The interaction between microbes, siderophores and minerals in podzol soil

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Abstract

Microorganisms play an essential role in the bioweathering of minerals in soil ecosystems to satisfy the nutrient demand for themselves and the surrounding plants. Microorganisms produce chelating agents like siderophores of low molecular masses (200 to 2000 Da), especially under iron-limiting conditions. One of the primary biogeochemical functions of siderophores in soil is to increase Fe bioavailability by promoting the dissolution of iron-bearing minerals.

Nonetheless, many studies have focused on the role of soil microorganisms in mineral weathering without considering the particular interaction between siderophores produced by these microorganisms and minerals. In the present thesis, two main questions were addressed: 1) Is there a relationship between soil horizon, mineral type and the distribution of siderophores in the boreal forest? 2) What are the biotechnological applications of siderophores in the environment?

To answer the first question, we worked on samples of bulk soil of the whole profile and soil attached to mineral surfaces collected in a field experiment, in which three different minerals (apatite, biotite and oligoclase) were inserted for two years in the podzol soil horizons (O (organic), E (eluvial) and B (upper illuvial)). The main aims were to a) determine the presence and concentration of hydroxamate siderophores in this soil and b) investigate the relationship between the presence of different minerals and the distribution of siderophores. For the second question, we discussed in a literature review the important roles and applications of siderophores in different environmental habitats.
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** Submitted to Microbial Biotechnology Journal

Front cover photo of boreal forest was taken by Cajsa Lithell, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences (SLU).
1. General introduction

Mineral weathering is the primary source of most essential elements for microorganisms and plants in soil ecosystems. Iron is an important element for the growth of almost all living microorganisms since it acts as a catalyst in various enzymatic processes, oxygen metabolism, electron transfer, and DNA and RNA synthesis (Touati, 2000; Verkhovtseva et al., 2001). Microorganisms produce siderophores with low molecular masses (200 to 2000 Da) as a chelating agent, especially under iron-limiting conditions (Schwyn and Neilands, 1987). The role of siderophores is primarily to scavenge iron, and also form complexes with other elements (i.e. Mo, Mn, Co and Ni) from the surrounding environment and make them available for microbial cells (Visca et al., 1992; Neilands, 1995; Duhme et al., 1998; Bellenger et al., 2008). Siderophores have three main functional groups, hydroxamate, catecholate and carboxylate, forming very strong complexes with iron. Siderophore studies started six decades ago when Neilands discovered the fungal ferrichrome type (Neilands, 1952). Since then, over 500 different types of siderophores have become known, 270 of which have been structurally characterized (Boukhalfa et al., 2002). So far, the historical development of siderophore studies under lab conditions have shown that some bacterial and fungal species can produce more than one type of siderophore (Wilhelm and Trick, 1994; Granger and Price, 1999; Cendrowski et al., 2004; Das et al., 2007), but still more research is needed that focuses on investigating siderophore production and function in natural environments.

1.1 Podzol soil

Podzol soils cover approximately 485 million hectares worldwide and are located mainly in the temperate and boreal regions of the Northern Hemisphere (Lundström et al., 2000). The podzol soil is the third most widespread in the European region, covering more than 0.5 million km² or 13.66% of its total area (Figure 1). Vast areas of podzols are found in the Scandinavian countries; for example they cover approximately 13.7 M ha or 60.4% of the forest land area of Sweden (Figure 1). This reference soil group is also present in 22 member states of the EU and is only absent in Hungary, Slovenia, Bulgaria, Malta and Cyprus.
A typical podzol profile consists of a litter layer (O), a leached ash gray eluvial mineral layer (E), an accumulation (illuvial) layer of organic matter in combination with Fe and Al (B), and the parent material (C) (Figure 2). Mineral composition of podzols is somewhat variable but is nearly always characterized by a predominance of quartz (Ugolini and Dahlgren, 1987). In cool, humid climates where leaching is intense, the parent material may originally have been of intermediate or even basic composition. The maximum Fe and Al content may occur at different depths in the B-horizon, depending on the genetic history of a particular soil (Mattson and Lönnermark, 1939). The mineral composition of the soil has been shown to affect the structure and physiological activities of the associated microbial communities (Boyd et al., 2007; Carson et al., 2009; Carson et al., 2007). Microorganisms have a significant effect on releasing the nutritional elements from minerals into the soil environment through the bioweathering process (Certini et al., 2004).
1.2 Microbial siderophores

Siderophores play an important role in the extracellular solubilization of iron from minerals and make it available to microorganisms (Lamont et al., 2002; Dale et al., 2004). Most of the bacterial siderophores are catecholates, and few are hydroxamates and carboxylates, whereas most fungal siderophores are hydroxamates (Schalk et al., 2011).

Hydroxamate siderophores are the most common group of siderophores, especially in natural samples. Hydroxamates form 1:1 complexes with ferric iron and the binding constants is in the range of $10^{22}$ to $10^{32}$. The ferric hydroxamate complexes are stable against hydrolysis and enzymatic degradation in the natural environment with pH above 1 (Winkelmann, 2007). Hydroxamates are produced by fungi, i.e. ferrichromes, coprogens and fusigenes, and by bacteria like ferrioxamines (Van der Helm and Winkelmann, 1994; Winkelmann and Drechsel, 1997; Winkelmann, 2007). Ferrichromes are the predominant siderophores of fungi, and are based on a cyclic hexapeptide structure (Figure 3) (Leong and Nielands, 1982; Deml et al., 1984). Examples of fungal species that produce ferrichrome type siderophores are *Ustilago sphaerogena* for ferrichrome (Emery, 1971), *Aspergillus fumigatus* for ferricrocin (Wallner et al., 2009) and *Neurospora crassa* for tetruglycylferrichrome (Winkelmann, 2007). Coprogens (Figure 3) are produced by some fungal species i.e. *Trichoderma spp.* and were first isolated from *Neurospora crassa* (Zähner et al., 1963). Fusigen (Figure 3) are produced by some species of fungi e.g. *Fusarium spp.* (Diekmann and Zahner, 1967; Sayer and Emery, 1968; Neilands, 1973). Ferrioxamine type siderophores (Figure 3) are commonly produced by many soil bacteria, such as *Erwinia, Nocardia, Streptomyces, Arthrobacter, Chromobacterium* and *Pseudomonas* species.
(Berner et al., 1988; Berner and Winkelmann, 1990; Gunter et al., 1993; Meyer and Abdallah, 1980; Muller and Raymond, 1984; Wei et al., 2007).

Ferrichrome: \( R_1 = R_2 = H, R_3 = \text{CH}_3 \)
Ferrichrome A: \( R_1 = R_2 = H \)
Ferricrocin: \( R_1 = H, R_2 = \text{CH}_2 \text{OH}, R_3 = \text{CH}_3 \)
Ferrichrysin: \( R_1 = R_2 = \text{CH}_2 \text{OH}, R_3 = \text{CH}_3 \)
Ferrirubin: \( R_1 = R_2 = \text{CH}_2 \text{OH}, R_3 = H \)
Ferrirhodin: \( R_1 = R_2 = \text{CH}_2 \text{OH} \)

Ferrichrome B: \( R_1 = H, R_2 = \text{CH}_3 \)
Ferrichrome D: \( R_1 = \text{COCH}_3, R_2 = \text{CH}_3 \)
Ferrichrome G: \( R_1 = H, R_2 = \text{CH}_2 \text{CH}_2 \text{COOH} \)

Coprogen: \( R_1 = H, R_2 = \text{COCH}_3, R_3 = R_4 = H \)
Neocoprogen I: \( R_1 = H, R_2 = \text{COCH}_3, R_3 = \text{CH}_3, R_4 = H \)
Neocoprogen II: \( R_1 = H, R_2 = \text{COCH}_3, R_3 = R_4 = \text{CH}_3 \)

Figure 3. Chemical structures of hydroxamate siderophores.
1.3 Siderophore content in soil ecosystems

The presence of siderophores in soil has been estimated by using microbial assays that revealed only the total concentration of hydroxamates, as well as ferrichrome-type siderophores. Powell et al. (1982) and (1983) were the first to use these assays to quantify the siderophores from water extracts of sandy clay soil. Powell et al. (1982) found that the total hydroxamate concentrations were relatively high in the 27–279 nM range, reported as desferrioxamine B equivalents, while Powell et al. (1983) estimated 34 nM of total hydroxamate siderophores by using the M. flavescens assay and 78 nM of ferrichrome type by the E. coli assay. Hydroxamate siderophore concentration of individual siderophores in Swedish podzolic forest soils has been measured previously (Holmström et al., 2004; Essén et al., 2006; Ali et al., 2011) by using high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS), in which much lower concentrations were estimated compared with microbial assays since individual siderophores were quantified. For instance, hydroxamates have been detected to be between 0.9-1.4 nM in soil solution and identified as ferrichrome and ferricrocin in the O-horizon of podzol soil (Holmström et al., 2004). Essén et al. (2006) also detected 0.1–12 nM of ferricrocin and 0.1-2.1 nM of ferrichrome in the podzol horizons and the lower concentrations of hydroxamates found in the lower horizons like the B- and C-horizons. Recently, Ali et al. (2011) found a small amount of ferricrocin while ferrichrome was only detected occasionally. The maximum concentration of ferricrocin was 3.74 nmol/kg soil in the O-horizon.

1.4 Scope of the thesis

For decades, mineral weathering by forest soil microorganisms has mainly been attributed to mycorrhizal fungi, largely overlooking the role of other associated soil microorganisms. As a consequence, little is known about the interaction between siderophores produced by soil microorganisms and minerals in forest soil. The present thesis focused on three main points: a) the relationship between soil horizon and the distribution of siderophores in the boreal forest soil b) the interaction between the siderophores concentration and the different minerals in the soil ecosystem c) the functions and applications of siderophores in different areas of environmental research.
The thesis is presented in two manuscripts:

**Manuscript I** “research article” aimed to a) determine the presence and concentration of hydroxamate siderophores in different soil horizons of podzol and b) investigate how the presence of different minerals may influence the concentration and distribution of siderophores.

**Manuscript II** “review article” aimed to emphasize the roles and biotechnological applications that the siderophores could play in applied environmental processes.

2. **Methods**

2.1 **Sampling site**

The sampling was carried out in September 2011 at a site (63°07′N, 16°70′E) in central Sweden in the vicinity of the village Bispgården (Figure 4). The site is located on a slope (angle 2°) at an altitude of 258 m above sea level and was forested with 80-yr-old Norway spruce (*Picea abies* L. Karst.) and Scots pine (*Pinus sylvestris*). Three different polished minerals, apatite, oligoclase and biotite (3X4 cm) had been inserted in O-, E- and B-horizons two years earlier, June 2009 (Manuscript I; Olofsson et al., in preparation). The soil samples for this study were collected from the bulk soil of the whole profile and soil attached to mineral surfaces from all the soil horizons and kept cold (+4 °C) until chemical characterization (Table 1) and further analysis.

![Figure 4. The location of the sampling area in Bispgården, Sweden and podzol soil profile.](image)
Table 1. Chemical characterization of soil samples of each horizon

<table>
<thead>
<tr>
<th>Podzol Horizons</th>
<th>C%</th>
<th>N%</th>
<th>Ca*</th>
<th>K*</th>
<th>Mg*</th>
<th>Na*</th>
<th>Fe*(total)</th>
<th>pH</th>
<th>Moisture content%</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>47</td>
<td>1.2</td>
<td>1.7</td>
<td>0.5</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
<td>4.4</td>
<td>26</td>
</tr>
<tr>
<td>E</td>
<td>0.93</td>
<td>0.03</td>
<td>1.7</td>
<td>0.5</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
<td>4.6</td>
<td>78</td>
</tr>
<tr>
<td>B</td>
<td>1.9</td>
<td>0.06</td>
<td>1.5</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>5.1</td>
<td>86</td>
</tr>
<tr>
<td>C</td>
<td>0.3</td>
<td>0.01</td>
<td>1.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.09</td>
<td>5.4</td>
<td>89</td>
</tr>
</tbody>
</table>

*Exchangeable cations
*a) Vestin et al., 2008, b) Manuscript I

2.2 Siderophore extraction and quantification in podzol soil

To estimate the content of the siderophores in the soil samples, we extracted the siderophores by two different methods; water extraction (dissolved) and methanol extraction (adsorbed) as described in Manuscript I and the analysis was performed using a HPLC-ESI-MS method developed by Holmström and Kalinowski (Manuscript, 2013). We quantified the concentration of the four main families of trihydroxamates (ferrichromes, ferrioxamines, coprogen and fusigen). The HPLC system (Ultimate 3000 RS, Thermo Scientific, US) was built up of two pumps with flow rate of 0.030 ml/min for the low pressure gradient pump and 0.15 ml/min of high pressure gradient pump and a column compartment set at 10°C. The columns and eluents used in this method were described in more detail in (Manuscript I). The standards were purchased from EMC microcollections (GmbH, Germany). The HPLC system was connected to a mass spectrometer (TSQ Quantum Access Max, Thermo Scientific, US) where the ferric complex of each individual hydroxamate siderophore was detected by selected ion monitoring (SIM) of the proton adducts [M + H]+ (Manuscript I).
3. Summary and discussion of the major results of manuscript I

3.1 Soil horizons and siderophores

The concentration of total dissolved (water soluble) ferrioxamines ranged between 2-7 pmol/g dry soil; 1-4 pmol/g dry soil for ferrichromes; 0-39 pmol/g dry soil for fusigen and 0-14 pmol/g dry soil for coprogens (Figure 5). Maximum concentrations of total dissolved ferrioxamines, fusigen and coprogens were all found in the E-horizon, while the maximum concentration of the ferrichromes was found in the B-horizon.

![Figure 5. Concentration of dissolved hydroxamate siderophore groups (ferrioxamines, ferriochromes, fusigen and coprogens) per each soil horizon.](image)

We performed principal component analysis (PCA) based on the composition of hydroxamate siderophore types in the bulk soil samples. The first principal component (PC1) correlated with soil horizon and the second one (PC2) correlated with individual hydroxamate types. As shown in the PCA biplot, the soil horizons have a strong influence on the hydroxamates diversity in which each soil horizon formed groups in the corners of the graph (Figure 6). In addition, all the individual ferrioxamines correlated to the E-horizon, while most of ferrichromes and coprogens correlated to the O-horizon except for tetraglyclferrichrome and fusigen which correlated to the C-horizon and ferrirhodon that correlated to the E-horizon.
Figure 6. Principal component analysis (PCA) ordination of hydroxamates in soil horizons. O, E, B and C referred to the soil horizons.

Thus the soil horizons have a great effect on the distribution of hydroxamates throughout the soil profile. That is in agreement with Bossier et al. (1988) and Nelson et al. (1988), who have suggested that the presence of siderophores in soil depends strongly on the chemical, physical and biological properties of the soil horizons. Possible explanations this relationship depend on three main factors: (1) chemical and mineralogical properties of podzol soils change with depth, which creates a number of different habitats for microorganisms throughout the soil profile (Fierer et al., 2003; LaMontagne et al., 2003; Rosling et al., 2003), in which the highest siderophore metabolic diversity were found in the O- and E-horizons compared to the B- and C-horizons, (2) The pH of the soil. However, the pH of the soil solution in our study were about 4-5.5, and at these pH conditions are the major part of iron soluble, so why would the microorganism have to produce siderophores when sufficient amounts of iron are already available? The answer could be that the microorganisms that produce hydroxamate siderophores
at these pH values have a great advantage over other non-siderophore producing microorganisms, due to the extreme acid stability of the siderophore molecules and are thus able to scavenge the needed iron from competing microorganisms and also protect themselves from the overdose Fe stress. (3) The variations of hydroxamates that we found in the soil horizons also indicate that there is a high diversity of forest soil microorganisms that produce a wide range of siderophores. For example, ferrioxamines can be produced by Streptomyces spp. (Das et al., 2007); ferrichromes by Aspergillus spp. (Charlang et al., 1981), Suillus variegates (Wallander and Wickman, 1999) and Microsporum spp. (Bentley et al., 1986); coprogens by Fusarium dimerum (Van der Helm and Winkelmann, 1994) and Epicoccum purpurascens (Frederick et al., 1981) and Histoplasma capsulatum (Burt, 1982). Thus, according to our findings that coprogens and fusigen had the maximum concentrations in all the investigated soil horizons, it could indicate that microorganisms like Fusarium spp., Epicoccum purpurascens and Histoplasma capsulatum are the most common species in that soil.

3.2 Mineral type and siderophores

We found that the concentration of hydroxamate siderophores in the soil attached to the polished mineral surfaces was higher than in the bulk soil by approximately 66%. The total dissolved (water extracts) hydroxamates found on apatite, biotite and oligoclase ranged between 20-83, 42-107 and 18-36 pmol/g dry soil, respectively (Figure 7) (Manuscript I). These findings could depend on the interaction between the siderophores and the specific chemical characteristics of the mineral surfaces. For instance, biotite had the maximum dissolved hydroxamate concentration in the O-horizon and thereafter decreased with the depth of the soil, whereas oligoclase had the maximum concentration in O-horizon followed by the B-horizon, while the concentration from the apatite surface increased gradually with the depth of the soil until it reached maximum in the B-horizon. Such interaction may depend on the binding between siderophores with elements on each mineral (Ehrlich, 1998). There are a number of factors that influence the conformation and bonding of attached siderophores on mineral surfaces such as siderophore’s architecture, charge, and hydrophobicity (Kraemer, 2004). There is also a limitation to the number of bonds that can form between the siderophore and a single Fe(III) ion in the inner coordination sphere at the mineral surfaces, that is why each mineral in our
experiment had a unique behavior with regard to each hydroxamate siderophore type as we mentioned before. The siderophore/mineral interaction can also be explained that the elemental composition of each individual mineral type induces different siderophore production by the presence of microorganisms. For instance, we can correlate the high content of potassium, iron and magnesium (4% K, 4.5% Fe and 7.8% Mg) on the biotite surface (Table 2) which are the main essential elements for fungal growth with the high concentration of ferrichrome siderophores which was found.

![Figure 7. Total dissolved hydroxamate siderophore concentration of soil attached to mineral surfaces per each soil horizon.](image)

<table>
<thead>
<tr>
<th>Minerals per each horizon</th>
<th>Total hydroxamates (pmol/g dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Apatite</td>
<td></td>
</tr>
<tr>
<td>Biotite</td>
<td></td>
</tr>
<tr>
<td>Oligoclase</td>
<td></td>
</tr>
<tr>
<td>Apatite</td>
<td></td>
</tr>
<tr>
<td>Biotite</td>
<td></td>
</tr>
<tr>
<td>Oligoclase</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Atomic percentage of selected elements in apatite, biotite and oligoclase obtained by energy-dispersive-X-ray spectroscopic analysis performed with SEM (Olofsson et al., in preparation).

<table>
<thead>
<tr>
<th>Element (%)</th>
<th>O</th>
<th>Si</th>
<th>P</th>
<th>Ca</th>
<th>Al</th>
<th>Mg</th>
<th>K</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apatite</td>
<td>56.7</td>
<td>0.9</td>
<td>11.9</td>
<td>18.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.24</td>
</tr>
<tr>
<td>Biotite</td>
<td>63</td>
<td>14.9</td>
<td>–</td>
<td>–</td>
<td>4.6</td>
<td>7.8</td>
<td>4.11</td>
<td>4.5</td>
</tr>
<tr>
<td>Oligoclase</td>
<td>63.1</td>
<td>19.5</td>
<td>–</td>
<td>1.5</td>
<td>8.2</td>
<td>–</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>
4. Short summary for the review of the roles and applications of siderophores

“Manuscript II”

Siderophores have received much attention in recent years because of their potential roles and applications in various areas of environmental research. For example, siderophores function as plant growth promoters (Yadav et al., 2011; Verma et al., 2011), biocontrol agents (Verma et al., 2011; Schenk et al., 2012) and bioremediation agents (Wang et al., 2011; Ishimaru et al., 2012), in addition to their valuable role in soil mineral weathering (Reichard et al. 2005; Buss et al., 2007; Shirvani and Nourbakhsh, 2010).

Potential roles are as follows:

- Most of soil microorganisms produce siderophores to promote the mineral weathering of insoluble phases. Siderophores provide an efficient Fe acquisition system due to their high affinity for Fe(III) complexation by means of mineral dissolution (Kraemer, 2004). In soils that are enriched with insoluble iron oxides, siderophores play an important role in iron dissolution, making it available for microorganisms and plants (Hersman et al., 1995). The mechanism is that the Fe-siderophore complex is formed at the mineral surface and is then transferred into the surrounding soil solution and becomes available for uptake by the cell membrane of microorganisms or plants (Kalinowski et al., 2000; Liermann et al., 2000; Kraemer, 2004).

- Marine bacteria can also produce siderophores and thereby play a significant role in the biogeochemical cycling of Fe in the ocean (Hutchins and Bruland, 1998; Granger and Price, 1999). This role depends on the competition between marine bacteria and phytoplankton for Fe by producing different types of siderophores that affect the Fe abundance and solubility in the marine environment (Tortell et al., 1999).

Biotechnological applications are as follows:

- Microbial siderophores can provide the plants with iron nutrition to enhance their growth when the bioavailability of iron is low in the soil (Crowley, 2006). Kloepper et al. (1980) were the first to show the role of siderophores in increasing plant growth. They found that different Pseudomonas species can improve plant growth by producing siderophores and protecting them from pathogens. Thus they classified these Pseudomonas species as plant
growth promoting bacteria. In addition mycorrhizal fungi can also be used as biofertilizer to enhance plant growth that depends on their production of siderophores (Van Schöll et al., 2008). Kloeper et al. (1980) were also the first to investigate the role of siderophores in the mechanism of biological control. This mechanism depends on the role of siderophores as competitors for iron in the soil that reduce the iron availability for the phytopathogens (Scher and Baker, 1982; Thomashow et al., 1990).

- The production of siderophores can be a powerful tool in a quick identification of microbes to the species level (Meyer and Stintzi, 1998; Meyer et al., 2002). Siderotyping is defined as the characterization of microbial strains by the siderophores they produce (Neilands, 1981). There are two different methods for siderotyping, the analytical by using high performance liquid chromatography (HPLC) coupled with mass spectrometry (HPLC-ESI-MS) and the biological methods by using molecular biology method based on the recognition of specific functional genes (Meyer et al., 2002).

- Siderophores have also many other applications including biocontrol of fish pathogens, bioremediation of metals and petroleum hydrocarbons, nuclear fuel reprocessing, optical biosensor and bio-bleaching of pulps, which were described in details in Manuscript II.

5. Conclusion and future perspective

Our field experiment succeeded in describing the relationship between the presence of siderophores, soil horizon and mineral type.

- A wide range of fungal hydroxamate siderophores (ferrichromes, coprogens and fusigen) and bacterial ones (i.e. ferrioxamines) were detected in a podzolic soil, however, fusigen and coprogens had the maximum concentration. These new results may change our previous knowledge that the most of the hydroxamate siderophores in soils are of ferrichrome type as determined and suggested in earlier studies.

- Our findings regarding the effect of the presence of different minerals on the concentration and distribution of hydroxamates in the soil make the mineral type as one of the factors affecting the siderophores content in the natural environment.

- The observation that the concentration of hydroxamates in the soil attached to the polished mineral surfaces was higher than the surrounding bulk soil may indicate that the microenvironment attached to the mineral surfaces is more active in producing
siderophores than the microorganisms in the bulk soil and thus influence the weathering of these minerals.

The significant roles and applications of the siderophores in various environmental habitats i.e. plant growth promoting, biocontrol and bioremediation processes and microbial ecology, make them a powerful tools in the environmental research.

Our next step is to gain greater insight into the siderophore-mineral interactions in soils by investigating the microbial diversity in the bulk soil and the soil attached to mineral surfaces which could have a major effect on the soil mineral weathering. This topic will be further investigated by metagenomic sequencing of soil DNA to see the whole microbial composition throughout the soil profile and on the different mineral surfaces. In addition, additional focus will be placed on the diversity of the siderophore producing microorganisms throughout the soil horizons and how their composition could affect the mineral weathering processes.

6. Acknowledgments

I would like to thank my main supervisor, Sara Holmström who offered me a challenging research project, encouraged and supported me throughout my research. Her knowledge and guidance was and will be very helpful for my PhD. I am very grateful to my co-supervisors, Nils Holm and Volker Brüchert who always give me good advice and valuable comments on my work.

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7. References


Manuscript I
Title: The effect of soil horizon and mineral type on the diversity of siderophores in soil

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Abstract

Iron is a key component of the chemical architecture of the biosphere. Due to the low bioavailability of iron in the environment, microorganisms have developed specific uptake strategies, like siderophores, which are operationally defined as low-molecular-mass biogenic Fe(III)-binding compounds, that can increase iron’s bioavailability by promoting the dissolution of iron-bearing minerals. In the present study, we aimed to investigate the composition of dissolved and adsorbed siderophores of the hydroxamate family in the soil horizons of podzol soil, and how it is affected by the presence of specific mineral types. Three different minerals (apatite, biotite and oligoclase) were inserted in the soil horizons (O (organic), E (eluvial) and B (upper illuvial)). After two years, soil samples were collected from both the bulk soil of the whole profile and from the soil attached to the mineral surfaces. The concentration of ten different fungal tri-hydroxamates within ferrichromes, fusigen and coprogens families, and five bacterial ones within the ferrioxamine family were determined in soil water (dissolved) and soil methanol (adsorbed) extracts along the complete soil horizon by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS), and hence the study is the most extensive of its kind. We found that the concentration of coprogens and fusigen were present in much higher concentrations in bulk soil than ferrioxamines and ferrichromes. On the other hand, the presence of the polished mineral completely altered the diversity of siderophores. In addition, each mineral had a unique interaction with the dissolved and adsorbed hydroxamates in the different soil horizons. Thus, siderophore composition in the soil environment is controlled by the chemical, physical and biological characteristics of each soil horizon, in addition to available mineral types.

Keywords: Apatite, Biotite, Coprogen, Ferrichrome, Ferrioxamine, Fusigen, Hydroxamates, Oligoclase, Podzol soil and Weathering.
1. Introduction

In the soil environment, the microbial communities that colonize mineral surfaces differ from those of the surrounding soil particles (Certini et al., 2004). Microbial attachment to mineral surfaces leads to the formation of a microenvironment that protects the microorganisms against environmental stress (Beveridge et al., 1997; Liermann et al., 2000b; Ojeda et al., 2006). In the microenvironments, mineral nutrients can be chelated directly from the soil minerals by certain microorganism or shared amongst the surrounding microorganisms (Brown et al., 1994; Rogers et al., 1998; Roberts Rogers et al., 2001; Bennett et al., 2001; Roberts Rogers and Bennett, 2004). Most soil microorganisms can promote mineral weathering by production of siderophores which are defined as low-molecular-mass Fe(III)-binding compounds. Siderophores provide an efficient Fe-acquisition system due to its high affinity for Fe(III) complexation by means of mineral dissolution (Kraemer, 2004). In soils that are enriched with iron oxide and clay silicate mineral phases, siderophores play a significant role in iron dissolution, making it available for microorganisms and plants (Hersman et al., 1995). There are different mechanisms for siderophore promoted iron dissolution (e.g., Holmén and Casey, 1996; 1998). The general mechanism is that the Fe-siderophore complex is formed at the mineral surface and is then transferred into the surrounding soil solution and thereby becomes available for uptake by the cell membrane of microorganisms or plants (Kalinowski et al., 2000a; Liermann et al., 2000a; Kraemer, 2004). Siderophores are either recycled or destroyed upon iron reduction, whereas the reduced iron Fe(II) that is not used by the cell can act as an electron donor in electron transport chains (Kalinowski et al., 2000b). The impact of siderophores on soil mineral weathering can be more effective compared to that of organic acids since siderophores form more stable complexes with Fe(III). Siderophores form 1:1 complexes with Fe(III), with constants ranging between $K=10^{30}$ and $K=10^{52}$ (Jalal and van der Helm, 1991; Matzanke, 1991), while the constants of oxalic and citric acids with Fe(III) are $K=10^{7.6}$ and $10^{12.3}$, respectively (Perrin, 1979).
Microorganisms produce a wide range of siderophore types. Most of the bacterial siderophores are catecholates, and some of them are trihydroxamates and carboxylates, whereas most of fungal ones are hydroxamates (Schalk et al., 2011). The trihydroxamate ferrioxamine produced by many soil bacteria, such as Erwinia, Nocardia, Streptomyces, Arthrobacter, Chromobacterium and Pseudomonas species (Berner et al., 1988; Gunter et al., 1993; Meyer and Abdallah, 1980; Muller and Raymond, 1984; Wei et al., 2007). While, most of the ferrichrome family produced by soil fungal species (i.e. Suillus granulatus, Fusarium spp and Aspergillus spp.), which is further divided into five groups depending on the side chain of the hydroxamate functional group: acetyl (ferrichrome, ferrichrome C, ferricrocin and ferrichrysin), malonyl (malonichrome), trans-b-methylglutaconyl (ferrichrome A), trans-anhydromevalonyl (ferrirubin) and cis-anhydromevalonyl (ferrirhodin) (Winkelmann and Huschka 1987; Renshaw et al., 2002).

Due to the importance of microbial siderophores in weathering and soil formation, the role of siderophores in the dissolution of iron minerals has been investigated intensively (Inoue et al., 1993; Watteau and Berthelin, 1994; Hersman et al., 1995; Hiradate and Inoue, 1998; Holmén and Casey, 1996, 1998; Kraemer et al., 1999; Liermann et al., 2000a; Kalinowski et al., 2000a; Stone, 1997; Reichard et al. 2005; Buss et al., 2007; Shirvani and Nourbakhsh, 2010). Hydroxamate siderophores produced by the ectomycorrhizal fungus Suillus granulatus have a high efficiency in the dissolution of goethite, where significant quantities (10^{-9} \text{ mol m}^{-2} \text{ h}^{-1}) of iron were mobilized in the presence of Suillus sp. because of their continuous production of siderophores (Watteau and Berthelin, 1994). Mineral dissolution is enhanced not only by siderophore-producing fungi but also by bacteria such as Bacillus sp., which have been documented to produce siderophores that promote the dissolution of the surface of hornblende (Buss et al., 2007). In addition, the dissolution of Fe from the hornblende that has been observed in the presence of siderophore-producing actinomycetes such as Streptomyces and Arthrobacter was higher.
than the dissolution of Fe by the synthetic desferrioxamine B siderophore (Kalinowski et al., 2000b). Therefore, the interactions between siderophores and iron minerals are directly related to the iron acquisition efficiency of living cells in the soil environment (Shirvani and Nourbakhsh, 2010). For example, fungal siderophores, such as dissolved ferrichrome and ferricrocin, have been found to play a significant role in changing the surface structure of biotite and increasing its dissolution in podzolic forest soil (Sokolova et al., 2010).

Few studies have discussed the concentrations of siderophore in podzolic soil solution (Powell et al., 1980, 1982; Buyer et al., 1993; Holmström et al., 2004; Essén et al., 2006; Ali et al., 2011) so many gaps still remain in understanding the relation between siderophore content and mineral weathering in the field. Due to the wide variation of the chemical properties (e.g. pH and mineral nutrients, etc.) and microbial composition of each horizon in the podzol soil, the present study aimed to answer several questions; how do the podzol soil horizon characteristics affect the concentration and diversity of hydroxamates? Could the presence of different mineral types change the concentration and diversity of hydroxamates? In which phase, dissolved or adsorbed, can siderophores be found in soil?

2. Materials and methods

2.1 Sampling site

Soil was sampled in September 2011 at a site (63°07′N, 16°70′E) in central Sweden in the vicinity of the village Bispgården. The site is located in a slope (angle 2°) at an altitude of 258 m above sea level and was forested with 80-yr-old Norway spruce (Picea abies) and Scots pine (Pinus sylvestris). The annual average precipitation is 700 mm, which is not acidic, and the annual average temperature is +2 °C. The bedrock in the area is granite and gneiss. The soil is a typical haplic podzol (FAO, 1990) and the soil
horizons in the studied soil profile have the following thickness: 28 cm for O (organic horizon), 9 cm for E (elluvial horizon) and 7 cm for the B (upper illuvial horizon). Three different polished minerals; apatite, oligoclase and biotite (3X4 cm) were inserted in O-, E- and B-horizon two years earlier, June 2009 (Olofsson et al., in preparation). The soil samples for this study were taken from the bulk soil of the whole profile and mineral surfaces (Figure 1) and kept cold (+4 °C) until further analysis. The chemical characterization of the soil samples of each horizon have been performed as shown in (Table 1).

2.2 Extraction of dissolved and adsorbed siderophores from soil

For extraction of dissolved siderophores, 1 g of air dried soil sample was added to 10 ml of Milli Q-water and shacked vigorously for 2 hours. The soil solutions were thereafter filtrated through 0.45 μm. While for extraction of adsorbed siderophores, 1 g of air dried soil sample was added to 10 ml of methanol and shacked for 2 hours. The soil solutions were then filtrated through 0.45 μm. The methanol filtrates were evaporated using rotary evaporation (Laborota 4001-efficient, Heidolph instruments), then the remaining residues were dissolved in Milli-Q water. The water extracts were pre-concentrated by freeze-drying (Scanvac cool Safe, 100-9 Pro). When all water in the sample had been evaporated a yellow-white, solid dust remained that was dissolved in 1 ml of Milli Q-water. To remove high molecular mass compounds (>3000 Da) centrifugal ultrafiltration using 3000 Da cutoff size filter devices were applied to all pre-concentrated extracts (methanol and water) (Nanosep 3K Omega, Pall, Mexico) and thereafter stored at -20°C until further analysis. The pre-concentration and purification method developed by Holmström et al., (2004).
2.3 Quantification and structure identification of the siderophores using HPLC-ESI-MS

Analysis of the extracted siderophores was performed using a method developed by Holmström and Kalinowski. (Manuscript, 2013) that is a modification of the method used by Duckworth et al. (2009). The HPLC system (Ultimate 3000 RS, Thermo Scientific, USA) was built up of two pumps with flow rate of 0.030 ml/min for the low pressure gradient pump and 0.15 ml/min of high pressure gradient pump and a column compartment (Dionex Ultimate 3000, Thermo Scientific, USA) set at 10°C. The injection volume of standards and samples were 100 µl. The pre-column was a Syncronis C18 (50 mm x 2.1 mm, particle size 1.7 µm, Thermo Scientific, USA) while the separation column was a Hypersil GOLD (100 mm x 2.1 mm, particle size 1.9 µm, Thermo Scientific, USA). The pre-column was eluted to the waste with mobile phase A (11 mM ammonium formate buffer, pH 4.0 and 1% v/v methanol) for on-line concentration and purification of the hydroxamate siderophores and then after 10 min followed by back flushing the pre-column towards the analytical column with a gradient of mobile phase B (11 mM ammonium formate buffer, pH 4.0 and 15% v/v acetonitrile) and mobile phase C (11 mM ammonium formate buffer, pH 4.0 and 5% v/v acetonitrile). The total analysis time was 60 min. The ferric complexes of the tri-hydroxamate siderophores, including ferrichromes, ferrioxamines, coprogenes and fusigen (Figure 2), were detected by selected ion monitoring (SIM) of the proton adducts [M + H]⁺, i.e. m/z 797.3 for Tetraglycyl Ferrichrome, 771.3 for Ferricrocin, 741.2 for Ferrichrome, 801.2 for Ferrichrysin, 1011.3 for ferrirubin, 1011.2 for Ferrirhodin, 1052.2 for Ferrichrome A, 614.2 for Ferrioxamine B, 672.2 for Ferrioxamine G, 656.3 for Ferrioxamine D, 654.3 for Ferrioxamine E, 682.5 for Neocoprogen II, 793.2 for Fusigen (lin.), 752.3 for Neooprogen I, and 821.2 for Coprogen on a triple quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Scientific, US).
2.4 Data analysis and statistics

The data were normalized and the principal component analysis (PCA) and one/two-way ANOVA were performed by using XLSTAT (http://www.xlstat.com/en/). The PCA was investigated for different parameters i.e., dissolved and adsorbed siderophore content in bulk soil with different soil horizons or/and mineral surfaces. The sum of Tetracycl Ferrichrome, Ferricrocin, Ferrichrome, Ferrichrysin, ferrirubin, Ferrirhodin and Ferrichrome A were calculated and was denoted as the total concentration of ferrichrome siderophores; the sum of Ferrioxamine B, Ferrioxamine G, Ferrioxamine D and Ferrioxamine E correspond to the total concentration of ferrioxamines; the sum of Neocoprogen II, Neooprogen I and Coprogen were denoted as the total concentration of coprogens and fusigen (lin.) represent the fusigen. We also calculated the sum of all the fifteen different types of siderophore that were analyzed in the present study as the total concentration of hydroxamate siderophores.

3. Results

3.1 Siderophore concentration and diversity in podzol soil

Dissolved and adsorbed ferrioxamines, ferrichromes, fusigen and coprogens concentration of podzolic soil samples were measured by HPLC-ESI-MS. The concentration of total dissolved ferrioxamines ranged between 2-7 pmol/g dry soil; 1-4 pmol/g dry soil for ferrichromes; 0-39 pmol/g dry soil for fusigen; 0-14 pmol/g dry soil for coprogens (Figure 4). Maximum concentrations of dissolved ferrioxamines, fusigen and coprogens were all found in the E-horizon, while the maximum concentration of the ferrichromes was found in the B-horizon, and there was a significant difference between the concentrations within the soil horizons (Table 2). When we calculated the total dissolved hydroxamates, we found that it ranged between 10-63 pmol/g dry soil and that the maximum concentration was found in the E-horizon (Figure 3). We performed principal component analysis (PCA) analysis of the samples based on their composition of dissolved hydroxamate siderophore types. The
first principal component correlated with soil horizon (PC1, eigenvalue 14%) and the second principal component correlated with hydroxamate types (PC2, eigenvalue 59%). As shown in the PCA biplot the soil horizons have a strong influence on the dissolved hydroxamates diversity where each soil horizon formed groups in the corners of the graph (Figure 5). In addition, all the individual ferrioxamines correlated to the E-horizon, while most of ferrichromes and coprogens correlated to the O-horizon except for tetracycl ferrichrome and fusigen that correlated to C-horizon and ferrirhodin to the E-horizon.

Adsorbed hydroxamates were present in lower concentration than the dissolved ones. The adsorbed total ferrioxamines ranged between 0-2 pmol/g dry soil; and the total ferrichromes varied between 0.3 to 2 pmol/g dry soil; 0-32 pmol/g dry soil for fusigen and 0-13 pmol/g dry soil for coprogens (Figure 7). Adsorbed hydroxamate siderophores were present in all investigated soil horizons, except for ferrioxamines that were completely absent in the C-horizon. The maximum concentration of adsorbed ferrioxamines, ferrichromes, fusigen and coprogens concentration were found in the E-horizon. When we calculated the total adsorbed concentration of hydroxamates for each soil horizon, we found that it ranged between 8-51 pmol/g dry soil and that the maximum total concentration was found in the E-horizon (Figure 6) as for the individual groups of hydroxamate siderophores. The PCA ordination was based on their composition of adsorbed hydroxamate siderophore types. The first principal component correlated with soil horizon (PC1, eigenvalue 11%) and the second principal component correlated with hydroxamate types (PC2, eigenvalue 70%). Based on the PCA, it was found that there is an even higher correlation between the diversity of adsorbed hydroxamates within each soil horizon (Figure 8) than for the dissolved ones (Figure 5). Therefore, most of the ferrichromes and all the ferrioxamines correlated to
the E-horizon. However, ferrichrome, fusigen and coprogens correlated to the O-horizon except tetracycl ferrichrome and ferrihodin that correlated to the B- and C-horizon, respectively.

3.2 Siderophore concentration and diversity on buried polished mineral surfaces

Dissolved and adsorbed hydroxamates were also extracted and quantified from the soil attached to the surfaces of the three different mineral types that had been buried for two years in the different horizons of a podzol soil. The diversity of hydroxamates was completely different compared to the bulk soil, and in addition the concentrations were much higher. We found that the total dissolved hydroxamates ranged between 20-83 pmol/g dry soil for apatite and the maximum concentration was found in the B-horizon; 42-107 pmol/g dry soil for biotite and the maximum concentration was found in the O-horizon; while 18-36 pmol/g dw soil for oligoclase and the maximum concentration was also found in the O-horizon as for biotite (Figure 9). As shown in Figure 10, fusigen was found to have the highest concentration for all investigated mineral types, where the concentration ranged between 6-60 pmol/g dry soil. The maximum concentration of fusigen was found on the biotite surfaces from the O-horizon (60 pmol/g dry soil), followed the B-horizon (43 pmol/g dry soil). Although the ferrichromes and ferrioxamines were found in low concentrations on all mineral types and no significant difference were found between their concentrations within the soil horizons (Table 2), ferrichromes showed a high concentration on the biotite surface in O-horizon 66 pmol/g dry soil that was higher than fusigen. The PCA ordination for these samples was based on all dissolved hydroxamate siderophore types along the first principal component (PC1, eigenvalue 53%), and the different mineral types within each podzol soil horizons along the second principal component (PC2, eigenvalue 17%). Figure 11 showed that the mineral type influenced the diversity of dissolved hydroxamates more than the type of soil horizon, which is quite different than the siderophores in the bulk soil that were affected by the soil horizon type. In addition,
ferrioxamine E and B correlated to the presence of the biotite and apatite in the E-horizon, while ferrioxamine G and D, ferrichrome and ferrirhodin correlated to apatite in O- and B-horizons. Tetracycl ferrichrome correlated to oligoclase in all the horizons and the rest of hydroxamates correlated to the presence of biotite in the O- and B-horizons.

We also found that the dissolved hydoxamates were higher than adsorbed ones in the soil from the mineral surfaces, which is the same behavior as in the bulk soil. The total adsorbed hydroxamates ranged between 35-64 pmol/g dry soil for apatite; 6-55 pmol/g dry soil for biotite; while 0.009-21 pmol/g dry soil for oligoclase, and the maximum concentration was found in E-horizon for all of the minerals (Figure 12). Fusigen and coprogens showed higher concentrations than ferrichromes and ferrioxamines for all mineral surfaces (Figure 13). Fusigen and coprogens were only absent on oligoclase in the O-horizon. The PCA ordination was based on all adsorbed hydroxamate siderophore types along the first principal component (PC1, eigenvalue 64%), and different mineral types within each podzol soil horizons along the second principal component (PC2, eigenvalue 17%). Based on PCA, it was found that not all the minerals influence the diversity of adsorbed hydroxamates, only apatite and biotite, and it was obvious that the E-horizon contained almost all the highest concentration of adsorbed hydroxamates (Figure 14). Therefore, tetracycl ferrichrome, ferrioxamine G and B, ferrichrome A, coprogen, neocoprogen II and fusigen correlated to the presence of apatite in the E-horizon, while the rest of the hydroxamates correlated to biotite in the same horizon. No correlation was found between the adsorbed hydroxamates and oligoclase.
4. Discussion

4.1 Siderophores in the soil environment

The presence of siderophores in soil have earlier been estimated by using microbial assays that revealed only the total concentration of hydroxamates, as well as ferrichrome-type siderophores. Powell et al. (1982) and (1983) were the first to use these assays to quantitate the siderophores from dried sandy clay loam soil-water extracts. Powell et al. (1982) found that the total hydroxamate concentrations were relatively high in the 27–279 nM range, reported as DFOB equivalents, while Powell et al. (1983) estimated 34 nM of total hydroxamate siderophores by using the *M. flavescens* assay and 78 nM of ferrichrome type by the *E. coli* assay. These studies which used microbial assays estimated much higher concentrations of the total dissolved hydroxamates than our present findings where the total concentration of trihydroxamate siderophores ranged between 10 and 63 nM using HPLC-MS. Even though we detected higher concentrations than other studies that have found to hydroxamates in the range between 0.09-0.75 nM of soil (Akers 1983) and between 0.2-0.5 nM of soil (Buyer et al. 1993).

Dissolved individual hydroxamate siderophores concentration in Swedish podzolic forest soils have been measured previously in a couple of studies (i.e. Holmström et al., 2004; Essén et al., 2006; Ali et al., 2011) by using HPLC-MS, in which much lower concentrations were estimated compared to microbial assays. Hydroxamates has been detected to be between 0.9-1.4 nM/g in soil solution identified as ferrichrome and ferricrocin in the O-horizon of podzol soil at Pottång, Sundsvall (Holmström et al., 2004) that is more in line to our findings. We found 0.06-1.1 nM/g soil of ferricrocin and 0-1.2 nM/g dry soil of ferrichrome in all podzol horizons, while Essén et al. (2006) detected higher concentrations 0.1–12 nM/g of ferricrocin and 0.1-2.1 nM/g of ferrichrome in the podzol horizons at four different locations in north and south of Sweden. Essén et al. (2006) findings agreed with our results that the lower concentrations of hydroxamates found in the lower horizons like the B- and C-horizons. The differences in hydroxamates concentration between these previous studies and our findings may be due to; 1) the
different geographical location where the samples were collected within Sweden, 2) the different analytical methods used in the HPLC-MS measurement, 3) the different methods used to obtain soil solution, i.e. previous studies are based on soil solution obtained by a centrifugation drainage technique (Giesler and Lundström, 1993) and in this study we used different soil/liquid extraction methods to acquire the siderophores. Recently, Ali et al. (2011) extracted the siderophores by two different methods using a phosphate buffer or a buffer-methanol mixture to extract siderophores from podzol soil samples of a location near to our sampling site at Bispgården. They found a little amount of ferricrocin while ferrichrome wasn’t detected constantly. The maximum concentration of ferricrocin was 3.74 nmol/kg soil obtained by the buffer extraction (dissolved siderophores) was found in the O-horizon, in contrast we detected the highest concentration of ferricrocin obtained by water extraction (dissolved siderophores) was 1.1 nmol/kg dry soil (recalculated from pmol/g) in the same horizon. The higher ferricrocin concentration that was detected by Ali et al. (2011) may be due to the different extraction methods used in these two studies.

It was earlier suggested that most of the hydroxamate siderophores in soils are ferrichromes especially ferrichrome and ferricrocin, which were widespread in most such environments (i.e. Moberg et al., 2003; Holmström et al., 2004; Essén et al., 2006; Winkelmann, 2007; Ali et al., 2011), but our findings in this present study detect a wide range of other fungal trihydroxamates (coprogens and fusigen) and bacterial ones (ferrioxamines) in the podzol soil. Thus, we suggest that however ferrichromes and ferrioxamines are more commonly to be found in the soil environment in low concentrations, in comparison to coprogens and fusigen that occur in higher concentrations in such environments. For instance, we detected Neooprogen II between 1.1-3.5 pmol/g dry soil; Neooprogen I 0-14.4 pmol/g dry soil and fusigen 0-38.8 pmol/g dry soil. These variations of hydroxamates that we found in the soil horizons indicate that there is a high diversity of forest soil microorganisms that
produce a wide range of siderophores. For example, ferrioxamines can be produced by *Streptomyces* spp. (Das et al., 2007); ferrichromes by *Aspergillus* spp. (Charlang et al., 1981), *Suillus variegates* (Wallander and Wickman, 1999) and *Microsporum* spp. (Bentley et al., 1986); coprogens by *Fusarium dimerum* (Van der Helm and Winkelmann, 1994) and *Epicoccum purpurescens* (Frederick et al., 1981) and fusigen by *Fusarium* spp. (Van der Helm and Winkelmann, 1994) and *Histoplasma capsulatum* (Burt, 1982). Thus, according to our findings that coprogens and fusigen had the maximum concentrations in the soil horizons, it could indicate that microorganisms like *Fusarium* spp., *Epicoccum purpurascens* and *Histoplasma capsulatum* are the most popular species in that soil.

### 4.2 Dissolved and adsorbed phases of hydroxamates

As previously discussed, most of the hydroxamates were detected in the dissolved phase of the soil solution extract. In the present study, we found not only dissolved hydroxamates but also adsorbed ones that ranged between 0-2 pmol/g dry soil for ferrioxamines, 0.3-2 pmol/g dry soil for ferrichromes; 0-32 pmol/g dry soil for fusigen; and 0-13 pmol/g dry soil for coprogens. The presence of adsorbed hydroxamates could be depending on physico-chemical properties of their functional group. Powell et al. (1980) presented earlier that some reservoirs of siderophores are adsorbed to soil organic matter. In addition, Haselwandter et al. (2011) reported that the siderophores could be dissolved or adsorbed regards to their susceptibility to degradation. We found that each specific hydroxamate family has a different behavior regards to the adsorption. The ferrichromes and ferrioxamines were found in a higher concentration in the dissolved phase than in the adsorbed phase. In comparison, the coprogens behaved in the opposite way, where they were more abundant in the adsorbed phase. These findings could be related to the different chemical structure of the specific hydroxamate functional group. The fusigens, ferrichromes, ferrioxamines and coprogens differ with regard to the characteristic bonds within the molecule that could form strong or weak bonds with the soil particles, i.e. three ester bonds in fusigens,
six peptide bonds in ferrichromes, five peptide bonds in ferrioxamines, and one ester plus two peptide bonds in the coprogens (Winkelmann, 2007). The different behavior of the investigated hydroxamate families could also be explained by their wide range of lipophilicity, in addition to the different electrical charge of the respectively siderophores, which will affect the various mobility of the siderophores in the soil profile (Winkelmann, 2007). The main factor affecting the siderophores adsorption capacity is the effect of soil solution pH on the siderophore charge, rather than the effect of the charge of the mineral and soil particles. The adsorption of some hydroxamates like DFO-B can stay over a wide pH range (4.0–7.5) in accordance with their stable positive charge. However at higher pH values the adsorption decreased following a decline in its positive charge (Kraemer, 2004; Siebner-Freibach et al., 2004). That may explain the quite high stable concentration of adsorbed hydroxamates which we found in the soil horizons where the soil pH ranged between 4.4 and 5.4. Thus, the hydroxamates still have their stable positive charge in that pH range. There were also some differences in the individual types of hydroxamates regards to their adsorption, i.e. FO-B had lower adsorption compared to FO-D. That agrees with what Kraemer et al. (1999) and Kraemer (2004) found about that the electrostatic repulsion between the hydroxamate and the positively charged mineral surface is resulting in lower adsorption of FO-B compared to FO-D at pH < 8. FO-B is a cationic species at pH < 8 due to protonation of the terminal amine group (pKᵦ₁ = 8.38), while FO-D is an acetyl derivative of FO-B which has no charge below pH 8.9. It has been suggested that the adsorption of hydroxamates in soil ecosystem are strongly influenced by their interactions with the solid phase like clay minerals that comprise a major part of the surface area in soils (Siebner-Freibach et al., 2004). On the other hand, some studies reports that hydroxamate siderophores are more commonly found in the dissolved phase in the soil since they consist of strong cyclic hexapeptides that make them highly resistant to the environmental degradation by some enzymes produced by plants (i.e., hydrolases and proteases), which affect the life time of the siderophores (e.g. Hider and Kong, 2010). In addition, some hydroxamates are
also resistant to the utilization of soil microorganisms and others not. For instance, pseudomonads isolated from soil is capable of degrading ferrichrome A and coprogen (Warren and Neilands 1964) and that may explain the very low concentration of ferrichrome A and the high content of adsorbed coprogen that were detected in our soil samples.

4.3 The behavior of siderophores in the presence of different minerals in the soil

In the other part of our study, we found that the concentration of hydroxamates in the soil attached to the polished mineral surfaces was higher than in the bulk soil with approximately 66%. As for the bulk soil, we found that the dissolved phase was greater than the adsorbed one for all investigated mineral types, even though the total dissolved and adsorbed hydroxamates showed a great variation between the different minerals. For example, the total dissolved hydroxamates found on apatite, biotite, oligioclase ranged between 20-83; 42-107 and 18-36 pmol/g dry soil, respectively, whereas the total adsorbed ones ranged between 35-64 pmol/g dry soil for apatite; 6-55 pmol/g dry soil for biotite and 0.009-21 pmol/g dry soil for oligioclase. These findings could be depending on the interaction between the siderophores and the specific mineral surfaces. For instance, when reacting trihydroxamate siderophores such as ferrioxamines with an iron containing minerals, only one Fe(III) center can be coordinated at a time (Cocozza et al., 2002) and the mineral dissolution reaction rates are controlled by the nature of the Fe(III)-siderophore complexes (Furrer and Stumm, 1986). Each polished mineral in our experiment also had a unique interaction with the dissolved and adsorbed hydroxamates in the different soil horizons. For instance, biotite had the maximum dissolved hydroxamates concentration in the O-horizon and thereafter decreased with the depth of the soil, whereas oligioclase had the maximum concentration in O-horizon followed by the B-horizon, while the concentration from the apatite surface increased gradually with the depth of the soil until it reached maximum in the B-horizon. On the other hand, the maximum adsorbed hydroxamates were found on the surface for all of the three different minerals in the
E-horizon compared to the O-horizon for the dissolved hydroxamate siderophores. The binding of strong complex formers like siderophores with cations on the minerals reduces the stability of the mineral surface structure and thereby enhancing the weathering process (Ehrlich, 1998) and thereby provide a highly efficient Fe acquisition system due to its high affinity and specificity for Fe(III) complexation (Kraemer, 2004). There are a number of factors that influence the conformation and bonding of attached siderophores on mineral surfaces, i.e. siderophore architecture, charge, and hydrophobicity (Kraemer, 2004). There is also a limitation to the number of bonds that can form between the siderophore and a single Fe(III) ion in the inner coordination sphere at the mineral surface, that is why each mineral in our experiment had a unique behavior regards to each hydroxamate siderophore type as we mentioned before. Our findings regards to the interaction between the siderophore and the three different minerals can be considered as a new knowledge of how siderophores concentration and type can differ according to the presence of different minerals during the weathering processes at natural conditions.

4.4 The diversity of siderophores regards to soil horizons

The PCA results verified that the soil horizons and mineral types strongly influenced the distribution of adsorbed and dissolved hydroxamates. That is in agreement with Bossier et al. (1988) and Nelson et al. (1988), who have suggested that the concentration of siderophores in soil depend strongly on the chemical, physical and biological properties of respectively soil horizon. Possible explanations of the affection of soil horizons and mineral types on hydroxamates depend on three main reasons; (1) chemical and mineralogical properties of podzol soils change with depth, which creates a number of different habitats for microorganisms throughout the soil profile (Fierer et al., 2003; LaMontagne et al., 2003; Rosling et al., 2003), in which the highest siderophore metabolic diversity were found in the O- and E-horizon comparing with B- and C-horizon, (2) the hydroxamate concentrations were also highly
correlated with the soil organic matter content, where the low clay soils yielded almost twice as much hydroxamates as did high clay soils suggesting that adsorption might be an important determinant of their concentration in bulk soil solution (Powell et al., 1982) and that can explain the high adsorbed hydroxamates detected in O- and E-horizon regarding to the high organic matter found in those horizons, (3) however, the pH of the soil solution in our study were about 4-5.5, and at these pH conditions are the major part of iron soluble and found as Fe(III), so why would the microorganism have to produce siderophores when sufficient amounts of iron is already available? The answer could be that the microorganisms that produce hydroxamate siderophores at these pH values have a big advantage over other non-siderophore producing microorganisms, due to the extreme acid stability of the siderophore molecules and are thus able to scavenge the needed iron from competing microorganisms and also protect themselves from the overdose Fe stress. In these pH ranges, the structures of natural tri-hydroxamate siderophores allow the formation of intra molecular hydrogen bonds. This bond is formed between the coordinating oxygen and the amide hydrogen enhances the stability of hydroxamate siderophores, which make them to be persistent (Matsumoto et al., 2001; Wittenwiler, 2007).

5. Conclusion

A wide range of fungal hydroxamates (ferrichromes, coprogen and fusigen) and bacterial ones (i.e. ferrioxamines) were detected in a podzolic soil and these new results may change our knowledge of that the most of the hydroxamate siderophores in soils are ferrichromes as indicated and suggested in earlier studies. For the first time have coprogens and fusigen siderophores been detected in podzolic soil, and they were found in higher concentrations than ferrichromes and ferrioxamines even if they are the most common ones throughout the soil profile. Each of the polished biotite, apatite, and oligioclase minerals had a unique interaction with the dissolved and adsorbed hydroxamates in the different soil horizons and that makes the mineral type as one of the factors affecting the siderophores concentration and diversity.
in the natural environment. The concentration of hydroxamates in the soil attached to the polished mineral surfaces was higher than in the surrounding bulk soil and this indicate that the microenvironment attached to the mineral surfaces more active in producing siderophores than the microorganisms in the bulk soil. The soil depth variability in concentration and composition of hydroxamate types in the bulk soil, in addition to the abundance and distribution of ferrichromes, ferrioxamines, fusigen and copregens on the different polished mineral surfaces in each horizon strongly suggest that our field experiment succeed to describe how much the behavior of siderophores can differ regards to the mineral type, and the soil horizon characteristics. The next step is to get a greater insight into the siderophore mineral interactions in soils by investigating the microbial diversity in the bulk soil and soil attached to mineral surfaces that could also have a major effect on the siderophore distribution.

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References


Figure captions

Figure 1. Field experiment set up and soil sampling; the minerals (A) apatite, (B) biotite and (O) oligoclase were inserted in replica within the soil horizons and (BS) bulk soil samples were also taken in replica.

Figure 2. Chemical structure of the analyzed hydroxamate siderophores.

Figure 3. Total dissolved hydroxamate concentration per each soil horizon.

Figure 4. Dissolved hydroxamate groups (ferrioxamines, ferriochromes, fusigen and coprogens) concentration per each soil horizon.

Figure 5. Principal component analysis (PCA) ordination of dissolved hydroxamates and soil horizons with total contribution of observation 73%. O, E and B referred to the soil horizons.

Figure 6. Total adsorbed hydroxamate concentration per each soil horizon.

Figure 7. Adsorbed hydroxamate siderophores (ferrioxamines, ferriochromes, fusigen and coprogens) concentration per each soil horizon.

Figure 8. Principal component analysis (PCA) ordination of adsorbed hydroxamates and soil horizons with total contribution of observation 82%. O, E and B referred to the soil horizons.

Figure 9. Total dissolved hydroxamate concentration of soil attached on mineral surfaces per each soil horizon.

Figure 10. Dissolved hydroxamate groups (ferrioxamines, ferriochromes, fusigen and coprogens) concentration of soil attached on mineral surfaces per each soil horizon.
Figure 11. Principal component analysis (PCA) ordination of dissolved hydroxamates and mineral type per each soil horizon with total contribution of observation 69%. O, E and B referred to the soil horizons. Ap, Bio and Olig referred to apatite, biotite and oligoclase.

Figure 12. Total adsorbed hydroxamate concentration of soil attached on mineral surfaces per each soil horizon.

Figure 13. Adsorbed hydroxamate groups (ferrioxamines, ferriochromes, fusigen and coprogens) concentration of soil attached on mineral surfaces per each soil horizon.

Figure 14. Principal component analysis (PCA) ordination of adsorbed hydroxamates and mineral type per each soil horizon with total contribution of observation 81%. O, E and B referred to the soil horizons. Ap, Bio and Olig referred to apatite, biotite and oligoclase.
Ferrioxamine B ($K_{Fe(III)}=30.5$)

Ferrioxamine E ($K_{Fe(III)}=32.4$)

Ferrioxamine G ($K_{Fe(III)}=30.8$)

Ferrioxamine D ($K_{Fe(III)}=30.6$)

Coprogen ($K_{Fe(III)}=30.2$)

Fusigen ($K_{Fe(III)}=31.2$)
Ferrichrome ($K_{Fe(III)}=29.1$)

Ferrichrome A ($K_{Fe(III)}=32.0$)

Ferrichrysin ($K_{Fe(III)}=30.0$)

Ferricrocin ($K_{Fe(III)}=30.4$)

Ferrirhodin ($K_{Fe(III)}=30.0$)

Ferrirubin ($K_{Fe(III)}=30.1$)

Tetraglycylferrichrome ($K_{Fe(III)}=30.2$)

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

Total hydroxamates (pmol/g dry soil)

Soil horizons

O

E

B

C
Figure 7
Figure 8
Figure 9
Figure 10
Biplot (axes F1 and F2: 68.84 %)

Figure 11
Figure 12
Figure 13
Figure 14
Table 1. Chemical characterization of soil samples of each horizon

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<th>C&lt;sup&gt;a&lt;/sup&gt; %</th>
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<th>K&lt;sup&gt;a&lt;/sup&gt; μmol/g</th>
<th>Mg&lt;sup&gt;a&lt;/sup&gt; μmol/g</th>
<th>Na&lt;sup&gt;a&lt;/sup&gt; μmol/g</th>
<th>Fe&lt;sup&gt;*(total)&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt; μmol/g</th>
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<td>E</td>
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*Exchangeable cations

<sup>a</sup> Vestin et al., 2008,  <sup>b</sup> The present study
Table 2. *P*-values derived from ANOVA for dissolved and adsorbed hydroxamtes in both bulk soil and mineral surfaces that vary with soil horizons and hydroxamate types.

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<td>Ferrichromes</td>
<td>Fusigen</td>
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<td>and coprogens”</td>
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Manuscript II
Title: Siderophores in Environmental Research: Roles and Applications

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Summary

Siderophores are organic compounds with low molecular masses that are produced by microorganisms and plants growing under conditions of low iron. The primary function of these compounds is to chelate the ferric iron from different terrestrial and aquatic habitats and thereby make it available for microbial and plant cells. Siderophores have received much attention in recent years because of their potential roles and applications in various areas of environmental research. Their significance in these applications is because siderophores have the ability to bind a variety of metals in addition to iron, and they have a wide range of chemical structures and specific properties. For instance, siderophores function as biocontrols, biosensors, and bioremediation and chelation agents, in addition to their important role in weathering soil minerals and enhancing plant growth. The aim of this literature review is to outline and discuss the important roles and functions of siderophores in different environmental habitats and emphasize the significant roles that these small organic molecules could play in applied environmental processes.

Key words: Bioweathering, Bioremediation, Biocontrol, Biosensor, Siderophores
1. Introduction

Iron is an essential element for the growth of almost all living microorganisms because it acts as a catalyst in enzymatic processes, including oxygen metabolism, electron transfer, and DNA and RNA synthesis (Aguado-Santacruz et al., 2012). It is the fourth most common element in Earth’s crust and often appears in primary minerals such as biotite, as well as secondary minerals such as goethite, hematite and ferrihydrite. Siderophores are chelating agents with low molecular masses (200 to 2000 Da) that are produced by microorganisms and plants, especially under iron-limiting conditions (Schwyn and Neilands, 1987). Marine organisms such as phytoplankton (Trick et al., 1983) and cyanobacteria (Armstrong and van Baalen, 1979) can also produce siderophores. The role of siderophores is primarily to scavenge iron, but they also take up other elements (i.e., Mo, Mn, Co and Ni) from the environment and make them available for microbial cells (Bellenger et al., 2008; Braud et al. 2009a; Braud et al. 2009b). Siderophores have three main functional groups, hydroxamate, catecholate and carboxylate, which form very strong complexes with iron. Hydroxamate siderophores have a 1:1 stability constant with iron that nears that of the Fe(III)-EDTA complex (10^{30}), whereas catecholates and carboxylates can form 1:1 complexes with stability constants near that of Fe(III)-EDDHA (10^{40}) (Robert and Chenu, 1992). More than 500 different types of siderophores are known, of which 270 have been structurally characterized (Boukhalfa et al., 2002). The formation of Fe(III)-siderophore complexes are affected by pH due to the competition for the free siderophore ligands between free protons and iron (Albrecht-Gary and Crumbliss, 1998). For instance, hydroxamates are resistant to hydrolysis above pH 1 and are not quickly degraded in a natural soil environment (Winkelmann, 2007), and catecholates are more stable at lower pH than hydroxamates (Budzikiewicz, 2004). In nature, iron has to compete not only against free protons for the siderophore binding sites but also against other metal ions.
such as divalent cations, including Cd\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Pb\(^{2+}\), and Zn\(^{2+}\) (Albrecht-Gary and Crumbliss, 1998); trivalent cations, including Mn\(^{3+}\), Co\(^{3+}\), and Al\(^{3+}\); and actinides, including Th\(^{4+}\), U\(^{4+}\) and Pu\(^{4+}\) (Peterson et al., 2004). There are several studies that have shown that siderophores have an impact on the mobility of these metal ions in the environment (e.g., John et al., 2001; Dahlheimer et al., 2007). Nature seems to employ siderophores not only in plant and microorganism nutrition and in the biological control of pathogens but also in other environmental applications. In this review, microbial and plant siderophores will be investigated with a primary focus on the roles, functions and applications of siderophores in different areas of environmental research.

2. **Microbial Siderophores**

In conditions of low iron, siderophores play an important role in the extracellular solubilization of iron from minerals, which make it available to microorganisms (Neilands, 1995; Winkelmann, 2007). Most of the bacterial siderophores are catecholates, and some of them are carboxylates, whereas most of fungal siderophores are hydroxamates (Schalk et al., 2011). Siderophores with mixed functional groups, such as azotobactin and yersiniabactin, are produced by *Azotobacter vinelandii* and *Yersinia pestis*, respectively (Haag et al., 1993). One of the most widespread bacteria in the environment, *Pseudomonas*, produce mainly over 50 types of pyoverdine siderophores (Figure 1), which contain both hydroxamate and catecholate groups (Boukhalfa and Crumbliss, 2002). In addition to pyoverdines, *Pseudomonas* can produce a variety of other siderophore types, such as pyochelin, salicylic acid, cepabactin, corrugatin, ferribactin, ferricrocins, ornibactin, pyridine-2-6-di-monothyoarboxylic acid, quinolobactin (Cornelis, 2010). Most of the fungal siderophores belong to the ferrichrome family, which is further divided
into five groups depending on the side chain of the hydroxamate functional group: acetyl (ferrichrome (Figure 1), ferrichrome C, ferricrocin and ferrichrysin), malonyl (malonichrome), trans-b-methylglutaconyl (ferrichrome A), trans-anhydromevalonyl (ferrirubin) and cis-anhydromevalonyl (ferrirhodin) (Winkelmann and Huschka 1987; Renshaw et al., 2002).

Microorganisms use different siderophore-mediated iron transport systems. There are two general mechanisms for siderophore transport in bacteria. The first mechanism depends on the interaction between the transporters and the Fe(III)-siderophore complex. Fe$^{3+}$ is separated from the siderophore and reduced to Fe$^{2+}$ by a cytoplasmic reductase enzyme, which makes it available to the microbial cell (Crowley et al., 1991). The second mechanism depends on bonding between the Fe(III)-siderophore complex and the cell surface receptor. Fe$^{3+}$ is thereafter separated and reduced to Fe$^{2+}$ or transported directly as Fe$^{3+}$ by the carrier (Dhungana and Crumbliss, 2001). Siderophores are either recycled or destroyed upon iron reduction, whereas the reduced iron (Fe(II)) that is not used by the cell can act as an electron donor in electron transport chains (Kalinowski et al., 2000b).

Gram-negative bacteria have outer membrane receptors like (TonB) at the cell surface that recognize Fe(III)-siderophore complexes, which are actively transported across the cell membrane through an energy-dependent system consisting of the outer membrane siderophore receptor proteins, periplasmic binding proteins and inner membrane transport proteins (see more details in Krewulak and Vogel, 2008; Noinaj et al., 2010). On the other hand, in both gram-positive and gram-negative bacteria, Fe(III)-siderophore complexes are also recognized by another membrane receptor proteins in which the complexes are transported into the cytoplasm by ABC-type transport proteins (Koster W. 2005; Braun and Hantke, 2011).
Fungi have four different mechanisms for siderophore-mediated Fe(III) uptake (van der Helm and Winkelmann, 1994); 1) In the shuttle mechanism, the Fe(III)-siderophore complex is transported across the cell membrane, where the iron is released from the ligand and the free siderophore is then recycled. This mechanism is, for example, used for transporting ferrichrome in some fungal species such as *Ustilago maydis* (Ardon et al., 1998). 2) In the taxicab mechanism, the Fe(III) from the extracellular siderophore is transferred across the cell membrane to intracellular ligands. This mechanism is used by *Rhodotorula* species (Winkelmann and Huschka, 1987). 3) In the hydrolytic mechanism, the whole Fe(III)-siderophore complex is transported into the cell and is subjected to several reductive and degradative processes to release the ferric ion. The Fe(III) is reduced to Fe(II) inside the cell and the siderophore is excreted again. This mechanism is used in the uptake of Fe(III)-triacetylfusarinine complexes by *Mycelia sterilia* (Adjimani and Emery, 1988). 4) In the reductive mechanism, the Fe(III)-siderophore complex is not transported across the cell membrane. The reduction of Fe(III) to Fe(II) occurs at the cell membrane, and then the reduced iron is taken up by the cell. This mechanism is used by *Ustilago sphaerogena* in the uptake of ferric iron from ferrichrome (Lesuisse et al., 1998).

3. **Plant Siderophores**

Iron is an essential micronutrient for plant growth (Kobayashi and Nishizawa, 2012). Under conditions of iron deficiency, graminaceous plants (e.g., barley and wheat) have developed an efficient strategy for acquiring Fe from insoluble sources (Kraemer et al., 2006). These plants secrete ferric iron-chelating compounds called phytosiderophores, which form specific strong complexes with Fe(III) (Ma, 2005). The phytosiderophores are hexadentate ligands that coordinate Fe(III) with their amino and carboxyl groups (Singh, et al., 2011). When released to
the rhizosphere, the phytosiderophores chelate iron from the soil by forming Fe(III)-phytosiderophore complexes, which can be subsequently transported across the root plasma membrane (Dell'mour et al., 2012). In comparison with the molecular mass of microbial siderophores (200 – 2000 Da), phytosiderophores range between 500 and 1000 Da (Neilands, 1981).

Mugineic acid (MA) is the most common and was the first phytosiderophore identified in barley by Takemoto et al. (1978) (Figure 1). It appears to be usually produced by graminaceous plants (Ma, 2005). The formation constant of the mugineic acid-Fe(III) complex is $K = 10^{19.8}$ (Gustafson and Martell, 1963), which is low compared to those of microbial siderophores such as ferrichrome ($K = 10^{29.1}$) (Schwarzenbach and Schwarzenbach, 1963), ferrioxamine B ($K = 10^{30.6}$) and ferric enterobactin ($K = 10^{52}$) (Harris et al., 1979). The mugineic acid (MA) family includes MA, 2'-deoxymugineic acid (DMA), 3-hydroxymugineic acid (HMA) and 3-epihydroxymugineic acid (epi-HMA) (Dell'mour et al., 2010). Plants such as barley and rye mostly produce hydroxylated phytosiderophores (mugineic acid and epi-hydroxymugineic acid), whereas maize and rice produce non-hydroxylated phytosiderophores (DMA) (Mori et al., 1991). Wheat only produces one siderophore type of the mugineic acid family, which is called DMA, and has a high tolerance to iron deficiency because of its high phytosiderophore production rates (Ma and Nomoto, 1996). Other types of phytosiderophores have also been identified from gramineous plants: avenic acid A from oats (Avena sativa) and distichonic acid from beer barley (Hordeum vulgare) (Nomoto et al., 1981). In general, several studies show that plant species such as barley, rye, and wheat, which produce a high concentration of phytosiderophores, are more resistant to iron deficiency than other species such as maize, sorghum, and rice that produce fewer phytosiderophores (e.g. Masuda et al., 2009; Kobayashi et al., 2010).
4. Role of siderophores in nature

4.1 Soil mineral weathering

In soils, the microbial communities that colonize mineral surfaces differ from the inhabitants of the surrounding soil (Certini et al., 2004). Microbial attachment to mineral surfaces leads to the formation of a microenvironment that protects the microorganisms against environmental stresses (Liermann et al., 2000; Ojeda et al., 2006). In microenvironments, mineral nutrients can be chelated directly from the soil minerals or shared amongst the surrounding microorganisms (Roberts Rogers and Bennett, 2004). Siderophores produced by soil microorganisms can promote the mineral weathering of insoluble phases. Siderophores provide an efficient Fe acquisition system due to their high affinity for Fe(III) complexation by means of mineral dissolution (Kraemer, 2004). In tropical soils that are enriched with insoluble iron oxide and clay silicate mineral phases, siderophores play a significant role in iron dissolution, making it available for microorganisms and plants (Shirvani and Nourbakhsh, 2010). There are different mechanisms for siderophore-iron dissolution (e.g., Holmén and Casey, 1996; 1998). The general mechanism is that the Fe-siderophore complex is formed at the mineral surface and is then transferred into the surrounding soil solution and becomes available for uptake by the cell membrane of microorganisms or plants (Kalinowski et al., 2000a; Liermann et al., 2000; Kraemer, 2004).

The impact of siderophores on soil mineral weathering can be more effective compared to that of organic acids because siderophores form more stable complexes with Fe. Siderophores form 1:1 complexes with Fe(III), with constants ranging between $K=10^{30}$ and $K=10^{52}$ (Jalal and
van der Helm, 1991; Matzanke, 1991), while the constants of oxalic and citric acids with Fe(III) are $K=10^{7.6}$ and $10^{12.3}$, respectively (Perrin, 1979).

Due to the importance of microbial siderophores in weathering and soil formation, the role of siderophores in the dissolution of iron minerals has been investigated intensively. Hydroxamate siderophores produced by *Suillus granulatus* have a high efficiency in the dissolution of goethite, where significant quantities ($10^{-9}$ mol m$^{-2}$ h$^{-1}$) of iron were mobilized in the presence of *Suillus* sp. because of their continuous production of siderophores (Watteau and Berthelin, 1994).

Mineral dissolution is enhanced not only by siderophore-producing fungi but also by bacteria such as *Bacillus* sp., which have been documented to produce siderophores that promote the dissolution of the surface of hornblende (Buss et al., 2007). In addition, the dissolution of Fe from the hornblende that has been observed in the presence of siderophore-producing actinomycetes such as *Streptomyces* and *Arthrobacter* was higher than the dissolution of Fe by the synthetic desferrioxamine B siderophore (Kalinowski et al., 2000a). Therefore, the interactions between siderophores and iron minerals are directly related to the iron acquisition efficiency of living cells in the soil environment (Shirvani and Nourbakhsh, 2010). For example, fungal siderophores such as ferrichrome and ferricrocin have a significant role in changing the surface structure of biotite and increasing its dissolution in podzolic forest soil (Sokolova et al., 2010). On the other hand, the Co dissolution from Co-bearing minerals such as heterogenite in the presence of the desferrioxamine siderophore was also found to be an effective way to increase Co bioavailability (Bi et al., 2010).

Microbial siderophores are not the only compounds that play an important role in mineral weathering; there are also studies that have shown that plant siderophores can play a significant role in these processes. For instance, plant-produced phytosiderophores increase the dissolution
of iron-containing minerals. Hiradate and Inoue (1998) found that phytosiderophores were more efficient in dissolving ferrihydrate compared to goethite. The series of dissolution experiments reported in the literature review by Reichard et al. (2005) elucidates the mechanisms and rates of dissolution of goethite that is promoted by phytosiderophores. They also report several plant growth experiments that were conducted to test the role of wheat in acquiring iron from the crystalline oxy-hydroxide goethite. Recently, genetic engineering has allowed us to observe that the expression of iron-phytosiderophore transporter genes in barley enhances its ability to dissolve iron from soil minerals (Gómez-Galera et al., 2012).

4.2 Biogeochemical cycling of iron in the ocean

The biogeochemical cycling of trace metals in the oceans has become a subject of great concern. Of all trace metals, Fe has received the most attention due to its very low concentration in the ocean, its influence on the carbon cycle and its importance to climate change (Boyd et al., 2007). Marine bacteria produce most of the organic Fe chelators in seawater and thereby play a significant role in the biogeochemical cycling of Fe in the ocean in which those bacteria compete with phytoplankton for Fe by producing different types of siderophores that affect the Fe abundance and solubility in the marine environment (Cordero et al., 2012). Marine siderophores include a hydroxyl-carboxylate functional group, provided either by citrate as in snychobactins, petrobactin, aerobactin and marinobactins (Figure 1) or by b-hydroxyaspartate as in aquachelins, loihichelins and alterobactin (Hider and Kong, 2010).

On the surface water, siderophores participate in the photochemical cycling of iron by forming Fe(III)-siderophore complexes that increase iron availability for plankton (Hunter and Boyd, 2007). There is also a relationship between the distribution of siderophores and the
biogeochemical cycles of iron in different sites in the Atlantic Ocean. Ferrioxamine G was found widely distributed in surface waters throughout the Atlantic Ocean, whereas ferrioxamine E had a more varied distribution, and both of ferrioxamines contribute to the fraction of iron solubility (Mawji et al., 2008; Amin et al., 2012). Siderophores have an important role in the complexation of iron in the marine environment, where the Fe siderophore complex contains 99% of dissolved Fe, which affects the iron biogeochemical cycle (Gledhill and Buck, 2012).

5. Biotechnological applications of siderophores

5.1 Enhancing growth and pathogen biocontrol of plants

Microbial siderophores provide plants with iron nutrition to enhance their growth when the bioavailability of iron is low (Crowley, 2006). Kloeper et al. (1980) were the first who found that different Pseudomonas species can improve plant growth by producing siderophores besides protecting them from pathogens, and they classified this group of bacteria plant growth-promoting bacteria. Siderophore-producing Pseudomonas putida can also be used as a seed inoculant to increase the germination rate, shoot and root length and chlorophyll content in maize, potato, peanut and other ground nuts. It functions in two different ways: indirectly by suppressing pathogens or directly through the secretion of phytohormones and vitamins or by increasing the mineral uptake by siderophores (Omidvari et al., 2010). There are also two ways to stimulate the growth of plants with siderophores produced by fluorescent Pseudomonads: by enhancing the availability of iron in the rhizosphere or by inhibiting plant pathogens by limiting iron uptake (Pathma et al., 2011; Gamalero and Glick, 2011). In addition to pyoverdine siderophores, other microbial siderophores such as ferrioxamine and rhodothorulic acid could also contribute to plant iron nutrition (Siebner-Freibach et al., 2003). There are also some
endophytic *Streptomyces* isolated from the roots of plants such as *Azadirachta indica*, which produce siderophores that promote the root and shoot growth, as well as seed germination (Verma et al., 2011).

In addition to bacteria, mycorrhizal fungi can also be used as biofertilizers to enhance plant growth. Mycorrhizal sorghum plants were shown to take up higher concentrations of iron than nonmycorrhizal plants (Caris et al., 1998). The role of ectomycorrhizal fungi associations in plant nutrition depends on their production of siderophores (Van Schöll et al., 2008). For instance, siderophores produced by ectomycorrhizal fungi have an important role in the mobilization of Al, Fe, K and Si in the soil, which enhances both mineral mobilization and plant growth (Van hees et al., 2006). Recently, the plant growth promoting activities of fungi were investigated, and siderophores produced by *Aspergillus niger*, *Penicillium citrinum* and *Trichoderma harzianum* were found to increase the shoot and root lengths of chickpeas (*Cicer arietinum*) (Yadav et al., 2011).

On the other hand, Kloeper et al. (1980) were also the first to demonstrate the role of siderophores in the biological control mechanism. This mechanism depends on the role of siderophores as competitors for iron that reduce the iron availability for the phytopathogen (Beneduzi et al., 2012). Several studies have reported the roles of siderophores in iron nutrition, plant growth promotion and the biological control of plant pathogens (e.g. Walsh et al., 2001; Whipps, 2001). Pyoverdines produced by Pseudomonads were found to control the wilt diseases of potato caused by *Fusarium oxysporum* (Schipper et al., 1987) and were also involved in the biocontrol of *Gaeumannomyces graminis*, which causes a deficiency of wheat and barley growth (Voisard et al., 1989). Siderophores produced by Pseudomonads were also observed to suppress the phytopathogens in peanuts and maize (Pal et al., 2001). Siderophores have been reported to
inhibit the spore germination of pathogenic microorganisms, thus suppressing disease (Crowley and Kraemer, 2007; Sindhu et al., 2009). There are many other bacteria species in addition to *Pseudomonads* that can be used as biocontrol agents. For example, the siderophore produced by *Bacillus subtilis* had a significant role in the biocontrol of *Fusarium oxysporum*, which causes the fusarium wilt of pepper (Yu et al., 2011). In addition, siderophores produced by *Azadirachta indica* had a high affinity for Fe$^{3+}$ that reduced the iron available to the pathogen and negatively affected the pathogen’s growth (Verma et al., 2011). Thus, siderophores have been suggested to be an environmentally friendly alternative to hazardous pesticides (Lemanceau et al., 2007; Schenk et al., 2012).

### 5.2 Biocontrol of fish pathogens

Siderophores can play a role in disease control of fish by limiting iron that is important in virulence and bacterial interactions (Junfeng and Zhenming, 2004). The pathogenic bacterium of Atlantic salmon, *Aeromonas salmonicida*, can produce harmful enzymes such as proteases and cholesterol acyl transferase to resist the defense mechanisms of the host (Ellis, 1999) and produce siderophores called “Transferrin” in order to compete with the host for iron and suppress its growth (Yano, 1996). *Pseudomonas fluorescens* strains were used as probiotics in fish farming (Gram et al., 1999) because its siderophores have been shown to inhibit the growth of *V. anguillarum* and *Aeromonas salmonicida* (Gram et al., 2001). *Bacillus* sp. has been recommended as a suitable strain for a biocontrol agent in fish intestines and culture water (Sugita et al., 1998). For instance, *Bacillus* sp. strain NM 12 can produce siderophores with a wide antibacterial spectrum that inhibit the growth of 62.5% of the 363 intestinal bacteria from several coastal fishes (Junfeng and Zhenming, 2004). In addition, siderophores produced by
Bacillus cereus can inhibit the growth of the fish pathogen Aeromonas hydrophila by 70% (Lalloo et al., 2010). Siderophores produced by Pseudomonas fluorescens have been shown to compete for iron and inhibit the growth of some fish’s bacterial pathogens such as Aeromonas salmonicida, Lactococcus garvieae, Streptococcus iniae, Vibrio anguillarum, Vibrio ordalii, Flavobacterium psychrophilum and Yersinia ruckeri (Brunt et al., 2007; Korkea-aho et al., 2011; Dimitroglou et al., 2011). Recently, siderophores produced by bacteria that were isolated from the fish intestinal tract were investigated for their ability to inhibit fish pathogens such as Aliivibrio logei, Aliivibrio salmonicida, Vibrio ichthyoeenteri, Vibrio anguillarum, Vibrio splendidus and Aeromonas salmonicida. Because they were highly efficient, these siderophore-producing bacteria were recommended for use as a biocontrol agent for fish pathogens (Lazado et al., 2010; Sugita et al., 2012).

5.3 Microbial ecology and taxonomy

Siderotyping can provide a quick and unambiguous identification of microbes to the species level and be a powerful tool in environmental research (Meyer and Stintzi, 1998; Meyer et al., 2002). Siderotyping is defined as the characterization of microbial strains by the siderophores they produce (Neilands, 1981). Siderophores can be important in the ecological fitness of the microorganisms that produce them (Bosen and Levy Frebault, 1992). There are two different methods for siderotyping, the analytical and the biological methods (Meyer et al., 2002). The analytical methods, such as high performance liquid chromatography (HPLC) or HPLC coupled with mass spectrometry (HPLC-ES/MS) are based on the physicochemical properties of siderophores, whereas the biological methods are based on the direct measurement of
siderophore-mediated iron in the microbial cells or using a molecular biology method based on the recognition of specific DNA sequences related to siderophores (Bach et al., 2000).

*Pseudomonas* produces fluorescent pyoverdine, its peptide chain varying among strains and species. This variability can be used to determine the relatedness of the strains (Chincholkar et al., 2005). Siderotyping analysis has been performed on 400 strains of fluorescent and non-fluorescent *Pseudomonas* sp. that are grouped into 28 taxa, including 15 well-defined species, each characterized by a specific siderophore (Meyer et al., 2002). Sixty-eight fluorescent *Pseudomonas* strains were identified using mass spectrometry analysis of their pyoverdines, and siderotyping was recommended as a helpful method for studying microbial diversity and taxonomy (Meyer et al., 2008). In siderotyping, the siderophores produced by *Pseudomonas* help identify species by allowing comparisons within the group of pyoverdines to evaluate the most related strains and thus help in phylogenetic studies (Meyer, 2010).

Several studies have found that siderophore production is specific to the genus level. For instance, pyoverdines are isolated only from *Pseudomonas* spp., ornibactin from *Burkholderia* spp. and mycobactin from *Mycobacterium* spp. (e.g. Meyer et al., 2002; Oelschlaeger et al., 2003; Mokracka et al., 2004; Bultreys et al., 2006). Mycobactin can be used as a chemotaxonomic marker for the identification of *Mycobacteria* to the species level, based on its chemical structure (Snow and White, 1969; Barclay and Ratledge, 1988). Moreover, from investigations conducted on *Rhizobia* and *Burkholderia* spp. (Meyer and Stintzi, 1998; Carson et al., 2000), it appears that siderotyping could be extended to the identification of many siderophore-producing microorganisms.
5.4 Bioremediation of the environmental pollutants

5.4.1 Metals

Metals play a vital role in the development of human civilizations (Jonhson et al., 2002), but the manufacturing industry, sludge applications, nuclear power stations and mining have led to metal pollution (Wasi et al., 2013). Siderophores are extremely effective at solubilizing and increasing the mobility of a wide range of metals such as Cd, Cu, Ni, Pb, Zn and the actinides Th(IV), U(IV) and Pu(IV) (Rajkumar et al., 2010) by forming stable complexes with them. This makes siderophores useful as bioremediation agents, which is important because bioremediation is a cost effective and environmentally friendly technique.

It is well known that siderophores form their most stable complexes with Fe(III); however, it has also been shown that some siderophores such as desferrioxamine chelate Co(III) better than Fe(III) (Neubauer et al., 2000). This diversity in metal chelation suggests that siderophores can act as metallophores (metal-specific ligands secreted by organisms for metal uptake). For example, A. eutrophus CH34 has a high capacity for metal solubilization from soil samples contaminated with Cd (Diels, 1997). Azotobacter vinelandii, which has been shown to produce the siderophores “azotochelin” and “azotobactin” in iron starvation conditions, had the ability to use the same siderophores for Mo and V acquisition (John et al., 2001). Kluyvera ascorbata decreased the heavy metal toxicity such as Ni, Pb, and Zn in soil samples from a metal-impacted wetland near Sudbury, Ontario (Burd et al., 2000). Siderophores were also used in model systems for the bioremediation of soils polluted by heavy metals and gave promising results (Kayser and Neubauer, 2005). For example, pyoverdines produced by Pseudomonas fluorescens that was used to mobilize metals from a uranium mine also had the ability to mobilize Fe, Ni, U and Co from the ore (Edberg et al., 2010). It was also reported that the siderophores produced by
bacteria participate in the mobility of arsenic from contaminated soil (Purakayastha, 2011). For instance, *Agrobacterium radiobacter* has the ability to remove approximately 54% of arsenic from contaminated soil (Wang et al., 2011).

Not only do siderophores from microbes contribute to metal bioremediation, but plant siderophores can also have a role in this process. There are many studies that have demonstrated that phytosiderophores are efficient in mobilizing of metals in soil (e.g. Rajkumar et al., 2009; 2010; 2012). Studies in both uncontaminated and contaminated soils have showed that phytosiderophores are more efficient at mobilizing Fe, Cu, Zn, Ni and Cd in the soil than synthetic chelators and microbial siderophores (i.e. Awad and Romheld, 2000; Singh et al., 2008). Plant siderophores have a high affinity for complexation with several metals in the following order (Cd$^{2+}$ > Ni$^{2+}$ > Pb$^{2+}$ > Sn$^{2+}$ > AsO$_4^{-2}$ > AsO$_2^{-1}$ > Mn$^{2+}$ > Co$^{2+}$ > Cu$^{2+}$ > Fe$^{3+}$) and very weakly binds Al$^{3+}$ and Cr$^{3+}$ (Ruggiero et al., 1999). The capacity of siderophores to form complexes with different metals could be used to develop processes for metal remediation from waste sites (Hernlem et al., 1999). Recently, the role of plants in the remediation of metal from contaminated soil, especially the ability of phytosiderophores to form complexes with toxic metals, has been investigated (Seth, 2012). Phytosiderophores produced by rice have the ability to mobilize Mn, Cd and Fe from the soil (Ishimaru et al., 2012).

### 5.4.2 Petroleum hydrocarbons

Petroleum hydrocarbons in marine ecosystems are one of the major environmental problems. Microorganisms could play an important role in the remediation of petroleum hydrocarbons from the marine environment (Das and Chandran, 2011). Siderophores participate in the biodegradation of petroleum hydrocarbons through indirect mechanisms, by facilitating iron for...
degradation by microorganisms and by mediating the decarboxylation reaction (Barbeau et al., 2002). Petrobactin (Figure 1) was the first structural characterization of a siderophore produced by the oil-degrading marine bacterium *Marinobacter hydrocarbonoclasticus* (Barbeau et al., 2002). There is also another sulfonated siderophore called “Petrobactin sulfonate” isolated from the same oil-degrading marine bacterium (Hickford et al., 2004). New synthetic methods were developed to allow easy access to a number of petrobactin homologues, which differed in both the polyamine and the dihydroxybenzene components. From the preliminary studies, it was suggested that using siderophores may prove to be a good strategy for oil-spill cleanup (Gardner et al., 2004; Leão et al., 2007). It was also shown that marine *Vibrio* species isolated from the Gulf of Mexico after the 2010 Deepwater Horizon oil spill could produce amphiphilic siderophores called “ochrobactins” that efficiently degrade petroleum hydrocarbons (Gauglitz et al., 2012).

5.5 Nuclear fuel reprocessing

Siderophores contain anionic hydroxamate or catecholate functional groups that form hard oxodonors, which strongly bind to Lewis acids, resulting in complexes with remarkably high stability constants (Harris et al., 1979). Because actinides form strong complexes with hard oxygen anions, it has been suggested that siderophores could bind actinides with a complexation constant estimated to be log$^{16}$ (Jarvis and Hancock, 1991). Desferrioxamine and enterobactin siderophores have been shown to solubilize hydrous plutonium oxide and uranite through a wide pH range (Brainard et al., 1992). The Purex process has been used commercially to reprocess irradiated nuclear fuel by solvent extraction techniques and separate uranium and plutonium for reuse from fission products such as technetium and neptunium (Taylor and May, 1999). In this
process, uranium and plutonium flow into the solvent and become contaminated with neptunium. Siderophores have been shown to allow for the selective removal of neptunium from the solvent phase (May et al., 1998, Taylor et al., 1998) and could be used in the Purex process to simplify the separation of the actinides (Joanna et al., 2002). A high UO$_2$ dissolution and solubility in the presence of siderophores was observed by using a batch and continuous flow stirred tank (Frazier et al., 2005). Desferrioxamine forms a complex with U(VI), where the hydroxamate functional group of desferrioxamine is similar to acetohydroxamic acid (AHA), a ligand that has been proposed for actinide complexation (Mullen et al., 2007). The time resolved laser-induced fluorescence spectroscopy with ultrafast pulses (fs-TRLFS) and ultraviolet visible-light (UV-Vis) techniques were used to examine complexation of UO$_2$ with pyoverdine, and researchers found high stability constants between pyoverdine and U. This suggests that pyoverdines could be used in uranium mobilization in contaminated environments such as mine waste disposal sites (Moll et al., 2008). Low concentrations of siderophores can potentially influence the dissolution of spent nuclear fuel, and there are no significant differences between using the synthetic desferrioxamine and the pyoverdine produced by Pseudomonas fluorescens (Johnsson et al., 2009; Marshall et al., 2010). Pyoverdines produced by Pseudomonas fluorescens can also contribute to the mobilization of U(VI), Np(V) and other metals from uranium mine waste (Edberg et al., 2010; Behrends et al., 2012). Based on these features and examples, siderophores have been proposed for the remediation of radioactive waste and reprocessing of nuclear fuel (Renshaw et al., 2002).
5.6 Optical biosensor

A biosensor is a bio-molecule coupled to an electrical device (such as a transducer, amplifier, or noise filter) in order to increase the signal to noise ratio that allows detection of various types of responses through specifically engineered systems (Gupta et al., 2007). The bacterium *Pseudomonas fluorescens* produces a yellow-green water-soluble fluorescent siderophore called pyoverdine. Pyoverdines are characterized by the following properties (Barerro et al., 1993): (a) they form a strong complex with Fe(III) and have a weak or negligible affinity for Fe(II), and (b) the Fe(III) complexes have very high stability constants (approximately $10^{32}$) (Kurtz and Crouch, 1991). These characteristics make pyoverdine a promising agent for the construction of optical biosensors (Pesce and Kaplan, 1990). To determine the iron bioavailability in oceanic water or soils, a siderophore with an exceptional Fe(III) binding constant would be an ideal choice for the molecular recognition element of the sensor (Chung Chun Lam et al., 2006). Siderophores provide the potential for good sensitivity and selectivity and provide a detection system that would mimic the biological uptake process (Ellerby et al., 1992). For example, the fluorescent azotobactin has been used as a chemosensor for determining Fe(III) quantity (Palanché et al., 1999). In addition, the concentration of iron present in the ocean has been determined by using a siderophore as a biosensor (Chung Chun Lam et al., 2006). In this study, they used parabactin that was produced by *Paracoccus denitrificans* as a biosensor by encapsulating it in sol-gel thin film on a quartz substrate. The seawater samples were analyzed by a flow cell that was mounted in the sample partition of the fluorescence spectrometer. The microbial siderophores can be used as biosensors for heavy metal analysis instead of the conventional instruments (Lavecchia et al., 2010). For instance, azotobactin produced by *Azobacter vinelandii* has been used as an optical biosensor for Fe(III) in a modified design that depends on the encapsulation of the azotobactin in
sol–gel matrices without significant loss of its fluorescence signal (Sharma and Gohil, 2010). Additionally, the Fe(II and III) specificity for the iron biosensor pyoverdin has been optimized by immobilizing it in three formulations of porous sol-gel glass (A, B and C), which contained various amounts of water added (Yoder and Kisaalita, 2011). In that study, the most specific and linear response for binding to Fe(II and III) was observed for pyoverdin immobilized in sol-gel C that had more water than A and B.

5.7 Bio-bleaching of pulps

The pulp and paper industry is a primary source of many environmental problems including global warming, human toxicity, ecotoxicity, photochemical oxidation, acidification, nutrification and solid wastes (Singh et al., 2008; Bajpai, 2010). The environmental problems of pulp and paper manufacturing result from the bleaching processes. Some pollutants are emitted into the air, while others are discharged in wastewater.

Brown-rot fungi are considered to be one of the most important groups of wood decaying microorganisms. There are many studies that have reported the production of catecholate and hydroxamate siderophores by wood decaying fungi (e.g., Machuca et al., 1999; Milagres et al., 2002). Siderophores isolated from the brown-rot fungus Gloephyllum trabeum have the ability to mediate the reduction of iron in redox cycling processes. The reduced iron can then react with hydrogen peroxide to generate oxygen radical species that depolymerize cellulose, hemicelluloses and lignocelluloses (Arantes et al., 2006; Arantes and Milagres, 2007). There are several characteristics of siderophores that could make them a powerful agent in the bleaching of pulp (Jellison et al., 1991). For instance, the siderophores produced by brown-rot fungus G.
trabeum can increase the cellulose degradation (Xu and Goodell, 2001). It was also observed that the siderophores produced by G. trabeum could degrade the pulps with 10.8% loss of viscosity, whereas P. medula-panis and T. versicolor siderophores degraded pulp with 13.6% and 14.4% loss, respectively (Milagres et al., 2002). The siderophores produced by Coriolus versicolor alter lignin structure to make it more susceptible to degradation (Wang et al., 2008). Siderophores are an effective agent in pulp treatment, where they can reduce 70% of the chemicals needed to bleach Kraft pulp (Bajpai, 2004).

6. Concluding remarks and future perspectives

The study of mineral-microbe interactions underscores the importance of microorganisms in making the Earth a suitable environment for all forms of life. In recent years, it has become clear that siderophores represent central organic compounds in iron uptake in many microorganisms and plants. Understanding the chemical structures of different siderophores and the membrane receptors involved in iron uptake has opened new areas for research. The importance of siderophores is obvious, and they play a significant role in both medical and environmental applications, even if there are many questions remaining to be answered. What is the specific role of microorganisms and plants in the selectivity of metal uptake by siderophores? Why do microorganisms secrete more than one type of siderophores to meet their mineral nutritional needs? Why is the concentration of siderophores in microbial cultures always higher than in the environment? What is the relative importance of the different siderophore structures involved in environmental applications? Can modified genetic methods such as labeled DNA be useful tools for the direct detection of siderophore functional genes in the environment?
More research is focusing on finding effective ways to use siderophores in bioremediation and biocontrol, which should enhance their application in the environment. An increased knowledge of the chemical structures and mechanisms of known siderophores should lead to various medical and environmental applications. Siderophore variability and their structural and functional characteristics in relation to microbial communities must be vigorously investigated to improve the role of siderophores in environmental applications. The molecular details of siderophore production and the metal uptake by microorganisms, as well as the relationship between siderophores and microbial structure, in environments with low iron bioavailability, i.e., oceans, and some soil conditions are still unknown. Combining metagenomics with detailed chemical analysis will reveal important information that could be used to improve the current environmental applications and develop new other applications for siderophores.

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Figure 1. Chemical structures of microbial and plant siderophores.