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Siderophores in forest soil solution

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Abstract. Siderophores in soil solution of coniferous forest soils have been chemically identified for the first time. We have identified the siderophores ferrichrome and ferricrocin in soil solution of the upper organic layer by High Performance Liquid Chromatography (HPLC) Mass Spectrometry (MS). The soil solutions were sampled from mor layers of podzolic soils from the south and the north of Sweden and from a mor layer overlying granitic rock and intensively colonised by ectomycorrhizal hyphae. Ferrichrome was found in nanomolar concentrations in all soil solutions investigated and ferricrocin only in the soil solution from the mor layer covering a rock and in the soil solution from the north of Sweden. The findings are discussed in relation to the possible role of fungal hyphae and siderophores in weathering minerals in podzolic soils under coniferous forests. Citric and oxalic acid are able to dissolve minerals via complexation of cations from the mineral. Siderophores should be, kinetically and thermodynamically, even more efficient complexing agents for trivalent cations than oxalic and citric acid. The present study provides direct evidence for the presence of siderophores in soil solution.

Abbreviations: B horizon – illuvial horizon; DFO – desferrioxamine; E horizon – eluvial horizon; HPLC – High Performance Liquid Chromatography; LMM – low molecular mass; LOD – limit of detection; MS – Mass Spectrometry; O horizon – Organic mor layer

Introduction

Several bacteria and fungi growing under iron deficient conditions can synthesise and excrete specific, relatively low molecular mass iron chelators called siderophores, from the Greek word for 'iron carrier', to circumvent the low solubility of iron (Powell et al. 1980; Bagg and Neilands 1987; Crowley et al. 1987; Matzanke et al. 1989). In the microbial community siderophores are universally present. Not all microbes create siderophores, because some do not require iron, but nearly all fungi secrete siderophores with the exception of budding and fission yeast (Neilands 1995). The production of siderophores increases in response to iron starvation (Szaniszlo et al. 1981). This phenomenon has been known for almost half a century. Siderophores are structurally diverse. The actual functional groups are hydroxamate, phenolate or catecholate, citrate (aerobactin and schizokinen) and oxazoline nitrogen (myobactins) (Matzanke et al. 1989). The hydroxamate and phenolate types are the main classes. A prototypical hydroxamate siderophore is

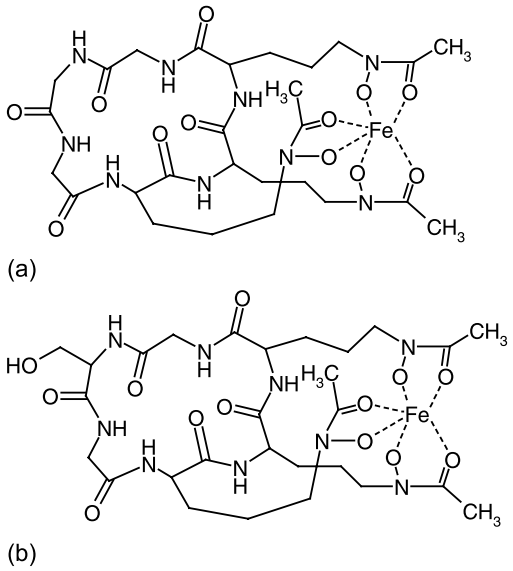

Figure 1. Structures of the two hydroxamate type siderophores of the ferrichrome family that was determined in the soil solutions of a boreal forest. (a) Ferrichrome ferric complex, a fungal hydroxamate siderophore with a stability constant of $K_f = 10^{29.1}$ with ferric(III) ions (Raymond et al. 1984). (b) Ferricrocin ferric complex, a fungal hydroxamate siderophore with a stability constant $K_f = 10^{30.4}$ with ferric(III) ions (Raymond et al. 1984).

ferrichrome, isolated in 1952 (Neilands 1952). Hydroxamate siderophores can be isolated from fungi, yeast and bacteria (Miller et al. 1985). Ferrichrome is a typical tri-hydroxamate siderophore. This type of siderophore is commonly synthesised by several fungal species. Fungi, which have been found to produce ferrichrome, include *Ustilago maydis*, *Ustilago sphaerogena*, *Aspergillus niger*, all examined *Penicillia* (Neilands 1984), *Neovissia indica*, *Lipomycetaceae* and the dermatophyte *Trichophyton mentagrophytes* (Winkelmann and Drechsel 1997). It has been found that ferricrocin is produced by *Aspergillus fumigatus* (Neilands 1984) and *Cenoccum geophilum* (Haselwandter and Winkelmann 2002).

Most hydroxamate siderophores are hexadentate ligands with three asymmetrical functional units, enabling 16 possible geometrical and optical isomers. All isomers are also theoretically possible for metal complexation. The ferrichrome family includes ferrichrome (Figure 1(a)), ferrichrome A, ferrichrome C, ferricrocin (Figure 1(b)), ferrichrysin, ferrirubin, ferrirhodin and malonichrome. Characteristic for the ferrichrome family is the tripeptide sequence of N^5-acyl-N^5-hydroxyl-L ornithine and a variable tripeptide sequence of short amino acids (Winkelmann and Drechsel 1997).

The abundance of iron in soils is 1–6% by weight and its solubility is dependent on pH. Iron (III) is insoluble under physiological conditions (Powell et al. 1980; Matzanke et al. 1989). To gain a selective growth advantage and bind iron, different organisms utilise specific siderophores. Iron availability to plant roots depends

on organic chelators and hydroxamate siderophores can be one of these. The occurrence of hydroxamate siderophores in aqueous extracts from grasslands, areas of mixed herbaceous vegetation and coniferous and deciduous forests across the USA has been determined using a bioassay (Powell et al. 1980). Ferrichrome-type siderophores can be estimated from soil-water extracts by an *Escherichia coli* bioassay (Powell et al. 1983). It has also been found that hydroxamate siderophores are to a large extent adsorbed to soil organic matter (Powell et al. 1980, 1982).

Mycorrhizal symbiosis with fungi is an important evolutionary strategy for roots of most plants to gain nutrients from the soil. It is known that fungal hyphal tips produce low molecular mass (LMM) organic acids and it is assumed that these acids promote weathering (Jongmans et al. 1997; Ahonen-Jonnarth et al. 2000; van Hees et al. 2000). A mechanism of weathering in soils of boreal coniferous forests has been proposed by Jongmans et al. (1997) and van Breemen et al. (2000). These authors suggested that mycorrhizal hyphae could corrode mineral grains in the E (eluvial) horizon of forest podzols. To corrode the mineral grains the hyphae must exude compounds, which are able to dissolve the mineral structure. The most probable mechanism is removal of trivalent ions from the mineral grain by complex formation. Mycorrhizal fungi exude citric and oxalic acid (Ahonen-Jonnarth et al. 2000) and these acids have been found in soil solution in micromolar concentrations (Westergaard Strobel et al. 1999; van Hees et al. 2000). It is assumed that the mycorrhizal hyphae, which live in symbiosis with trees and obtain their carbon from these host plants, can promote dissolution of mineral grains, and supply minerals directly to their host tree (Jongmans et al. 1997).

The purpose of this project was to determine hydroxamate siderophores in natural soil solution by developing a pre-concentration method and using HPLC-MS for the determination.

Materials and methods

Sites

Soil was sampled at three sites.

Pottäng, mor; the mor layer covering a granite rock was sampled at Pottäng, Alnö, Sundsvall, Sweden (62°24′N, 17°30′E). Twenty-five-year-old Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) were growing with moss and lichens in the undergrowth on podzolized soil.

Heden, mor; the organic mor layer, O-horizon, was sampled at Heden, Svartberget research park, 70 km NW of Umeå, Sweden (64°14′N, 10°46′E). The site was forested with Scots pine (*P. sylvestris*) and Norway spruce (*P. abies*) about 70 years old. The field layer consisted of dwarf shrub *Vaccinium myrtillus* and mosses. The soil was podzolized and the soil horizons had a thickness of about: O 5 cm, E (eluvial horizon) 5 cm and B (illuvial horizon) about 30 cm.

Horröd, 0–5 cm; the 0–5 cm of the E-horizon was sampled at Horröd, 10 km SW of Hässleholm, Sweden (56°05′N, 13°39′E). The site was forested with Norway

spruce (*P. abies*) about 80 years old. The soil was a haplic podzol and the soil horizons had a thickness of about: O 7–10 cm, E 6 cm (uncontinuous) and B about 40 cm (top 10 cm Bh).

Sampling

Mor layer covering a granite rock with mycorrhizal hyphae was collected and then filled into cylindrical sampling cups.

At the sites Heden and Horröd a pit of about 1 m^3 was dug. The mor organic soil layer was removed and collected. After removing the surface vegetation from the mor layer it was placed into cylindrical sampling cups. The 0–5 cm depth E-horizon samples were taken by driving soil sampling cups horizontally into the layer. The sampling cups were made of a PVC-tube with an inner diameter of 4.6 cm and a volume of about 120 cm^3. After sampling, the cups were sealed with a tight-fitting polyethylene lids.

The centrifugation drainage technique described by Giesler and Lundström (1993) was used to obtain soil solutions. A Beckman J2-HS centrifuge with JA-14 rotor (Spinco division, Palo Alto, Ca, USA) was used for that purpose. The soil samples stored at 4 °C were centrifuged within 24 h after sampling at a speed of 14,000 rpm for 30 min. The centrifugates were filtered through a hydrophilic 0.45 µm filter (Millex-HV, Millipore) before preconcentration according to the method described below.

Soil solution preconcentration

To remove high molecular mass compounds, 50 ml of each soil solution were ultrafiltered using a stirred cell, Amicon 8050 (Beverly, MA, USA) with a 4000 Da filter (PES 4, INTERSEP, UK). The filtration was stopped when the filter became dry. Before freeze-drying the filtrates were frozen in special freeze-drying glass jars.

A CHRIST Alpha 1–4 (Osterode am Harz, Germany) was used for freeze-drying. When all water in the sample had been evaporated a yellow-white, solid dust remained. The solid residue was dissolved in a minimum of water, about 1 ml. The ferric hydroxamate siderophores were determined using the HPLC and HPLC-MS methods described below.

Chemicals

Ferrichrome ($C_{27}H_{45}N_9O_{12}$), isolated from cultures of *U. sphaerogena* with a molecular mass of 687.7 g/mol, was purchased from Sigma, iron (III) chloride-6-hydrate and sodium acetate from Merck. Ammonium acetate was bought from Amresco and Amberlite XAD-16 was purchased from Fluka, methanol and acetonitrile from Lab-Scan.

All chemicals and solvents were of analytical grade and all water used was ion exchanged and passed through a Millipore-Q system (Millipore).

High Performance Liquid Chromatography with UV detection (HPLC-UV)

HPLC separations were performed using a Shimadzu LC-10 HPLC system equipped with a column oven and a diode array detector (215 and 425 nm). A C_{18} reversed phase column (Supelcosil LC-ABZ, 5 μm, and 4 × 150 mm) and a flow rate of 1 ml/min was used. The isocratic separations used methanol:15 mM acetate buffer pH 4.7 (10:90) or acetonitrile:15 mM acetate buffer pH 4.7 (7.5:92.5) (v/v) as eluting solvents.

High Performance Liquid Chromatography with Mass Spectrometry detection (HPLC-MS)

HPLC-MS was carried out on a Series 200 LC HPLC instrument (Perkin-Elmer SCIEX Instruments, PE Applied Biosystems) and an API 3000 LC/MS/MS triple-quadrupole instrument (Perkin-Elmer SCIEX Instrument, PE Applied Biosystems). The same C_{18} reversed phase column (Supelcosil LC-ABZ, 5 μm, and 4 × 150 mm) was used as in the other HPLC system. The positive ion electrospray mode was used. The 1 ml/min HPLC flow rate was split and 200 μl/min flowed into the PE Sciex Turbo-Ion Spray source.

Both isocratic (methanol:15 mM acetic buffer pH 4.7 (10:90)) and gradient separation was applied. The gradient separation used a 2 min isocratic step at 10% methanol followed by an 8 min linear gradient step to 60% methanol and a final third 2 min isocratic step at 60% methanol. All solvents contained 15 mM acetate buffer, pH 4.7.

The HPLC-method used for analysis of siderophores is a modification of the method of Konetschny-Rapp et al. (1988). The system of Konetschny-Rapp can separate most fungal ferric hydroxamate siderophores.

Results and discussion

Soil solution pre-concentration

The ultrafiltration through the 4000 Da filter had a recovery of about 100% and the freeze-drying process had a recovery of about 85% (Table 1). The ultrafiltration decreased the void volume peak in the chromatogram. Combination of the two pre-concentration techniques, with first ultrafiltration and then freeze-drying, was found suitable for natural samples. For preservation of biological products freeze-drying is a reliable process. The freeze-drying process stabilises easily degradable compounds and does not change the structure of the substances of the original

Table 1. A solution of 2 µM desferrioxamine (DFO) and ferrichrome ferric complexes standards was concentrated 25 times by freeze-drying and then determined by isocratic HPLC on a C_{18} reversed phase column (Supelcosil LC-ABZ, 5 µm, 4 × 150 mm) using 10% methanol and 15 mM acetate buffer, pH 4.7. The flow rate was 1 ml/min, with 50 µl injection volume and detection at 425 nm. To get the recovery the obtained area units were compared with those for a 50 µM standard solution of the ferric complexes of DFO and ferrichrome.

Sample	50 µM standard (area units)	Pre-concentrated sample (area units)	Recovery (%)
DFO	353982	292248	82.6
Ferrichrome	406339	346438	85.3

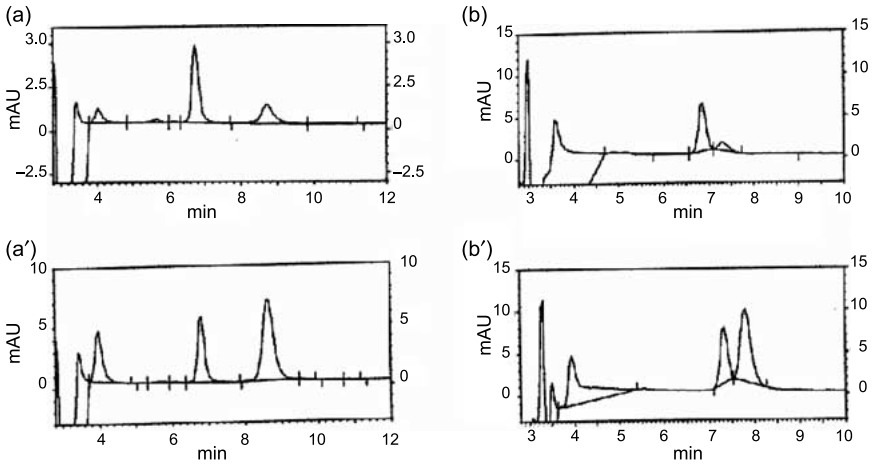

Figure 2. Soil solution sample from Heden analysed by HPLC. Concentrated 25 times by ultra filtration and freeze-drying. (a) Concentrated sample using methanol:15 mM acetate buffer pH 4.7 (10:90) as an eluting solvent. (a′) Sample as in (a) spiked with 2 µM DFO and 2 µM ferrichrome ferric complex eluted with same mobile phase. Retention time for DFO 4.0 min and ferrichrome 8.6–8.7 min. (b) Concentrated sample using acetonitrile:15 mM acetate buffer pH 4.7 (7.5:92.5) as an eluting solvent. (b′) Sample as in (b) spiked with 2 µM DFO and 2 µM ferrichrome ferric complex eluted with same mobile phase. Retention time for DFO 3.2–3.5 min and ferrichrome 7.3–7.8 min. The HPLC analysis was performed using a Shimadzu LC-10 HPLC system with a C_{18} reversed phase column (Supelcosil LC-ABZ, 5 µm, 4 × 150 mm) and detection at 215 nm.

sample (Franks 1998). Freeze-dried material is easily dissolved by addition of some water.

Identification and determination

A ferric ferrichrome standard was employed at concentrations from 0 to 250 nM ($n = 7$). The calibration graph of the ferrichrome (m/z 741.2) standards was linear

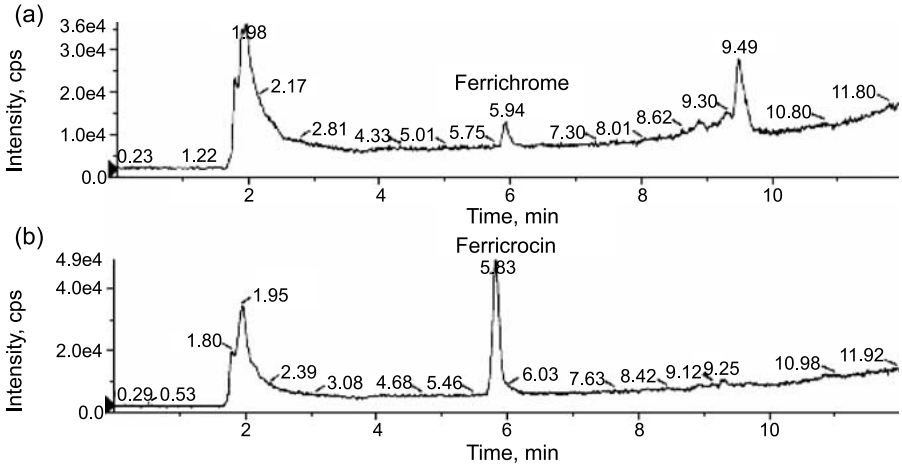

Figure 3. Chromatograms from Selected Ion Monitoring of soil solution of a mor layer (Heden) (a) m/z 741.2 amu indicates ferrichrome (ferrichrome $+ \mathrm{H})^+$ and (b) m/z 771.3 amu indicates ferricrocin (ferricrocin $+ \mathrm{H})^+$ in the sample. The analysis was performed on a PE Sciex API 3000 triple-quadrupole mass spectrometer using a C_{18} reversed phase column (Supelcosil LC-ABZ, 5 µm, 4 × 150 mm) with a gradient: 2 min isocratic step at 10% methanol followed by an 8 min linear gradient step to 60% methanol and a final third 2 min isocratic step at 60% methanol. All solvents containing 15 mM acetate buffer, pH 4.7.

and had a correlation coefficient of 0.9998. Each standard was injected twice; the two lowest were injected three times. The limit of detection (LOD), calculated as the concentration corresponding to three times the background noise, was in the nanomolar range for the pre-concentrated (about 25 times) soil solution samples. Thus, for the samples the LOD was determined to be 0.05 nM.

O-horizon centrifugates from Heden and soil solution from the E-horizon from Horröd were analysed using the HPLC and the HPLC-MS methods mentioned above. Ferrichrome and ferricrocin in the mor layer solution were identified, having the same retention time as standards, detected both by UV/VIS absorption and by mass spectra. The same retention times were also found for samples and samples spiked with ferrichrome and ferrioxamine when the composition of the eluent was changed from containing methanol to acetonitrile (Figure 2).

In Figure 3 (a) and (b) the presence of ferrichrome and ferricrocin is shown in mor layer solution from Heden, by selected ion monitoring for the $(\mathrm{M} + \mathrm{H})^+$ of these compounds using an HPLC gradient of methanol and buffer. Chromatograms from selected ion monitoring of two adducts of ferrichrome, $(\mathrm{M} + \mathrm{H})^+$ and $(\mathrm{M} + \mathrm{Na})^+$, for the mor layer solution from the mor layer covering a granite rock sampled at Pottäng are shown in Figure 4(a). For further identification of ferrichrome in the unknown samples, the samples were spiked with ferrichrome standard. The spiked samples (Figure 4(b)) showed enlarged peaks, which confirmed the presence of ferrichrome in the samples.

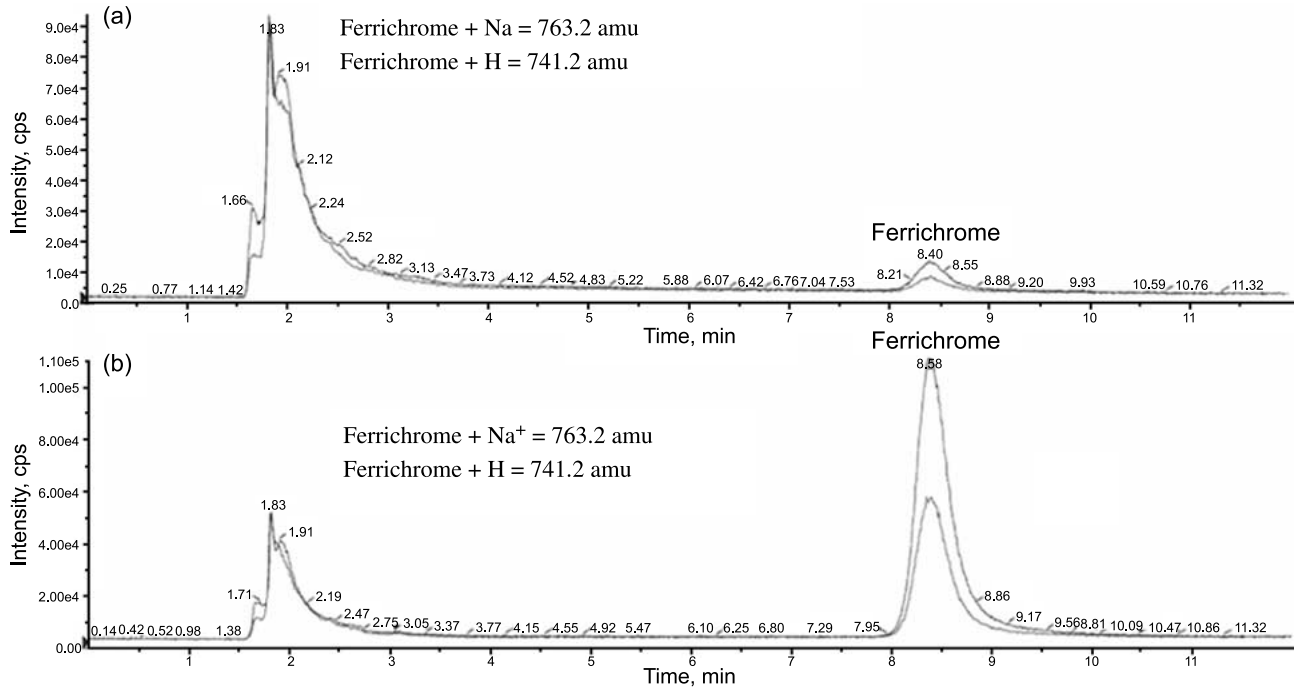

Figure 4. Chromatograms from Selected Ion Monitoring of (a) soil solution of a mor layer overlying granitic bedrock and (b) the same sample of soil solution with ferrichrome added. The analysis was performed on a PE Sciex API 3000 triple-quadrupole mass spectrometer using a C_{18} reversed phase column (Supelcosil LC-ABZ, 5 μm, 4 × 150 mm) with a mobile phase consisting of 10% methanol in 15 mM acetate buffer, pH 4.7.

Table 2. Ferrichrome in soil solution and mycorrhizal solution determined from HPLC/MS data.

Sample	Soil solution, ferrichrome (nmolar)[a]
Horröd, 0–5 cm	1.4 ± 0.1
Heden, mor	1.1 ± 0.3
Pottäng, mor	0.9 ± 0.01

[a]Concentrations corrected for the preconcentration, concentrations ± confidence limits (95%).

Ferrichrome was found in nanomolar concentrations in all soil solutions investigated (Table 2) and ferricrocin only in the solution from the mor layer covering a rock and in the mor layer solution from the north of Sweden, Heden.

The presence of siderophores in soil has previously only been inferred from bioassays by growing the hydroxamate siderophore auxotrophic bacterium *Microbacterium flavescens* JG-9 in water extracts of soil (Powell et al. 1980; Reid et al. 1984). *M. flavescens* JG-9 is a mutant strain unable to produce its own siderophore and can only grow when provided with an external source of hydroxamate type siderophores. The concentrations of siderophores in soil solutions by this bioassay were estimated to 10^{-7} – 10^{-8} M (10% soil moisture) in aqueous extracts from grasslands, areas of mixed herbaceous vegetation and coniferous and deciduous forests across the USA. The *M. flavescens* JG-9 bioassay has also been used by Powell et al. (1982) to establish the presence of hydroxamate siderophores in soils from 49 sites representing 22 different taxonomic subgroups of soil. The obtained concentrations ranged between 228 and 279 nM in aqueous extracts (10% soil moisture) (Powell et al. 1982). These concentrations are sufficient to affect nutrient uptake by plants (Powell et al. 1980). However this bioassay can only reveal the total concentration of hydroxamate siderophores in solution. Ferrichrome-type siderophores can also be estimated from soil-water extracts by an *E. coli* bioassay. If the soil-water extracts contain ferrichrome type siderophores the presence of EDDHA (ethylenediamine[di(o-hydroxyphenylacetic)acid]) which reduces available iron, stimulates the growth of *E. coli* K-12 strains containing the ferrichrome membrane transport protein FhuA. The concentrations of ferrichrome-type siderophores in the soil-water extracts established with this bioassay were approximately 78 nM compared to 34 nM for the same soil when determined by the *M. flavescens* JG-9 bioassay (Powell et al. 1983). In our experiment we could determine the concentrations of the individual siderophore ferrichrome see Table 2. The concentrations of ferrichrome were in the nM range. The low concentrations in soil solution can be the result of adsorption of hydroxamate siderophores to the soil (Powell et al. 1980, 1982; Cline et al. 1983). The centrifugation drainage technique described by Giesler and Lundström (1993) that was used to obtain soil solution at a speed of 14,000 rpm for 30 min may not be enough to drain the pores possibly containing siderophores. An ultra-centrifugation technique might be needed to drain the micropores.

In pure culture experiments by Haselwandter et al. (1992) the ericoid mycorrhizal fungi *Hymenoscyphus ericae* and *Oidiodendron griseum* have been found to exude ferrichrome type siderophores. In addition the ectomycorrhizal fungus *Cenococcum geophilum* has been found to exude ferricrocin (Haselwandter and Winkelmann 2002).

Siderophores are primarily recognised to be of fundamental importance for iron acquisition by microbes and plants. However, experiments on their ability to dissolve minerals have also been performed. It is known that weathering of microcline and biotite is stimulated by ectomycorrhizal infection of *P. sylvestris* plants (Wallander et al. 1997; Wallander and Wickman 1999). Beech plants infected by the ectomycorrhizal fungus *Laccaria laccata* contained more P, Mg, Fe and K than non-infected plants (Leyval and Berthelin 1989). Hydroxamate siderophores produced by the ectomycorrhizal fungus *Suillus granulatus* as well as the siderophore desferrioxamine mesylate were more efficient in dissolving goethite than citrate and oxalate, showing the impact of siderophores in weathering of this mineral (Watteau and Berthelin 1994). The release rate of Fe from hornblende was enhanced when the bacterial genus *Streptomyces* was present, probably due to exudation of a catecholamide siderophore that may be a mixed ligand siderophore (catechol-hydroxamate) (Kalinowski et al. 2000; Liermann et al. 2000). The hydroxamate siderophore desferrioxamine mesylate also enhances the release rates of Fe as well as Al and Si from hornblende (Liermann et al. 2000).

If fungal hyphae create corroded microsites in mineral grains, these are likely to be formed by exudation of chemical compounds, which interact with the mineral structure and dissolve it. Citric, oxalic and fumaric acids have been found in soil solution (Westergaard Strobel et al. 1999; van Hees et al. 2000). These and other LMM organic acids in podzolic soil solution were determined to be in the range of $<$1–1100 μM and 25–45% of the dissolved Al in the soil solution was bound to these LMM organic acids (van Hees et al. 2000). In laboratory experiments the dissolution rate of feldspars has been shown to increase in the presence of organic acids (van Hees et al. 2002). However even if these acids probably play a role in creating corroded microsites, siderophores are stronger complexing agents because of their extraordinarily high complex formation constants. The chemical identification of siderophores in soil solution in the present study indicate that these compounds exist in boreal forest podzols, and further functional studies of their possible role in mineral weathering are now an important research priority.

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