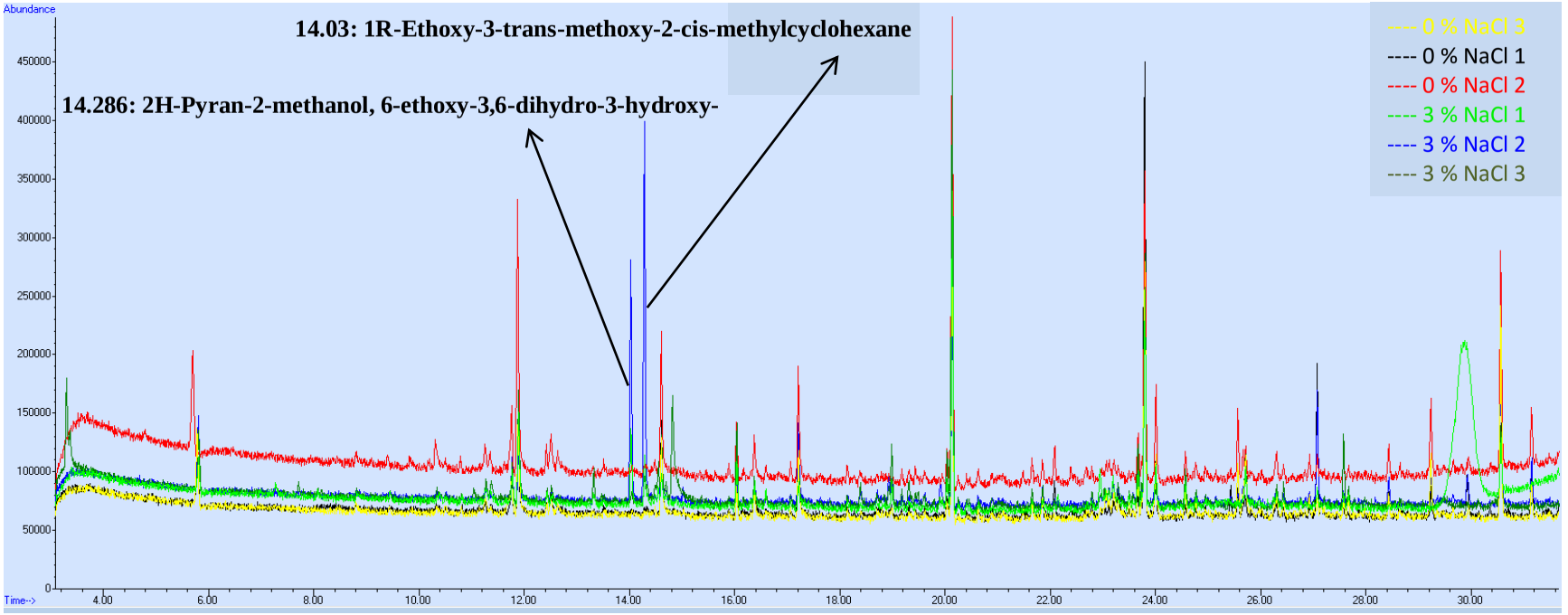


Figure 50: Volatiles organic compounds profile of *G. nigrescens*, after 15 days of incubation on media amended with 0 and 3 % of NaCl.

4.5. Conclusion

This study represents a preliminary research on the metabolic activities of fungi regarding chlorine. Even though the results obtained did not allowed to assess if fungi would be good candidates to develop a biotechnological method for the removal of chlorine from corroded iron, some interesting information was obtained in this chapter about fungal physiology regarding chlorine. In fact, SEM-EDS investigation allowed to observe precipitates composed of potassium and chlorine inside the biomass of *B. bassiana* when exposed to 10 mM $FeCl_2$. However, this phenomenon cannot be considered a resistance mechanism against chlorine since no substantial chlorine uptake was detected by IC. In addition, spectroscopic quantification of glycerol revealed that *F. culmorum* and *Alternaria* sp. accumulate glycerol when exposed to 3 and 5 % of NaCl, while *B. bassiana* employed another resistance mechanism to deal with the same salt concentrations. Glycerol was also measured in fungal biomass exposed to an iron source. Glycerol amount decrease in cultures amended with iron. Further investigation is required, but a link between these two compounds can be hypothesised. Finally, the presence of NaCl in the culture medium stimulated the production of different organic volatile compounds in fungi able to tolerate cholorine (*P. tuberculosus*), as well as in strains isolated from hypersaline environments such as G. *nigrescens*.

4.6. Perspectives

All the data set obtained during the GS-MS measurements has to be carefully analysed in order to better understand the influence of chlorides ions in the production of volatile compounds. In addition the nature and the role of the produced compounds should be assessed. Finally, to develop a biotechnological approach to remove chlorine through fungal volatilisation the following steps are required:

Step 1: Screening of all the volatile compounds and evaluating the production of halogenated organic compounds using NaCl as a source of chlorine

Step 2: Inoculate the interesting fungi (able to produce halogenated organic compounds) in medium amended with different sources of soluble as well as solid phase chlorinated iron compounds

Step 3: Test the ability to volatilize chlorine of the suitable fungi from corroded iron objects contaminated by chlorides ions

Step 4: Prepare artificially corroded iron samples and treat them with fungi and other methods usually employed for the removal of chlorine, and test the behaviour of the object in presence of high humidity after the treatments.

4.7. Supplementary information

	Site 1: Exploited salt flats	**Site 2:** Unexploited salts flats
GPS coordinates	N: 36°53.262'; E: 027° 10.243'	N: 36° 53.091; E: 27° 10.674
Temperature [°C]	29.6	29.2
pH	7	7-8

Supplementary table 1: GPS coordinates, temperature, and pH measures recorded on the sampling site 1 and 2.

4.8. References

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CHAPTER 5

General conclusions

5.1. Chapter 2

The study of the interaction between iron and the anaerobic bacterium *Desulfitobacterium hafniense* allowed to validate the concept of the use of bacteria to passivate corroded iron objects. In fact, for the first time, biogenic mineral production was successfully used to convert a part of the corrosion layer into more stable compounds. As a summary, the Figure 51 present the general idea, as well as the microbial activity involved during the proposed biotechnological treatment of corroded iron. During this treatment bacteria converted the main part of an unstable corrosion layer into more stable biogenic minerals through reductive iron dissolution. This bacterial activity produced a homogeneous and continuous layer of stable biogenic minerals covering almost all of the remaining original corrosion layer (Figure 51).

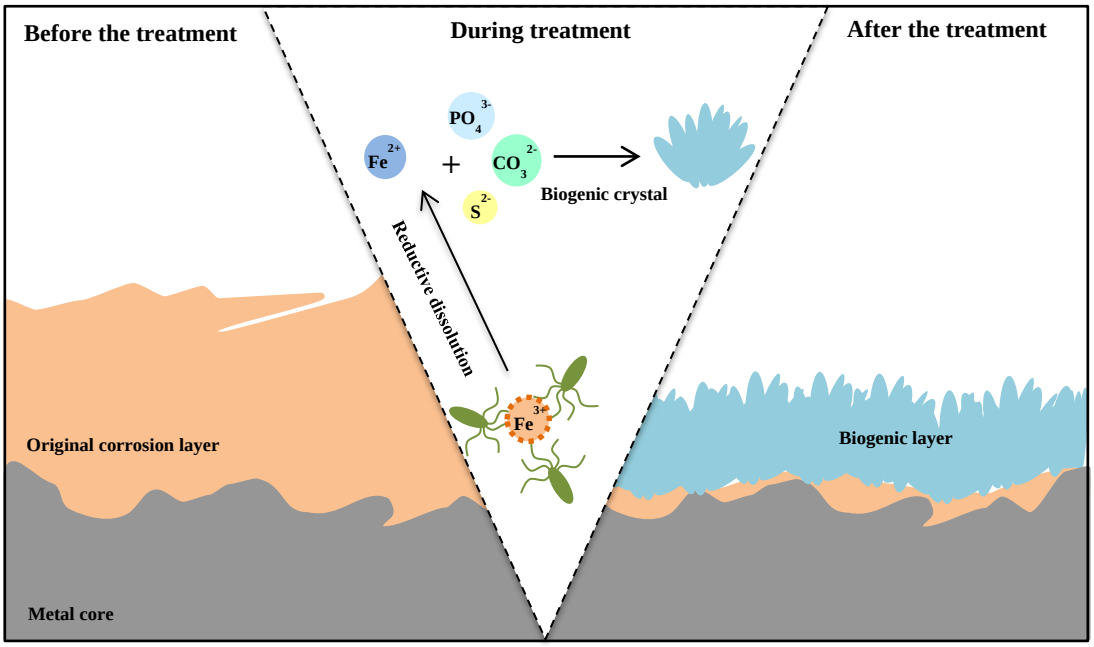

Figure 51: Schematic representation of the cross-section of corroded iron before and after the bacterial treatment studied, as well as the microbial induced modifications ongoing during the treatment.

Nevertheless, after this study two important points need to be discussed in order to develop an efficient treatment for corroded iron objects. The first is the evaluation of the efficiency of chlorine extraction that will influence the long-term stability of treated objects. In fact in this study bacterial reductive dissolution of the iron corrosion layer and the consequent conversion of unstable corrosion compounds into stable biogenic minerals were studied to indirectly remove chlorine from the corrosion layer. Even though the production of stable biogenic

minerals was successfully achieved, it was not possible to quantify the amount of chloride extracted. In fact, an efficient method to quantify chlorides released or still present in the objects at the end of the treatment needs to be tailored to our system. Two main approaches are proposed in literature, the first is to simply quantify chlorides released in the medium during the treatment through spectrophotometry or with a chloride-specific electrode (Rimmer, Watkinson et al. 2012). However, this technique was not possible in our case since objects contained a few amount of chloride, and this element was also added as trace element in the culture medium. The second, relies on complete acidic digestion of the object after treatment with 5 M nitric acid and quantification of chloride in the obtained solution with a chloride-specific electrode (Rimmer, Watkinson et al. 2012). This could be a valuable method that should be tested.

For the future the development of an efficient method for the quantification of chlorides extraction would be really important to validate this biotechnological treatment. Using a more simple growth medium devoid of chlorinated compounds will probably enable to determine chlorides released in the medium during the bacterial treatment compared to abiotic control. Some tests were performed (Kooli W. data not shown) with acidic digestion and chlorides quantification with HPLC, nevertheless the amount of acid used for the digestion impaired chlorides quantification. Then, the use of the chloride-specific electrode would be an option.

The second important point is the different type of iron objects and the consequent specific requirements. In fact after this study it can be concluded that each type of corroded iron object need a different approach. For instance, iron phosphates production is suitable for the stabilization of corroded iron surfaces that will be exposed to outdoor environments and that require protective systems against moisture and oxygen exposure. On the other hand, such protection is not necessary in case of archaeological iron objects exposed to controlled atmospheres in museums. Moreover, in our study vivianite caused blue staining of a part of the archaeological objects. Then, for this type of objects, iron phosphates production has to be avoided in favour of black minerals, such as magnetite, in order to respect the public perception of that iron colour should be.

Results obtained in this chapter demonstrated that biogenic mineral production is a useful metabolic activity for the development of a biotechnological approach for the stabilisation of corroded iron. An important point is the stratigraphic analysis of the corrosion layer after the bacterial treatment. This approach, allowed to discover the presence of a potentially detrimental layer composed of sulphur, localized between the original corrosion layer and biogenic crystals. Even if the effect of this element on the corrosion layer is still under evaluation, previous studies affirm that sulphur compound could lead to an increase of corrosion (Little, Ray et al. 2000). Therefore, in the future Na_2S has to be replaced with other reductive molecules without sulphur.

In order to assess the long-term behaviour of the biologically treated samples the following analyses are still necessary:

1. Ageing procedures
2. Adherence/leaching tests
3. Comparison with currently used treatments
4. Impedance measurements, to verify the protectiveness of the biogenic layer

On the other hand, a critical aspect of this technique is the complexity of the culture medium used during the treatment and the interaction of its components with the iron matrices, leading to the formation of undesired compounds, such as elemental sulphur. In order to solve this issue other experiments should be carried out with different medium composition, and with other anaerobic bacteria.

5.2. Chapter 3

The study of the interaction between alkalitolerant and halotolerant fungi with iron allowed to determine that fungi are not the best candidates for the development of a passivation treatment for corroded iron through the formation of stable biogenic minerals. However, promising results were obtained in the biocleaning test, and important information regarding fungal physiology and their role in iron dynamics was acquired.

As a conclusion, Figure 52 summarizes the fungal metabolic activities that are important for iron dynamics in soils. This thesis has contributed to this scheme by showing that even though iron reduction was not found to be a widespread fungal ability; it was demonstrated for the fungus *Agaricus lignicola* that probably uses this ability for wood degradation. This study allowed also to acquire information about iron biogenic particles production. In fact, the precipitation of biogenic minerals seemed to be inhibited by alkaline pH and manganese addition in the culture medium, and the production of EPS chelating iron was observed in presence of soluble as well as solid iron phases. In addition, iron uptake was shown for *Alternaria* sp. that accumulates substantial amounts of this metal in its biomass.

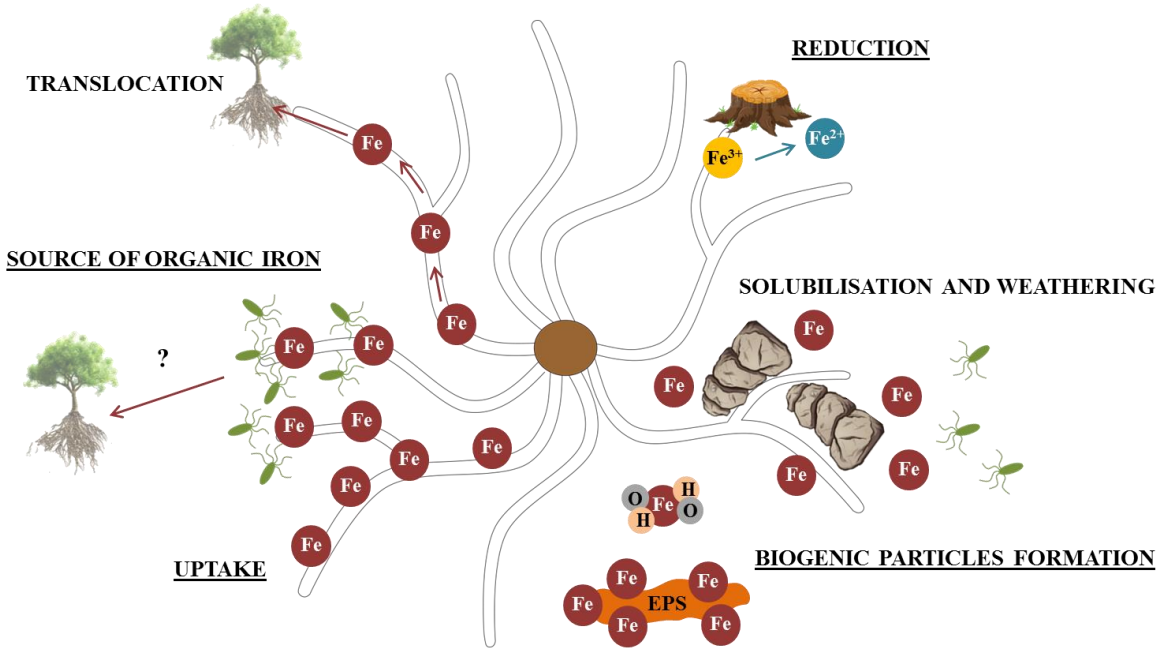

Figure 52: Schematic representation of the fungal abilities reported in literature as well as discovered during this research.

Furthermore, in this study we demonstrated that fungal dead biomass chelating iron could be exploited by bacteria as a source of bioavailable iron. This phenomenon revealed an unexplored microbial interaction that could be exploited for the development of a biotechnological approach to improve plant growth (Figure 52).

After this study it can be concluded that fungi have an important role in the dynamics of iron in soil. Indeed, even though fungi are reported to influence metals cycling through solubilisation and biogenic minerals production (Gadd 2007, Gadd 2010), the most important metabolic activities in the case of iron seems to be solubilisation, uptake and production of extracellular polymeric substances (EPS) able to chelate iron.

Fungal metabolic activities regarding iron are a particularly exciting research field but are still underexplored. Fragmentary information is available with focus on specific topics such as fungal pathogens, yeast, mycorrhizal fungi, and wood-degrading fungi. However, an overall background regarding the interactions between fungi and iron does not yet exist. In addition to this, most of the interest is dedicated today to the study of the interactions between fungi and toxic metals and, although in some cases also iron is included, it can only be supposed that information available for toxic divalent and trivalent metals could be applied for iron. Because iron is a key element in soil dynamics, and generally in the environment, and as fungi are significant weathering agents, it would be productive to put more effort into the study of the metabolic abilities of fungi regarding iron. As a consequence, a better understanding of this domain would allow exploitation of the metabolic abilities of fungi for the development of biotechnological applications in fields where iron needs to be preserved or removed.

Results obtained in this chapter demonstrated that the most useful fungal metabolic activity regarding iron from a biotechnological point of view is iron uptake. Two different biotechnological approaches could be proposed exploiting this fungal capacity.

5.2.1. Fungal biocleaning of corroded metal surfaces

It is widely recognized that in fungi toxic metals uptake is a phenomenon mainly caused by passive adsorption on the fungal wall components (melanin and chitin). For this reason in biotechnology fungal dead biomass is frequently used for the removal of toxic metals for example from contaminated water (Murugesan, Sathishkumar et al. 2006). The use of dead biomass could be really convenient also for our treatment, reducing the influence of iron

toxicity on fungal growth, as well as increasing the rapidity of the treatment. As a consequence, this technique will allow the application of fungal biocleaning also on other metal surfaces, more toxic for fungi, such as copper, tin, and silver. In addition through acidic washing the biomass could be reused for several cleaning procedures, reducing costs (Ahluwalia and Goyal 2007). To develop a biocleaning performed with fungal dead biomass the following steps will be required.

1. Verify the adsorption abilities of fungal dead biomass of *Alternaria* sp. for iron and other metals
2. Produce fungal dead biomass (culture, washing, freeze-drying)
3. Study the fungal dead biomass application procedure (delivery system)

5.2.2. Fungal Fe-fertilizer

On the other hand, as previously described, fungal iron uptake could also be used for the development of new Fe-fertilisers to improve plants growth on iron-deficient soils. For this purpose the following experiments will be required:

1. Analyse the production of other siderophores
2. Study the abilities of other bacterial strains to use fungal dead biomass chelating iron as a source of bioavailable iron
3. Study the influence of fungal dead biomass chelating iron on plants

5.3. Chapter 4

The preliminary results obtained in this chapter did not allow to assess if fungi would be valuable candidates for the development of a stabilization method for corroded iron based on a direct chloride extraction. To answer to this question further research is necessary. In fact the ability to volatilise chlorine could be an interesting metabolic activity exploitable for this purpose. However, it has to be mentioned that most of the halogenated organic compounds are toxic or considered to be harmful greenhouse gases (Anke and Weber 2006). Therefore, in order to develop a biotechnological treatment aiming to directly remove chlorides through fungal volatilisation without producing harmful substances, biodegradation of these compounds by fungi could also be considered. In fact, fungi are not only able to produce chlorinated organic compounds, but they are also reported to be able to degrade these substances (Moore, Gut et al. 2005). Then the idea is to set up an experimental procedure allowing a sequential volatilisation of chlorine from the object, followed by fungal degradation of the volatile halogenated compounds.

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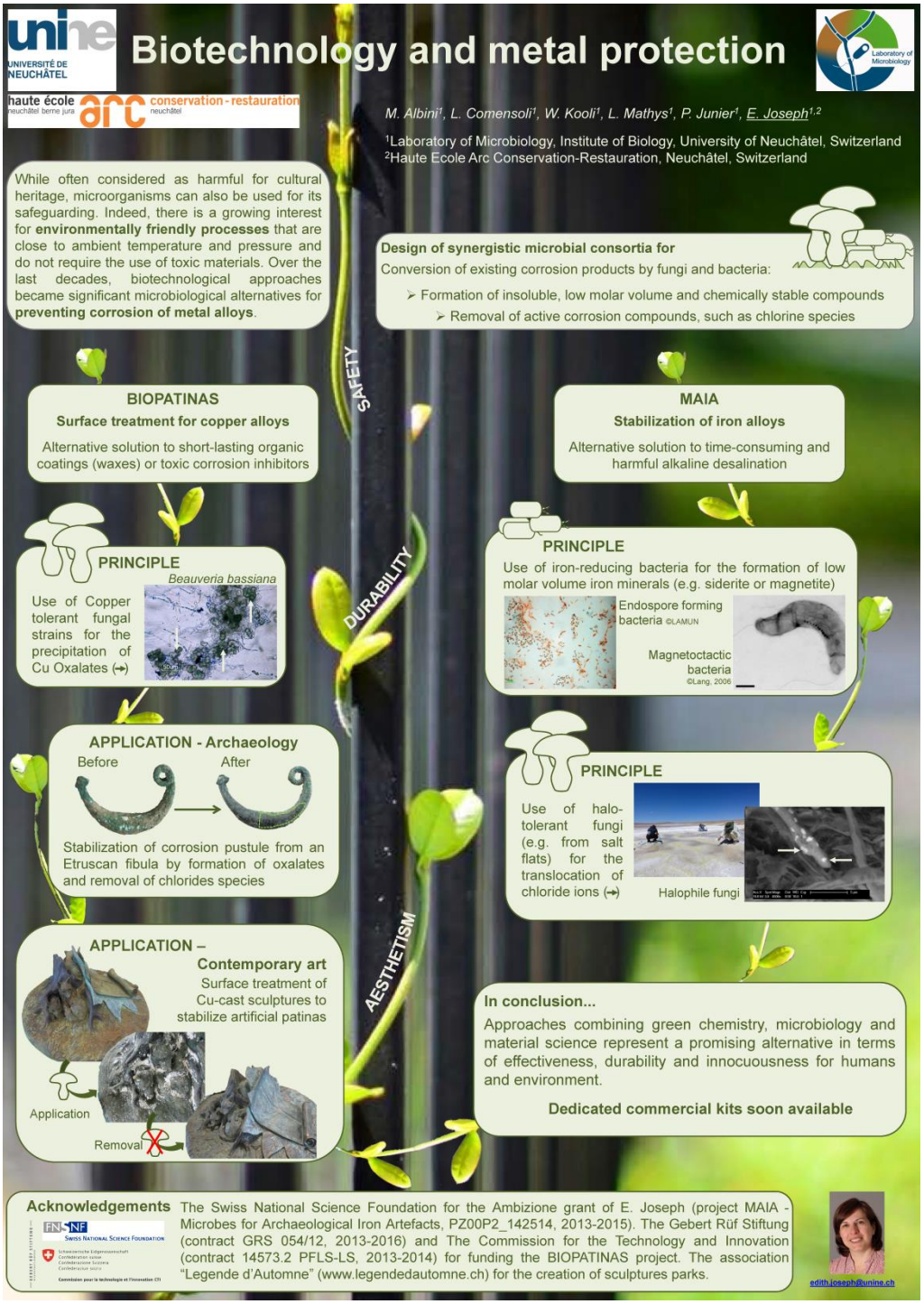
PRESENTATIONS AT NATIONAL OR INTERNATIONAL CONFERENCES

1. Poster presentation at ISME Conference, Seoul, South-Korea, August (24.-29.), 2014.



2. Oral presentation IMC10 Congress, Bankgog, Thailand, August (3.-8.), 2014.

3. Poster presentation at Annual Assembly of the Swiss Society of Microbiology (SSM), Lugano, Switzerland, Mai (28.-29.), 2015.

CAN BACTERIA AND FUNGI BE USED TO PRESERVE ARCHAEOLOGIACAL IRON OBJECTS?

L. Comensoli[a]; J. Maillard[b] P. Junier[a] E. Joseph[a,c]

[a]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland.
[b]ENAC-Laboratory for Environmental Biotechnology, Swiss Federal Institute of Technology (EPFL), CH-1015 Lausanne
[c]aute Ecole Arc Conservation-Restauration, Neuchâtel, Switzerland

1. Why archaeological iron objects need to be preserve?

When buried archaeological findings develop **corrosion layer** that depend on the soil conditions (i.e. humidity, pH, temperature, salts present).
Once excavated, the environmental conditions change and the corrosion layer can not be longer stable.

The most problematic element for these artefacts is **chlorine**. Interacting with iron ions, oxygen and humidity, chlorine causes **irreversible damages** to the objects.
Therefore, without any intervention these objects can **lose their shape** and be **irrevocably damaged** (fig. 1).

Did you recognize the object?

Figure 1: Untreated archaeological iron nail aspect.

2. Why do we need to develop new conservation-restauration methods?

The most common approach used to stabilize archaeological iron is the immersion in anoxic aqueous solutions **of alkaline sulphide** in order to diffuse out the **chloride ions**.

Time-consuming
Average of chlorine extraction only 75%
Production of high amount of toxic wastes that have to be neutralized
Problem to handle large findings (fig .2)

Imagine to handle an alkaline bath for this ship!

Figure 2: Untreated shipwreck, Ireland

3. What is the innovative solution propose by this study?

Use bacteria to reduce the corrosion layer and to produce stable iron minerals.

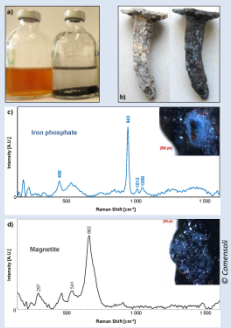


Figure 3: a) Control and liquid cultures of Desulfitobacterium hafniensis after 3 days of incubation, b) nails before and after the bacterial treatment, c) and d) Raman spectra of the biominerals found on the bacterially treated nails.

The bacterium *Desulfitobacterium hafniensis* was able to reduce iron citrate (fig. 3a) and also the corrosion layer of real archaeological iron nails (fig. 3b).

The biominerals produced was identified as a mix of **iron phosphate** and **magnetite** (figs 3c and 3d).

Use fungi to remove chlorine from archaeological iron objects.

Chlorine resistance mechanisms of several fungi were studied. Preliminary results show that *Beauveria bassiana* is able tolerate high amount of chlorine (until 35g/L), and accumulate chlorine inside the hyphal network. Indeed aggregates containing potassium an chlorine were found (figs 4a-c)

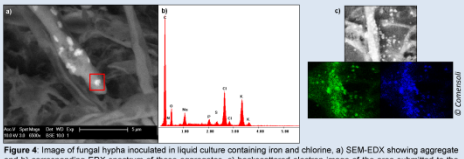


Figure 4: Image of fungal hypha inoculated in liquid culture containing iron and chlorine, a) SEM-EDX showing aggregate and b) corresponding EDX spectrum of these aggregates, c) backscattered electron image of the area submitted to the elemental mapping with elemental distribution of chlorine (green) and potassium (blue).

We can conclude that microbes are a valuable alternative to the conventional conservation-restauration methods for iron restauration. Indeed with bacteria we successfully produce stable iron mineral on the object surface. While with fungi, first results prove the ability of *B. bassiana* to uptake chlorine.

Contact:
lucrezia.comensoli@unine.ch

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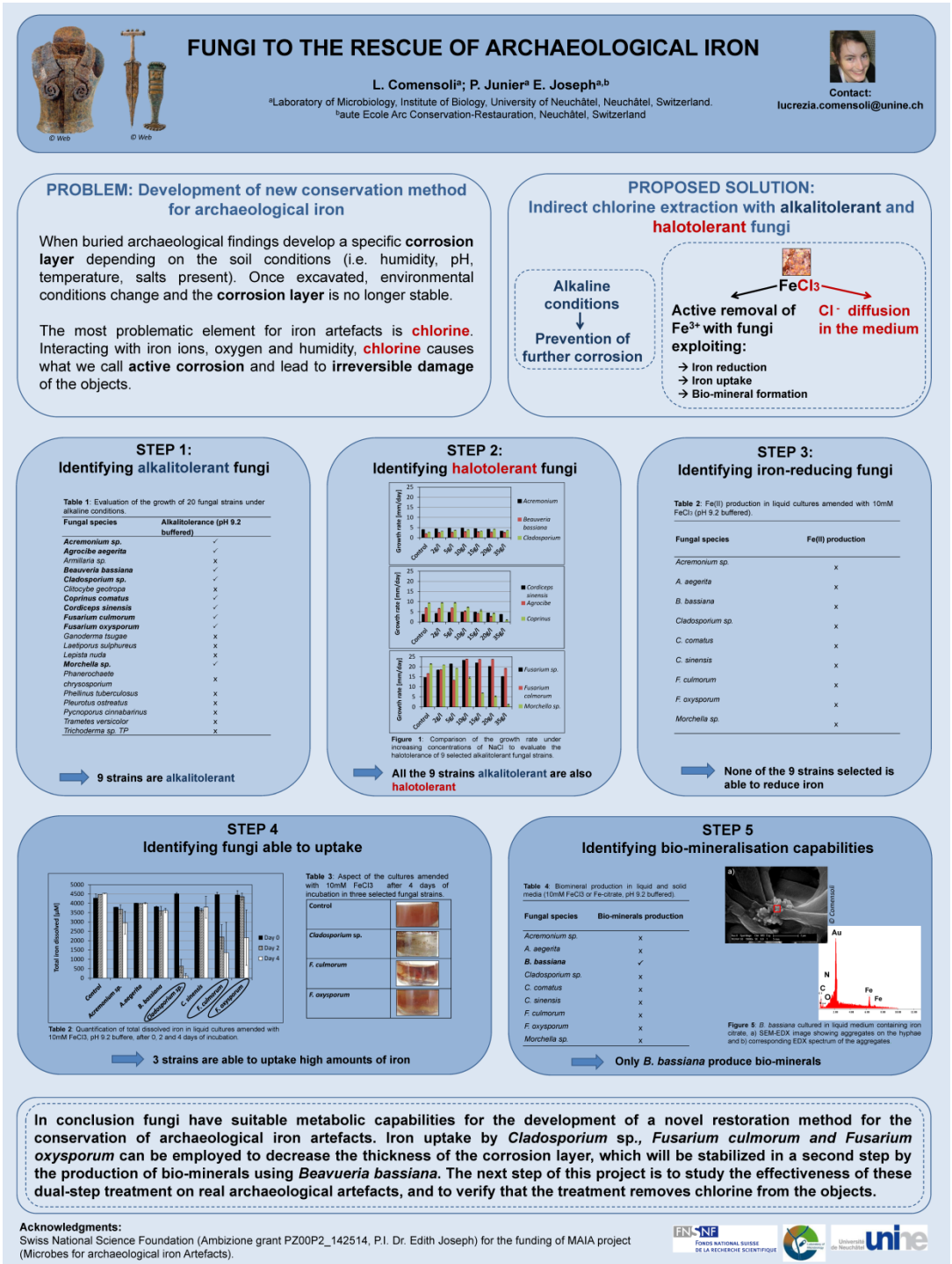
4. Oral presentation at FEMS Conference, Maastricht, Netherland, June (7.-11.), 2015.

5. Oral presentation at Swiss meeting of Microbial Ecology, Ascona, Switzerland, September (10.-12.), 2015.

6. Oral presentation at International Workshop: Green conservation of cultural heritage, Rome, Italy, October (27.-28.), 2015

7. Poster presentation at the Annual Assembly of the Swiss society of Microbiology (SSM), Bern, Switzerland, June (13.-16.), 2016.



8. Oral presentation at Zurich Mycology Symposium, Zurich, Switzerland, January (27.), 2016.

9. Poster presentation at ISME Conference, Montreal, Canada, August (21.-26.), 2016

The role of fungi in the geochemical cycle of iron

Lucrezia Comensoli[1], Saskia Bindschedler[1], Pilar Junier[1] and Edith Joseph[1,2]

[1] *Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, 2000 Neuchâtel, Switzerland*
[2] *Haute Ecole Arc Conservation-Restauration, 2000 Neuchâtel, Switzerland*

Iron is the second element most abundant on Earth; nevertheless, only a small part of it is bioavailable. Microbes have developed different strategies in order to gain access to this element, and then their influence on iron geochemical cycling is significant. Although most studies have focused on bacteria, microbial diversity is much larger than that and a group often overlooked is fungi.

The aim of this study was to elucidate fungal metabolic abilities regarding iron in order to better understand the cycle of iron in soil, biogenic mineral production and iron uptake in selected fungal strains.

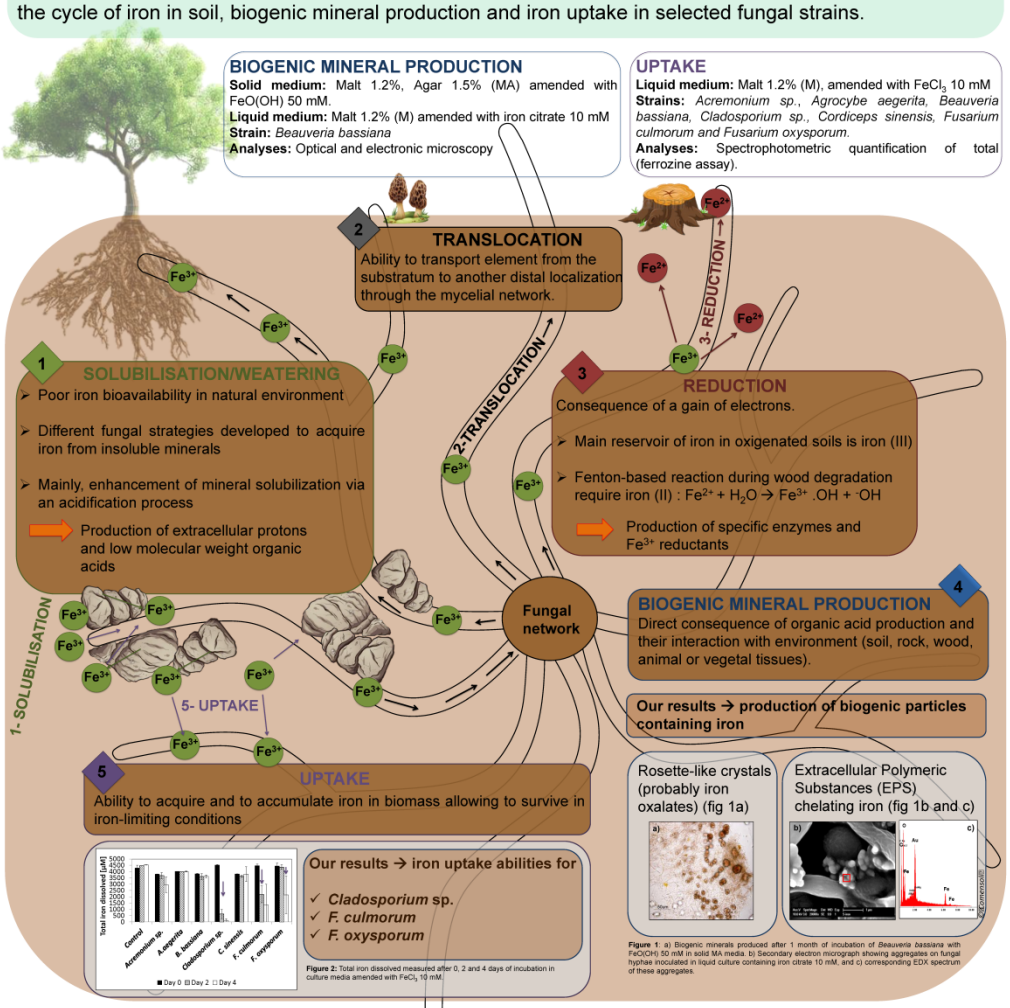


Figure 1: a) Biogenic minerals produced after 1 month of incubation of *Beauveria bassiana* with FeO(OH) 50 mM in solid MA media. b) Secondary electron micrograph showing aggregates on fungal hyphae inoculated in liquid culture containing iron citrate 10 mM, and c) corresponding EDX spectrum of these aggregates.

Figure 2: Total iron dissolved measured after 0, 2 and 4 days of incubation in culture media amended with $FeCl_3$ 10 mM.

Conclusion

Fungal metabolic activities regarding iron are a particularly exciting research field but still lacking of knowledge. Fragmentary information is available with focus on specific topics like plant pathogenic strains, yeast, mycorrhizal fungi, and wood-degrading fungi and an overall background regarding the interactions between fungi and iron does not exist. Iron cycling in soil is a really important phenomenon since it can influence microbial growth and organic matter degradation. This study proves the ability of fungi to produce organic iron sources in soil via the production of organic particles like EPS chelating iron or by the uptake in their biomass.

lucrezia.comensoli@unine.ch

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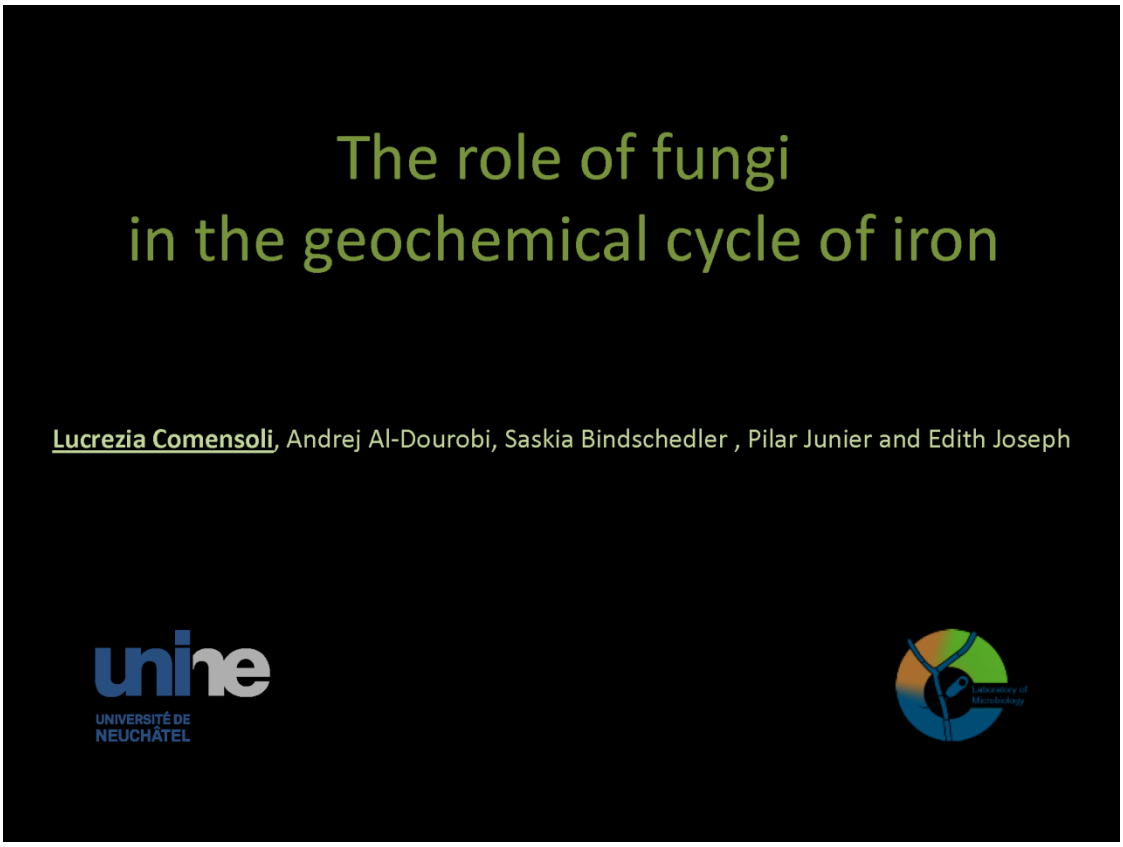
10. Oral presentation at Zurich Mycology Symposium, Zurich, Switzerland, January (23.), 2017.



CURRICULUM VITAE

Personal information

First name/Surname Lucrezia Comensoli

Date of birth 11 September 1987

Nationality Suisse

Address Oberer quai 112
2503 Biel

Tel. +41 76 248 60 45

E-Mail lucrezia.comensoli@gmail.com

Professional activities

Clarity – Patience – Organisation – Reliability

June 2017-September 2013 **Mycoexpertises** for Shire Neuchâtel
Quality control
Microscopic identification of moulds
Report performing

July-November 2016 **Lecturer** in mycology (Replacement of a maternity leave)
Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.
Planning of curses and lecturing
Exam design and correction

July 2016-May 2013 **Scientific and teaching assistant** in microbiology.
Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.
Student coaching, teaching, practical preparation

February-July 2014 **Lecturer** in mycology (Replacement of a maternity leave)
Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.

April 2013 **Internship** at the Cantonal microbiology center, Ticino, Switzerland.
Bacterial characterisation by 16S gene sequencing and MALDI-TOF mass spectrometry.

February-March 2013 **Internship** at the wastewater treatment consortium of Bellinzona, Ticino, Switzerland.

April-Mai 2012 **Scientific and teaching assistant** in microbiology
Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.

October-December 2011 **Scientific and teaching assistant**, in microbiology,
Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.

Education *Knowledge – Resoluteness - Precision*

June 2017-May 2013 **PhD in Microbiology**
Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.

Thesis project Applied microbiology, Biotechnology.
Study of the interaction between microbes (fungi and bacteria), iron and chlorine, in order to develop a new biotechnological restoration method for iron surface.

2010 – 2012 **Master of Science in Bio-geosciences**
Institute of Biology, Faculty of Sciences, Universities of Neuchâtel and Lausanne Switzerland.

Specialisation Geo-microbiology

Thesis project Applied Microbiology, Biotechnology.
Study of the fungal resistance mechanisms to copper. Improvement of a biotechnological treatment for copper based alloy artefacts.

2007 – 2010	**Bachelor of Sciences in Biology.** Institute of Biology, Faculty of Sciences, University of Neuchâtel, Switzerland.
2003 – 2007	**Diploma** Bellinzona, Switzerland.
Specific option	Latin and ancient Greek

Language skills

Discussion – Collaboration - Cooperation

Italian	Mother tongue
French	C2
English	B2 (IELTS Exam)
German	B1

Competences

Microbiology, Biotechnology, Ecology, Metal corrosion, Metal passivation.

Bio-alteration of metals and biogenic mineral production.

Fungal and bacterial metabolism.

Fungal organic matter degradation (wood degradation).

Fungal bacterial interaction.

Metal corrosion.

Technical skills

Morphological identification of moulds.

Sampling, isolation, and identification of fungi and bacteria.

Physiological and metabolic analysis.

Optical and scanning electronic microscopy.

Raman and FTIR Spectroscopy.

Publications

Comensoli L., Kooli W., Albini M., Maillard J., Wörle M., Junier P. and Joseph E. (2017). Evaluation of two biotechnological treatments for the stabilisation of corroded iron with Raman spectroscopy. Microchemical Journal. *In preparation*.

Kooli W., **Comensoli L**., Maillard J., Gelb A., Albini M., Junier P. & Joseph E. (2017). Production and control of biogenic minerals for the protection of corroded iron objects. Nature communication. *In preparation*.

Comensoli L., Maillard J., Albini M., Sandoz F., Junier P., Joseph E. (2017). Use of bacteria to stabilize archaeological iron. Applied and Environmental Microbiology. *In press.*

Comensoli L., Bindschedler S., Junier P. and Edith E. (2016). Iron and fungal physiology: a review of biotechnological opportunities. Advances in Applied Microbiology, 98:31-60.

Albini M., **Comensoli L.**, Kooli W., Junier P. and Joseph E. (2016). Microorganisms for safeguarding cultural heritage. International Journal of Conservation Science. *In press.*

Joseph E., Bindschedler S., Albini M., **Comensoli L.**, Kooli W., Mathys L. (2016). Microorganisms for safeguarding built heritage. The Fungal Community: Its Organization and Role in the Ecosystem, Eds. J. Dighton, J. White, P. Oudemans CRC press Taylor and Francis group. *In press.*

Albini M., **Comensoli L.**, Brambilla L., Beuret D. E., Kooli W., Mathys L., Letardi P., Joseph E. (2016). BIOPATINAS: Innovative biological approaches for metal conservation. Special issue of the Eurocorr WP21, Materials and Corrosion, 67, No. 2.

Joseph E., Letardi P., Albini M., **Comensoli L.**, Kooli W., Mathys L., Domon B. E., Brambilla L., Cevey C., Bertholon R., Job D., Junier P. (2014). Innovative biological approaches for metal conservation. Conference Proceedings of EUROCORR 2014, European Corrosion Congress. Pisa, Italy. 8th-12th September. DECHEMA e.V., Frankfurt and AIM – Associazione Italiana di Metallurgia, Milano, 2014; ISBN 9783897461598, 1-10.

Domon B. E., Mathys L., **Comensoli L.**, Brambilla L., Albini M., Cevey C., Bertholon R., Junier P., Joseph E. (2014). Biopatines: des champignons au service des alliages cuivreux. In Conference Proceedings of JOURNEES DES RESTAURATEURS EN ARCHEOLOGIE « restaurer l'ordinaire, exposer l'extraordinaire : du site au musée » Arles, France. 16th-17th October 2014.

Joseph E., Letardi P., **Comensoli L.**, Simon A., Junier P., Job D. & M. Woerle. (2013). Assessment of a biological approach for the protection of copper alloys artefacts. Conference Proceedings of Metal 2013, Interim Meeting of the ICOM-CC Metal WG. Edinburgh, Scotland. 16th-20th September 2013. 203-207.

Congresses

Comensoli L., Bindeschler S., Al-Dourobi A., Junier P. and Joseph E. (2017). The role of fungi in the biogeochemical cycle of iron. Zurich Mycology Symposium, 23th January. (Oral presentation).

Comensoli L., Bindschedler S., Junier P. and Joseph E. (2016). The role of fungi in the biogeochemical cycle of iron**.** 16th International Symposium on Microbial ecology (ISME) Montreal, Canada, 21th-26th of August. (Poster presentation).

Comensoli L., Albini M., Kooli W., Junier P. and Joseph E. (2016). The art of survival to the rescue of artworks. Zurich Mycology Symposium, 27th January. (Oral presentation).

Comensoli L., Junier P. and Joseph E. (2016). Fungi to the rescue of archaeological iron. 74th Annual Meeting of the Swiss Society for Microbiology, Bern, Switzerland, 13th-16th June. (Poster).

Comensoli L., Maillard J., Junier P. and Joseph E. (2015). Can bacteria and fungi be used to preserve archaeological iron objects? International Workshop: Green conservation of cultural heritage, Rome, Italy, 27th-28th October. (Oral presentation).

Comensoli L., Maillard J., Junier P. and Joseph E. (2015). Can microbes be used to preserve archaeological iron objects? SME: 6th Swiss Microbial Ecology Meeting, Monte Verità, Ascona, Switzerland, 10th-12th September. (Oral presentation).

Comensoli L., Maillard J., Junier P. and Joseph E. (2015). Can we exploit bacteria and fungi to preserve archaeological iron objects? FEMS: 6th Congress of European Microbiologist, Maastricht, Netherland, 7th-11th June. (Oral presentation).

Comensoli L., Junier P. and Joseph E. (2015). Fungi to the rescue of archaeological iron. Can bacteria and fungi be used to preservearchaeological iron objects? 73th Annual Meeting of the Swiss Society for Microbiology, Lugano, Switzerland, 28th-29th Mai. (Poster).

Joseph E., Albini M., **Comensoli L.,** Kooli W., Mathys L., Junier P. (2014) Innovative biotechnological approaches for metal protection. IMC10: The 10th International Mycological Congress, Bangkok, Thailand, 3rd-8th August. (Joseph E., invited speaker represented by **Comensoli L**.).

Albini M., **Comensoli L**., Kooli W., Mathys L., Junier P., Joseph E. (2014). Biotechnology and metal protection. 15th International Symposium on Microbial Ecology (ISME), Seoul, South-Corea. (Poster).