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Tian, Zhaomo

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ZHAOMO TIAN
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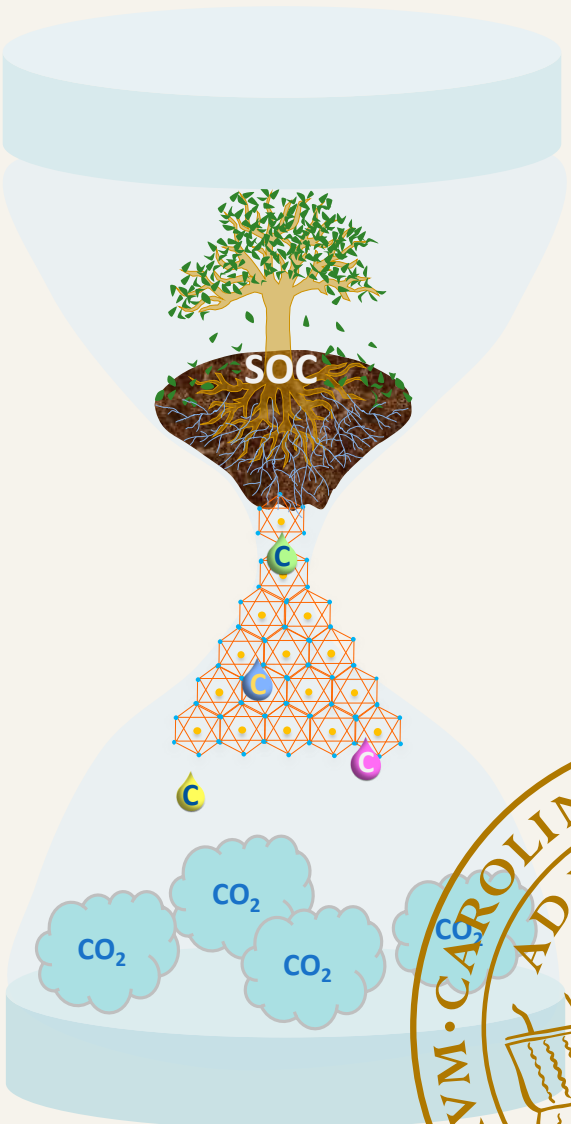

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Zhaomo Tian

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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University of Massachusetts Amherst, USA

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Properties and fungal decomposition of iron oxide-associated organic matter

Abstract

Soil organic matter (SOM) stores the largest quantity of C in terrestrial ecosystems. Most SOM can be decomposed by microorganisms and released as CO_2 into the atmosphere. The remaining fraction of SOM can be resistant against microbial decomposition over centuries to millennia. Changes in the magnitude of this stable SOM pool can have a substantial effect on the atmospheric CO_2 concentration and thus the extent of global warming.

In this thesis, the formation properties and persistence of mineral-associated organic matter (MAOM) were investigated in respect to adsorption-desorption properties and enzymatic/fungal availability of iron oxide mineral-associated OM. The effects of environmental factors on these processes were also tentatively evaluated. These studies revealed that MAOM is highly variable in terms of chemical composition, desorption rate and availability to enzymes and fungi. The composition of MAOM depends on the chemical composition of source DOM and the order in which DOM is exposed to mineral surfaces (Paper I). It can be further influenced by the fungal processing of DOM, either via modifications of sizes and chemical structures of DOM, or by the secretion of fungal metabolites (Paper II). Accordingly, factors affecting the fungal processing of DOM, such as the ammonium concentration, also have an impact on the composition of MAOM (Paper III). MAOM could be more dynamic than previously thought, as supported by the findings that the iron oxide mineral-associated proteins can be hydrolysed by a fungal enzyme (Paper IV) and N contained in iron oxide mineral-associated proteins can be assimilated by a common ectomycorrhizal fungus (Paper V). Another novel finding associated with the bioavailability of iron oxide mineral-associated proteins is that the proteolysis of proteins occurs directly at the mineral surfaces without a prior desorption step of the substrate protein (Paper V). This supports the idea that the enzyme-substrate (ES) complexes crucial for proteolysis are formed at the mineral surfaces. Any factor influencing the formation of such ES complexes can have a profound effect on the proteolysis of mineral-associated proteins. As a result, the bioavailability of iron oxide mineral-associated proteins depends on the protein surface coverage, co-adsorption of competitive ligands, and fungal secretion of mineral-surface reactive metabolites (Paper IV and V).

Key words: SOM persistence, formation of MAOM, fungal decomposition of MAOM, adsorption and desorption, mineral-surface reactive metabolites, environmental conditions

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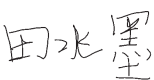
Signature  Date 2019-11-11

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MADE IN SWEDEN

It is the second tear that makes kitsch kitsch

-Milan Kundera, The unbearable Lightness of Being

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III. Wang, T., **Tian, Z.**, Tunlid, A. and Persson, P. (2019). Influence of ammonium on formation of mineral-associated organic carbon by an ectomycorrhizal fungus. Appl. Environ. Microbiol., 85(10), e03007-18.

IV. **Tian, Z.**, Wang, T., Tunlid, A. and Persson, P. Proteolysis of iron oxide-associated bovine serum albumin (BSA). Submitted.

V. Wang, T., **Tian, Z.**, Tunlid, A., Persson, P. Nitrogen acquisition from mineral-associated proteins by an ectomycorrhizal fungus. Submitted.

Author contributions

I. **Z.T.** and P.P. conceived and designed the experiments; **Z.T.** and T.W. performed the experiments; **Z.T.** and P.P. analyzed the data; **Z.T.** and P.P. wrote the paper with input from all authors.

II. T.W., P.B., A.T. and P.P. conceived and designed the experiments; T.W. and **Z.T.** performed the experiments and analyzed the data with input from P.B., A.T. and P.P.; T.W. wrote the paper with input from all authors.

III. T.W. designed the experiments under the supervision of A.T. and P.P.; T.W. and **Z.T.** performed the experiments and analyzed the data; T.W. wrote the paper with input from all authors.

IV. **Z.T.** and T.W. designed the experiments under the supervision of A.T. and P.P.; **Z.T.** and T.W performed the experiments; **Z.T.**, T.W. and P.P. analyzed the data; **Z.T.** and P.P. wrote the paper with input from all authors.

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Zhaomo Tian – Z.T.
Tao Wang – T.W.
Per Persson – P.P.
Anders Tunlid – A.T.
Per Bengtson – P.B.

Abbreviations and acronyms

ATR	Attenuated Total Reflectance
BET	Brunauer− Emmett−Teller
BSA	Bovine Serum Albumin
DOM	Dissolved Organic Matter
ECM	Ectomycorrhizal
ES	Enzyme-Substrate
FC	Forest Cold
FH	Forest Hot
FL	Forest Litter
FTIR	Fourier Transform Infrared Spectroscopy
H. pinastri	*Hydnomerulius pinastri*
IR	Infrared
LMW	Low-Molecular-Weight
MAOM	Mineral-Associated Organic Matter
MCR-ALS	Multivariate Curve Resolution-Alternating Least Squares
OM	Organic Matter
P. involutus	*Paxillus involutus*
SCM	Soil Continuum Model
SEC	Size Exclusion Chromatography
SIPT	Simultaneous Infrared and Potentiometric Titration
SOM	Soil Organic Matter
SSA	Specific Surface Area
TN	Total Nitrogen
TOC	Total Organic Carbon
XRD	X-ray Diffraction

Popular science summary

Despite more than 200 years of soil science, it is still unclear why some organic compounds in soils are able to persist over centuries to millennia without being rapidly respired by soil microbes and released to the atmosphere as CO_2. It is now believed that soil minerals play an essential role in retarding the decomposition rate of soil organic matter.

This is because soil organic matter, including chemically labile organic carbon compounds, can be protected from microbial decomposition once the organic compounds are adsorbed onto soil minerals, preserving them in soils for a very long time. The formed organic matter-mineral associations contribute to the persistence of soil carbon and any change related to this process will correspondingly regulate the atmospheric CO_2 concentration. Therefore, understanding the role of minerals in protecting soil organic matter against microbial decomposition will advance our knowledge of the processes that control global C cycling, and enable us to better predict the response of soil organic matter to climate change.

Adsorption of organic matter onto minerals is considered to be a major pathway to form organo-mineral associations. As soil organic matter is a heterogeneous mixture of organic compounds with varying mineral-binding affinity, and as the adsorption capacity of mineral surfaces is not infinite due to the limited adsorption sites, organic compounds with higher adsorption affinity will outcompete organic compounds with lower affinity to mineral surfaces, causing fractionation. Because mineral-associated organic compounds are more resistant to microbial decomposition, we need to identify what components of organic matter show a high affinity for mineral surfaces enabling them to be preferentially retained on minerals, and what factors control this process. The organic matter used in this study was extracted from forest litter by water, and then adsorbed on goethite which is an iron oxide mineral commonly found in soils. I found that the formation and properties of organo-mineral associations were affected by the organic matter composition and concentration, time of adsorption, intermolecular interactions between the organic components and solution pH, and in general carboxylate functional groups played an important role.

In order to study if the adsorption is reversible, the reverse process - desorption - was studied. A slow or negligible desorption was found throughout the studies, indicating that organic matter is strongly bound to the mineral surfaces.

As soil organic matter is continuously being decomposed by microorganisms, I studied whether microbial decomposition of organic matter will also affect the adsorption-desorption processes. I compared the adsorption of organic matter before and after fungal decomposition and found an enhanced adsorption of

organic matter after fungal decomposition. This enhanced adsorption was attributed to the modification of organic matter via depolymerization and oxidation, and via fungal secretion of metabolites.

Next, to answer the question why and to what extent minerals can protect soil organic matter against microbial decomposition, I studied the decomposition of iron oxide mineral-associated proteins by a fungal enzyme as well as by an ectomycorrhizal fungus. Protein was used as a representative for soil organic matter to decrease the complexity of the studied system, because it is ubiquitous in the soil and is an important N source. I found that a fraction of iron oxide mineral-associated proteins can be degraded either by the enzyme or by the fungus. The experimental data support that the formation of enzyme-substrate complexes on the iron oxide mineral surfaces is essential for the occurrence of the degradation of mineral-associated organic substrates, but this formation is restricted by the adsorption of enzymes to the vacant surface sites of minerals. Such restriction can be counteracted by microbial secretion and subsequent adsorption of mineral-surface-reactive metabolites, preventing enzyme adsorption.

Overall, this thesis advances our understanding of the formation and properties of organo-mineral associations. It further also points to a large variability in bioavailability of mineral-associated organic matter which depends on the microbial activity and environmental conditions, providing new insights into soil C persistence.

Introduction

Soil organic matter persistence in soil

Soil organic matter (SOM) is all the non-living organic constituents present in soil, including dead animal, plant and microbial tissues and fragments which are formed through incomplete microbial decomposition of above- and belowground organic residues (Trumbore 1997, Cotrufo et al. 2015). Soil organic matter stores the largest quantity of C in terrestrial ecosystems (Cotrufo et al. 2015, Sokol et al. 2019). Most SOM can be decomposed by microorganisms and released as CO_2 into the atmosphere. The remaining fraction of SOM can be resistant against microbial decomposition over centuries to millennia (Dungait et al. 2012, Sokol et al. 2019). Changes in the magnitude of this stable SOM pool can have a substantial effect on the atmospheric CO_2 concentration and thus the extent of global warming. Substantial research efforts into the dynamics of the SOM pool have therefore been initiated over the last few years (Sokol et al. 2019).

The classical view on long-term persistence of SOM attributed the persistence of these organic molecules to their inherent chemical recalcitrance, e.g., level of aromaticity. More recent studies, however, indicate that SOM stability is not completely correlated with its chemical composition, as presumably recalcitrant OM compounds such as aromatics can be decomposed at a fast rate if they are accessible to microbes with appropriate catabolism (Schmidt et al. 2011, Lehmann and Kleber 2015). Instead, emerging conceptual models highlight the importance of environmental controls on the formation of persistent SOM. These models propose that the persistence of SOM arises from complex interaction between the SOM components and the biological and physiochemical environment (Schmidt et al. 2011). This current view acknowledges the essential role of mineral-associated organic matter (MAOM) in the persistence of soil C (Lehmann and Kleber 2015). Some of the empirical evidence that emphasizes the importance of MAOM is: 1) a positive correlation between SOM storage and the reactive mineral content (Kaiser and Guggenberger 2000); 2) an inverse correlation between soil organic carbon turnover rates and abundance of reactive minerals (Torn et al. 1997, Masiello et al. 2004); c) a reduced microbial mineralization rate of mineral-associated organic C (Jones and Edwards 1998, Kalbitz et al. 2005).

A few mechanisms that could explain the persistence of SOM by its adsorption to minerals have been proposed. One plausible mechanism is that the formation of multiple strong chemical bonds between OM and minerals inhibits the mineralization of C. This could be due to the binding of crucial OM functional groups, needed for enzyme recognition and functioning, to mineral surfaces (Chevallier et al. 2003). Besides, changes in the conformation and/or electron distribution of organic molecules upon adsorption may inhibit microbes to detect or decompose substrates (Khanna et al. 1998, Kalbitz et al. 2005). The second conceivable mechanism is that minerals inhibit enzyme activity directly. This can occur due to (1) enzyme adsorption and immobilization, which causes a decreased efficiency of enzyme-substrate interactions (Allison and Jastrow 2006, Lammirato et al. 2010); (2) OM adsorbs into small pores (diameter < 20nm) and is effectively protected from microbial decomposition due to size exclusion of hydrolytic enzymes (Mayer 1994). Apart from these mechanisms, the reduced bioavailability of adsorbed OM can be caused by its decreased solubility and ability to diffuse towards microbial cell membranes compared to OM in solution (Marschner and Kalbitz 2003).

Formation of mineral-associated organic matter: Dissolved organic matter adsorption mechanisms

The SOM component that is quantitatively relevant for the adsorption and formation of MAOM is dissolved organic matter (DOM), which is the OM contained in the soil aqueous phase and operationally defined as OM molecules with sizes less than 0.45 μm (Kleber et al. 2015). The main origin of terrestrial DOM is plant litter material partially decomposed by microbes and subsequently leached into soil solution (Kaiser and Kalbitz 2012, Cotrufo et al. 2015). Also microbial exudates and necromass may contribute to DOM (Liang et al. 2017, Sokol and Bradford 2018). The typical broad compound classes of DOM consist of: carbohydrates, phenol-aromatics, lipids and nitrogen-rich compounds (Chenu et al. 2015, Paul 2016).

The adsorption of DOM to mineral surfaces and the subsequent formation of MAOM occur in both soil and aquatic systems (Kaiser and Guggenberger 2000, Kleber et al. 2015). However, the adsorption mechanisms are not completely understood. In general, six mechanisms involved in the adsorption of DOM on minerals have been proposed, including: ligand exchange, electrostatic interaction, polyvalent cation bridging, hydrophobic interaction, van der Waals interaction and hydrogen bonding (Lutzow et al. 2006, Philippe and Schaumann 2014). The energy associated with these bonds decreases from several hundred kilojoules per

mole (kJ mol^{-1}) to a few kJ mol^{-1} with ligand exchange often leading to more stable bonds than the rest of binding modes (Kleber et al. 2015). Evidence has shown that upon adsorption, DOM can attach to multiple adsorptive sites on minerals through different mechanisms simultaneously (Jardine et al. 1989, Kahle et al. 2003, Sodano et al. 2016). The relative importance of each mechanism to adsorption is governed by mineralogy, the characteristics of DOM and the environmental parameters (Lutzow et al. 2006, Kleber et al. 2015, Han et al. 2016). Important mineral properties include the type of surface functional groups, surface charge, reactive surface area, surface structure and porosity. Characteristics of DOM controlling adsorption are: concentration, functional group composition, electric charge, solubility, pKa, molecular size and weight. Environmental parameters such as solution composition, pH, ionic strength, diffusion, moisture and temperature largely influence the adsorption reactions as well. A combination of these properties in a specific system will determine the actual mechanisms involved in adsorption. Gu et al. (1994) found that at low pH, ligand exchange was the main mechanism for adsorption of organic carboxyl and phenolic OH groups to hydrous oxides (Gu et al. 1994). This is probably because low pH generally favors the formation of inner-sphere complexes, i.e. the formation of a direct bond between mineral surface and adsorbed molecules (Boily et al. 2000). In Ca^{2+} and Mg^{2+} enriched alkaline soils, or Fe^{3+} and Al^{3+} enriched acidic soils, polyvalent cation bridging can be the dominant mechanism responsible for binding negatively charged polysaccharides secreted by microbes with surfaces of expandable layer silicates. Weaker interactions such as hydrophobic interactions, van der Waals interactions or hydrogen bonding are often found between the uncharged or non-polar groups of OM and surfaces of non-expandable layer silicates (Lutzow et al. 2006).

The adsorption of DOM in subsoils, which are usually rich in Al and Fe (hydr)oxides, has been proposed to play an important role in SOM persistence. The adsorption capacity of these reactive mineral surfaces is not infinite however (Gu et al. 1994, Kaiser and Guggenberger 2007). Consequently, DOM components with higher adsorption affinities will outcompete those with lower affinities for the limited adsorption sites, causing adsorptive fractionation of DOM (Oren and Chefetz 2012, Heckman et al. 2013, Lv et al. 2016). Preferential adsorption of high molecular weight fractions (Wang et al. 1997, Hur and Schlautman 2003), hydrophobic fractions (Kaiser and Zech 1997, Guo and Chorover 2003), aromatic moieties and carboxylic rich fractions have been reported (McKnight et al. 1992, Gu et al. 1994, Wang et al. 1997, Lv et al. 2016). Particularly, when the concentration of DOM continuously increases, fractionation may induce displacement of previously adsorbed SOM molecules by strongly competing ones in solution (Kaiser and Zech 1998).

However, the adsorptive fractionation may not only result from the competition among DOM components for a limited number of mineral surface sites. Several studies indicated that OM adsorbs to mineral surfaces in a patchy pattern. A portion of adsorbed OM does not directly adsorb to mineral surfaces but instead binds to OM that is already adsorbed, forming multilayers of adsorption at mineral surfaces (Kaiser and Guggenberger 2003, Eusterhues et al. 2005, Wang and Xing 2005). This partially supports a recently proposed zonal model suggesting that DOM adsorbs to mineral surfaces in a zonal sequence (Kleber et al. 2007). This zonal model was proposed taking into consideration the large chemical diversity of DOM (Ohno et al. 2014, Lv et al. 2016) in which a continuum of hydrophobicity can be found. This continuum ranges from hydrophobic and nonpolar compounds dominant in alkyl and aromatic functional groups, to hydrophilic compounds containing predominantly hydrophilic and polar functional groups such as carboxyls and amines. The zonal model divides the adsorbed organic matter into three discrete zones **(Fig. 1)**: 1. A contact zone, containing molecules in direct contact with the mineral surface; 2. A hydrophobic zone that is created as a result of the amphiphilic properties of molecules in the contact zone and through interactions with the hydrophobic ends of these molecules and other adsorbing hydrophobic molecules; 3. An outer kinetic zone that is formed via comparatively weak interactions with the part of the hydrophobic zone facing the solution and possessing a more hydrophilic character. This multilayer structure of adsorbed OM illuminates another reality for the adsorptive fractionation: whereas adsorption of molecules in the contact zone may be strictly limited by available sites at the mineral surfaces, the density of molecules distributed over the other zones are controlled by interactions between the organic components and only indirectly by available mineral surface sites. Thus the adsorptive fractionation should also be controlled by competitive effects among the organic components in the outer zones.

The zonal model also suggests that different zones are characterized by different exchange rates with the soil solution, ranging from slow in the contact zone to fast in the kinetic zone. This is presumably because bond strength between OM and mineral surfaces decreases from the contact zone to the kinetic zone. In this view it is mainly the slowly exchanging components of MAOM that are protected from further decomposition.

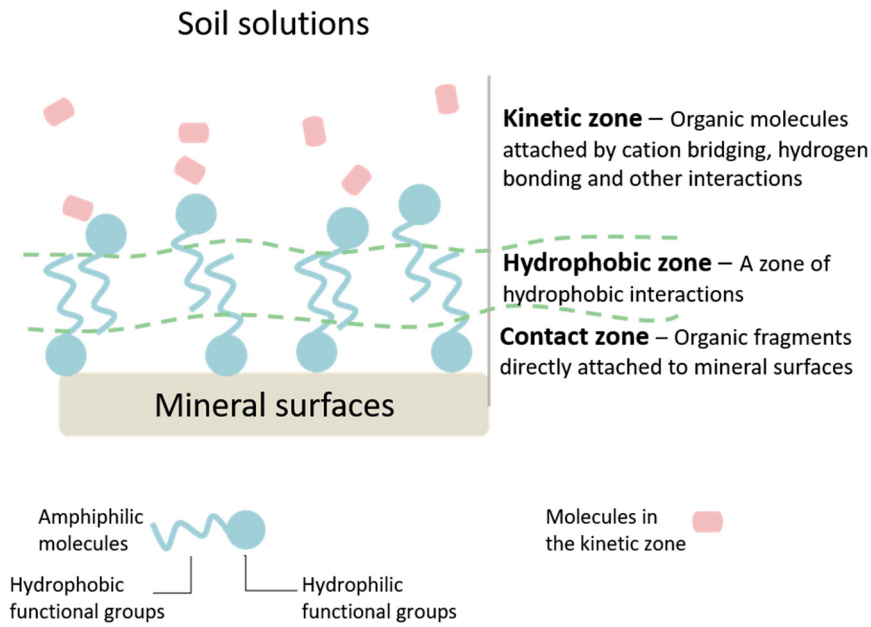

Fig. 1 A schematic representation of the conceptual model of organo-mineral interactions.
After Kleber, M., Sollins, P., Sutton, R., 2007. A conceptual model of organo-mineral interactions in soils: self-assembly of organic molecular fragments into zonal structures on mineral surfaces. Biogeochemistry 85, 9–24.

Microbes contribute to the formation and stabilization of mineral-associated organic matter

Microorganisms play two contrasting roles in regulating soil C fluxes: respiring CO_2 to the atmosphere, and transforming C into a form that is more resistant to decomposition (Liang et al. 2017). The latter is mainly realized by two major pathways: (i) *ex vivo* transformation (involving the action of extracellular enzymes), including depolymerization and oxidation of SOM, resulting in SOM which is more resistant to microbial decomposition, and (ii) *in vivo* turnover pathways that, via the assimilation of organic matter, biosynthesis, growth and death, result in the release of stable microbially-derived materials such as necromass and metabolites (Cotrufo et al. 2015). Because soil C cycling is eventually the consequence of microbial growth and activity, the mechanisms involved in microorganism-mediated processes that lead to soil C persistence need to be understood.

The *ex vivo* pathway is described in a recently proposed soil continuum model (SCM) **(Fig. 2)**, which views SOM as a range of organic molecules that are

continuously being processed into smaller molecules by decomposing microorganisms (i.e., depolymerization). This is accompanied by an increasing degree of oxidation of the decomposition products, which increases their water solubility and, possibly, also their reactivity toward mineral particles and propensity to be incorporated into aggregates (Lehmann and Kleber 2015). Thus, the SCM emphasizes the important role of microbial decomposition in the formation of stable SOM. Though there is earlier evidence showing the enhanced adsorption after decomposition due to changes in the chemical properties of DOM (e.g. increased hydrophobicity) (Hunt et al. 2007), direct and complete evidence to support the SCM is still missing.

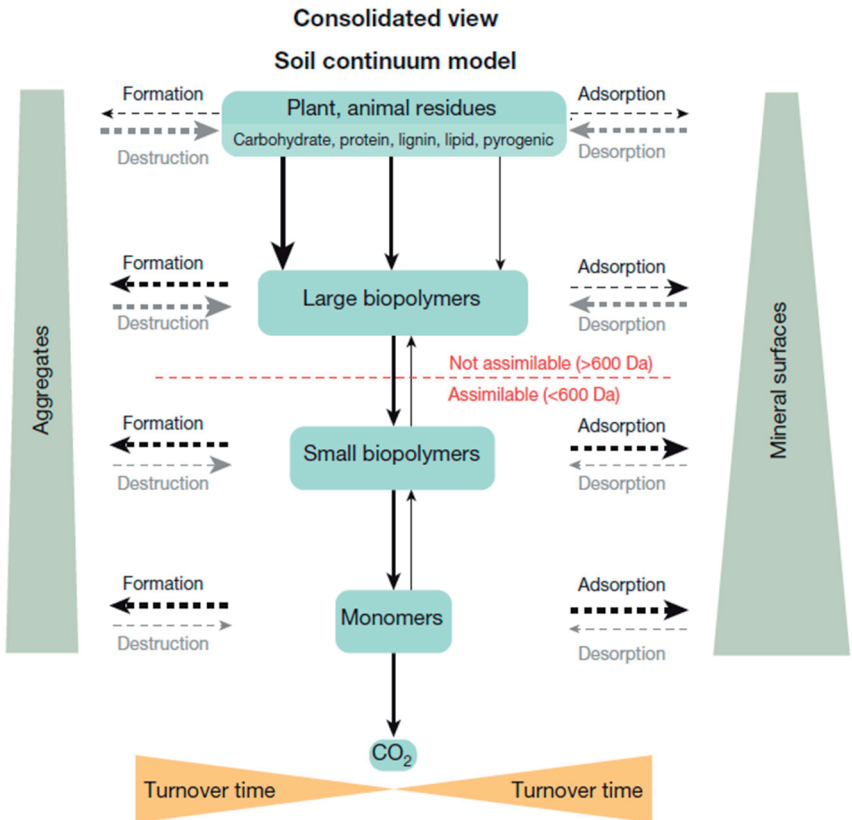

Fig. 2 Soil continuum model (SCM).
The proposed SCM emphasizes the continuous microbial processing of SOM and the protection of OM from decomposition by soil minerals. Dashed arrows denote mainly abiotic transfers, solid arrows denote mainly biotic transfers; thicker lines indicate more rapid rates; larger boxes and ends of wedges illustrate greater pool sizes; all differences are illustrative. All arrows represent processes that are a function of temperature, moisture and the biota present. Reprinted from Lehmann and Kleber (2015) with the permission from Springer Nature.

Historically, the formation of stable SOM has been less considered a result of the *in vivo* pathway but mostly of the *ex vivo* pathway. However, the *in vivo* pathway has recently received increasing amounts of attention thanks to modern analytical tools such as synchrotron-based spectroscopy becoming more readily available. Using these techniques, the chemical nature of mineral associated-OM has been found to be mainly of microbial origin rather than of plant origin. (Lehmann et al. 2007, Solomon et al. 2012). Moreover, physical fractionation methods have corroborated these findings by showing that microbially-derived C increased as particle density increased (Sollins et al. 2009). These studies emphasize the importance of microbially-derived compounds to form intimate associations with clay minerals, thereby possibly protecting them from further microbial decomposition.

Bioavailability of mineral-associated organic matter

The release of OM from mineral surfaces is often considered as a necessary prerequisite in order for molecules to be accessible for microbial degradation (Kleber et al. 2015). Such OM release can be caused by (1) desorption due to local disequilibrium, (2) displacement due to competitive adsorption and (3) mineral dissolution (Kleber et al. 2015). In accordance with this, Keiluweit et al. (2015) recently demonstrated that the strong protective capability of MAOM can be effectively counteracted by plant exudates-promoted mineral dissolution. On the other hand, some studies have indicated that microorganisms may degrade MAOM directly at the surfaces of minerals (Kaiser and Kalbitz 2012, Zimmerman and Ahn 2011).

Proteins are a substantial nitrogen source in soils (Chenu et al. 2015) provided that they can be hydrolysed into bioavailable small peptides or amino acids. Proteinaceous compounds often exhibit a strong affinity to soil mineral surfaces and a major fraction of soil proteins typically occurs associated to minerals (Aufdenkampe et al. 2001, Omoike and Chorover 2006). These associations limit the accessibility of soil extracellular enzymes to the substrate proteins (Chevallier et al. 2003). Accordingly, adsorbed proteins can have much longer turnover times than proteins in soil solutions or in particulate organic matter (Jilling et al. 2018). This is the reason why mineral-associated proteins rarely have been considered as an important source of bioavailable N (Jilling et al. 2018). At the same time, studies indicate that at least a part of the mineral-associated protein-N can be utilized by microorganisms (Pinck et al. 1954, Turner et al. 2017). Given the large size of the mineral-associated protein pool, proteolysis of even a small fraction could provide a substantial contribution to bioavailable soil N (Jilling et al. 2018).

It has been shown that protein adsorption by several different minerals is largely an irreversible process (Rabe et al. 2011). Consequently, direct proteolysis of mineral-associated proteins is expected to be the main process governing the microbial availability of this N pool. Indeed, proteolytic enzymes hydrolyse multilayers of adsorbed proteins on gold or functionalized silicon substrates, and the extent and rates of these reactions are sensitive to surface properties such as electrostatic charge at the water-solid interface (Foose et al. 2008, Feller et al. 2011). However, experimental evidence for proteolysis of proteins adsorbed onto reactive soil minerals such as secondary (hydr)oxides of aluminium and iron is scarce.

Aims

The aims of my PhD project were to understand the formation and composition of MAOM and to study the stability of MAOM against fungal decomposition. To achieve these aims, I investigated:

1. the time-resolved adsorption and desorption properties of MAOM. In order to mimic a soil situation where organic matter, mineral particles and an aqueous phase are simultaneously present, MAOM formation from DOM (leaching in situ from forest litter in the presence of mineral particles) was examined **(Paper I)**.
2. how fungal decomposition of DOM affects the formation and composition of MAOM **(Paper II)**. The central question was whether decomposition will enhance the formation of MAOM and if so, why? As N availability plays a critical role in the cycling of SOM, a follow-up study examined the effects of NH_4^+ addition on the fungal decomposition of DOM, and the consequences for the formation of MAOM **(Paper III)**.
3. whether a purified protease is capable of hydrolysing iron oxide-associated proteins **(Paper IV)**. If so, how do proteases access iron oxide-associated proteins and what are the factors affecting this accessibility? The enzyme used in this study was isolated from a soil-inhabiting fungus.
4. whether an ectomycorrhizal (ECM) fungus can acquire N from iron oxide-associated proteins and the mechanisms underlying this activity **(Paper V)**. This objective was an extension of objective 3 but was aimed to better assess the bioavailability of N in N-limited boreal forest soils. In these ecosystems, the mycelia of ECM fungi proliferate extensively into the deeper soil layers where mineral-associated proteins are common. The capacity of ECM fungi to decompose and assimilate N from mineral-associated proteins may largely affect the accessibility of this N pool to forest trees.

Main results and conclusions

Adsorption and desorption properties of iron oxide mineral-associated organic matter

Recent studies suggest that the structure, composition and kinetic properties of MAOM largely affect its resistance to microbial decomposition (Kleber et al. 2007). It is therefore crucial to characterize these properties of MAOM. I applied an infrared (IR) spectroscopic technique to monitor the in-situ adsorption and desorption of DOM at the water-goethite interface in real-time. The composition of the MAOM components and their corresponding adsorption and desorption rates were resolved from the IR data sets by means of multivariate curve resolution-alternating least squares (MCR-ALS) **(Paper I)**.

The formation of MAOM was compared between DOM directly leaching in situ from forest litter (FL) and DOM pre-extracted from the same forest litter with either cold (FC) or hot (FH) water. MAOM formed from FC was in general more similar to that from FL according to IR spectra **(Fig. 3)**. However, there was a tendency for the aromatic component in MAOM to be more pronounced when it was formed from FL at pH 4. This could partially be attributed to the strong adsorption of the aromatic compounds to goethite, increasing the leaching of this type of organic molecules from litter, specifically enriching the MAOM with aromatic compounds compared to pre-extracted FC. In contrast, MAOM formed from FH is substantially different from that leached from litter. The main difference is the high concentration of carbohydrates in MAOM derived from FH **(Fig. 3)**. The similarity between MOAM formed from FL and FC, and the larger difference between MOAM formed from FL and FH, were reinforced by desorption results as well. The desorption of MAOM showed that FL and FC displayed more similar desorption rates which were slower than FH. Moreover, the desorption from FL- and FC-goethite associations were dominated by similar OM components and were different from the OM components desorbed from FH-goethite associations.

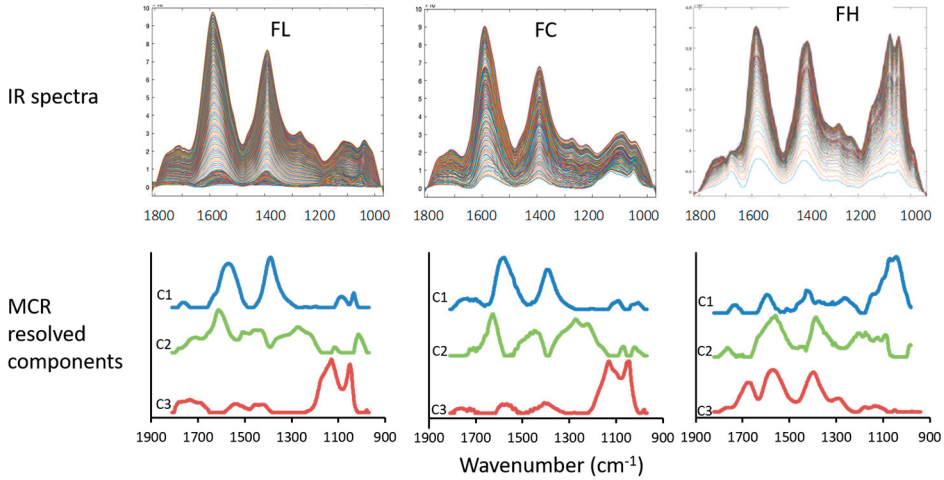

Fig. 3 Adsorption of FL (left), FC (middle) and FH (right) on goethite as a funtion of time at pH 4.
Shown are IR spectra data sets (top row) and the corresponding spectra of three components resulting from MCR-ALS anaysis (bottom row). Extracted from paper I.

Comparatively fast adsorption rates and slow desorption rates of MAOM were observed, suggesting that the exchange of initially formed MAOM with newly added DOM is slow. This emphasized the importance of the initial DOM composition for the build-up of MAOM. Interestingly, a weak adsorption continued to occur when DOM concentrations were further increased to the saturation point of goethite adsorption sites **(Fig. 4).** This may result from the re-arrangement of the already adsorbed organic compounds, creating new adsorption sites for incoming organic molecules. This may lead to the confinement of some adsorbed organic molecules into smaller spaces at the mineral surface and result in a patchy distribution of OM on mineral surfaces.

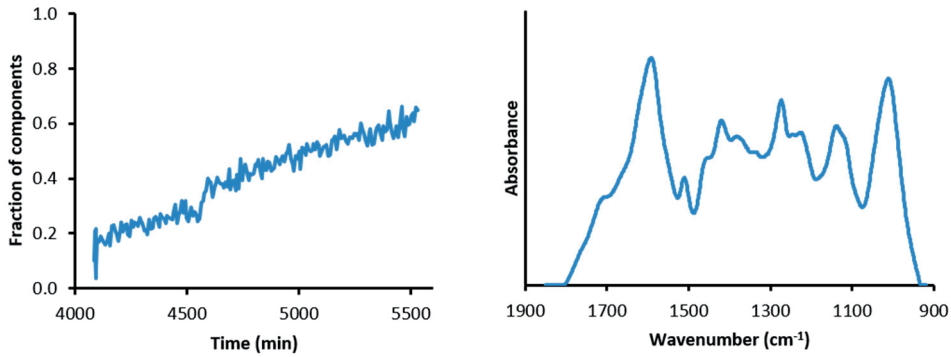

Fig. 4 A MCR resolved component showing the continued increase of adsorption close to the saturation of goethite adsorption sites.
(Left) Evolution over time of the concentration of the adsorbing component normalized to the total adsorption at the end-point. (Right) IR spectrum of the MCR component. Shown is an example from a FC sequential adsorption experiment at total organic carbon (TOC) concentration of 506 µmol m^{-2} and pH4. Extracted from paper I.

Overall, these results complement previous adsorption studies (which have mainly probed the changes of OM composition in solution), with direct, time-resolved observations of the formation of MAOM at water-goethite interface. The main conclusion is that the adsorption and desorption properties of MAOM formed on goethite from in-situ leached forest litter can be explained by a limited set of components (2-3), despite the chemical diversity of DOM. In agreement with previous studies (Avneri-Katz et al. 2017), our results showed the preferential adsorption of lignin-like aromatics and carbohydrates. Beyond this general agreement, our study demonstrated a strong contribution from carboxylate functional groups to both adsorption and desorption processes at all studied pH values.

Fungal decomposition of dissolved organic matter and the effects on the formation of iron oxide mineral-associated organic matter

In **paper I,** the effects of DOM composition and concentration on the adsorption and desorption properties of MAOM were investigated. In **paper II**, I continued this research by exploring how fungal decomposition of DOM affects the formation of MAOM. DOM decomposition was studied using an ECM fungus, *Paxillus involutus* (*P. involutus*), and a saprotrophic brown-rot fungus, *Hydnomerulius pinastri* (*H. pinastri*). Both fungi are widespread in boreal forests, are phylogenetically closely related (both are members of the Paxillaceae family)

but play very different ecological roles (Leake et al. 2002). Saprotrophic brown-rot fungi decompose cellulose from dead trees (Lundell et al. 2014). ECM fungi, on the other hand dominate in deeper soil layers that are enriched in more decomposed and oxidized SOM. In these soil layers, saprotrophic fungi cannot efficiently acquire C needed for growth and they are taken over by ECM fungi, which obtain their energy from their host plants. In exchange for photosynthates, ECM fungi supply the host plants with nutrients, such as N and P (Lindahl et al.,2007, Lindahl and Clemmensen, 2016).

After decomposition by both fungi, a decrease in molecular size (depolymerization) and an increase in the average oxidation state of C in DOM was observed. This resulted in an increased reactivity of DOM toward goethite surfaces **(Fig. 5)** and agrees well with the view of SOM formation and stabilization proposed by the SCM (Lehmann and Kleber 2015). In addition, these fungi secreted substantial amounts of aromatic metabolites that made up a significant fraction of the modified DOM. Some of the metabolites were mineral-surface reactive and also contributed to the enhanced formation of goethite-associated OM **(Fig. 5)**. These findings further also fit well into the conceptual framework proposed by Liang et al. (2017) that puts emphasis on the formation of stable SOM driven by both *ex vivo* and *in vivo* pathways described earlier.

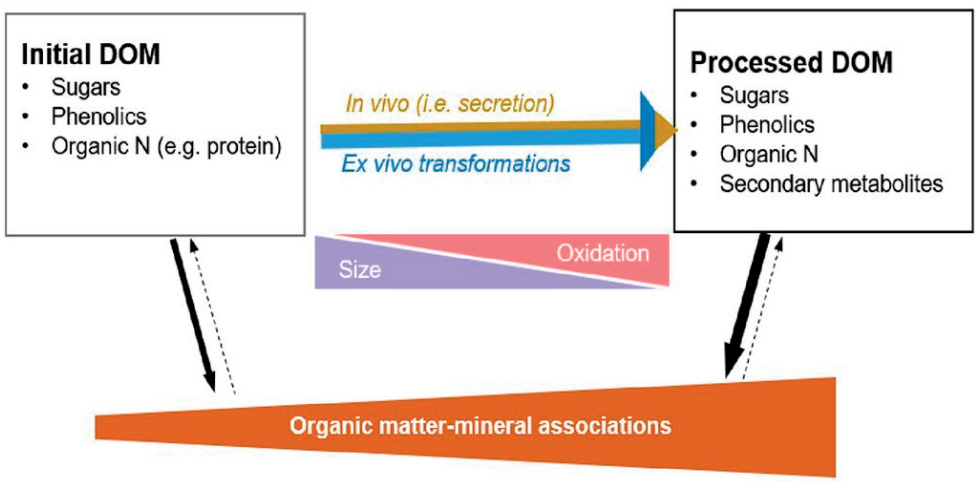

Fig. 5 Conceptual model describing the formation of MAOM influenced by fungal decomposition of DOM. Fungal processing of DOM can contribute to the formation of MAOM via two different pathways: extracellular transformation (*ex vivo*), including oxidation and depolymerization of compounds in DOM, and the secretion of fungal metabolites (*in vivo*). Both modifications can enhance the retention of the processed DOM on minerals. The relative importance of each mechanism is indicated by solid-arrow thickness. Dotted arrows indicate the desorption process. Figure from Paper III. Reprinted from Wang et al. (2019) with open copyright.

Effects of N availability

Soils in boreal forest ecosystems have experienced increases in N inputs through intentional N fertilization and unintentional, atmospheric N deposition. Significant declines in the biomass and altered community composition of ECM fungi have been reported in response to N fertilization (Treseder 2004, Lilleskov et al. 2011), but less is known about how N addition affects the decomposition activity of ECM fungi. Results showed that NH_4^+ amendments decreased the assimilation of organic N, but the overall production of mineral-associated organic C was not significantly affected by NH_4^+ additions. However, both the *ex vivo* and the *in vivo* pathways leading to the formation of mineral-associated C were affected, but their responses to increased NH_4^+ levels were different **(Paper III)**. The decreased acquisition of organic N at higher NH_4^+ levels was not accompanied by changes in the degree of oxidation of the DOM. Instead, the extent of depolymerization increased. Moreover, increasing NH_4^+ levels resulted in a decreased secretion of C compounds but an increased secretion of N-containing compounds. The shifts in DOM processing pathways changed the chemical composition of MAOM, which will affect the properties of MAOM and, thus, ultimately, the persistence of SOM. The major changes related to the NH_4^+ additions are summarized in **Table 1**.

Table 1 Effects of increased NH_4^+ levels on DOM processing pathways and on the formation of iron oxide mineral-associated organic C.

''↑'' denotes an increase of the measured parameter between the processed organic matter (incubated for 7 days) and the initial DOM. Within a row, the number of arrows indicates the magnitude of these changes. Extracted from paper III.

Analysis	DOM	DOM+N
Formation of mineral-associated C		
Total organic C	↑	↑
Fungal C	↑↑	↑
Ex vivo transformation		
Depolymerization	↑	↑↑
Oxidation	↑	↑
In vivo turnover		
N secretion	↑	↑↑
C secretion	↑↑	↑

Enzymatic hydrolysis of iron oxide mineral-associated proteins

Proteins associated with mineral particles need to be hydrolysed into amino acids or small peptides in order to become available to microbes and plants. However, the strong associations between proteins and soil minerals restrict such proteolytic reactions (Chevallier et al. 2003). Previous studies have shown that adsorption of proteases by minerals decreases their proteolytic activity (Zimmerman and Ahn 2011). Moreover, it has been suggested that strong adsorption of enzymes to mineral surfaces limits their activity if their substrate is also adsorbed on the minerals. This physical disconnection between substrate and enzyme results in a low rate of formation of enzyme-substrate (ES) complexes necessary for proteolysis (Fontana et al. 1993). In this thesis, I combined size exclusion chromatography (SEC) and in-situ IR spectroscopy to investigate if and how a model protein (bovine serum albumin - BSA) is accessible to an aspartic fungal protease when the protein is associated with iron oxide minerals. We also identified the factors that suppress enzyme adsorption to iron oxides and promote the formation of ES complexes.

Results showed that the ES complexes required for proteolysis to proceed were formed at the iron oxide surfaces. Proteolysis of the iron oxide-associated BSA occurred directly at the mineral surface without the need for initial desorption of BSA. Concurrently, the protease was adsorbed to vacant iron oxide surface sites, significantly slowing down the rate of proteolysis (**Fig. 6)**. This inhibiting effect could be counteracted by the presence of pre-adsorbed phosphate **(Fig. 7A vs. D)** or by increasing the BSA coverage on minerals **(Fig. 7A vs. B)**. Increasing protease concentration, increased the probability of interactions between BSA and the protease and thus enhanced the proteolysis as well **(Fig. 7A vs. C)**.

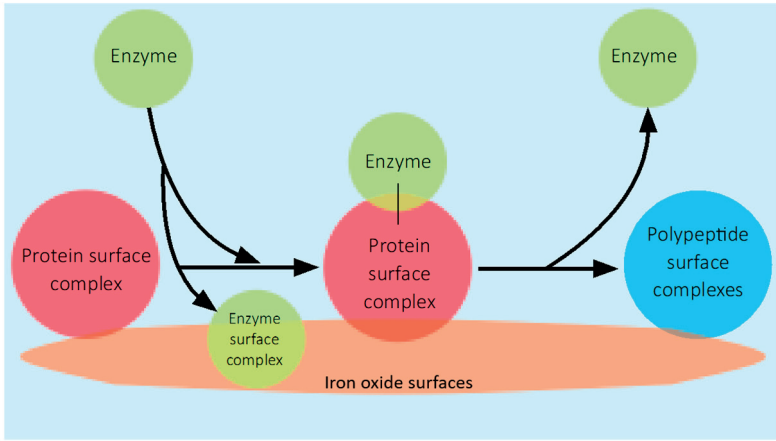

Fig. 6 A schematic representation of proteolysis of iron oxide-associated proteins via the formation of ES complexes at the iron oxide surfaces.
Figure from Paper IV.

These results challenge the notion that desorption of the substrate is a prerequisite for enzymatic decomposition of mineral-associated organic matter (Mikutta et al. 2007). Furthermore, the results highlight a large variability in the proteolysis of mineral-associated proteins which involves the formation of essential ES complexes. The formation of ES complexes depends on the interplay between different interacting factors such as competitive adsorption between enzymes and ligands, the level of particle aggregation, and the effect of ions on the enzyme activity. This points to a large complexity in bioavailability of mineral-associated proteins. Whether soil proteins are adsorbed to mineral surfaces or not will not be a precise indicator for their bioavailability as an N source.

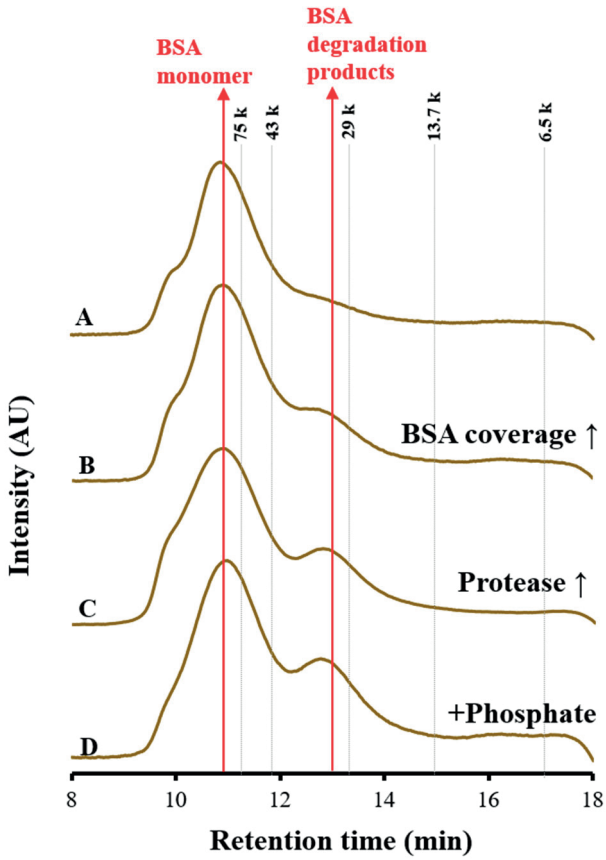

Fig. 7 Area normalized size exclusion chromatograms of the proteolysis of iron oxide-associated BSA
Extracted from Paper IV.

Fungal decomposition of iron oxide mineral-associated proteins

In **Paper IV,** we have shown that an aspartic fungal protease hydrolyses iron oxide-associated BSA via direct formation of ES complexes at the mineral surface. Still, it is unknown whether such mineral-associated proteins are bioavailable. Accordingly, we tested the ability of the ECM fungus *P. involutus* to acquire N from iron oxide-associated proteins by incubating *P. involutus* with iron oxide-associated BSA in microcosm systems **(Paper V)**. This study is especially relevant in N-limited boreal forest ecosystems where trees rely on ECM fungal symbionts to obtain N from soil.

Our results show that *P. involutus* secreted extracellular proteases which hydrolyse the iron oxide-associated BSA and that the degraded products were assimilated by the fungus. The fact that no BSA was desorbed, neither under disequilibrium conditions nor through ligand exchange reactions, indicated that N was assimilated directly from iron oxide-associated BSA without initial desorption of BSA. This agrees with the study in **paper IV**, showing that the desorption of OM is not necessary for microbial decomposition and nutrient acquisition.

We also found that the metabolites secreted by the fungus significantly increased the assimilation of N from adsorbed BSA **(Fig. 8)**. We have shown that the secreted metabolites are surface-reactive **(**in **Paper II and III).** Thus we attribute this effect mainly to metabolite adsorption preventing protease adsorption, thereby increasing the formation of ES complexes and the overall efficiency of the proteolytic reactions **(Fig. 9B vs. A)**. These results agree with the promotion of proteolysis of iron oxide-associated proteins by co-adsorption of phosphate, as exemplified in the previous section and the findings in **paper IV**.

Overall, the results demonstrate the ability of ECM fungi to generate extracellular proteases in combination with surface-reactive metabolites, which potentially make them efficient in hydrolysing mineral-associated proteins. This implies that mineral-associated organic N may be an important and overlooked N source for boreal forest trees.

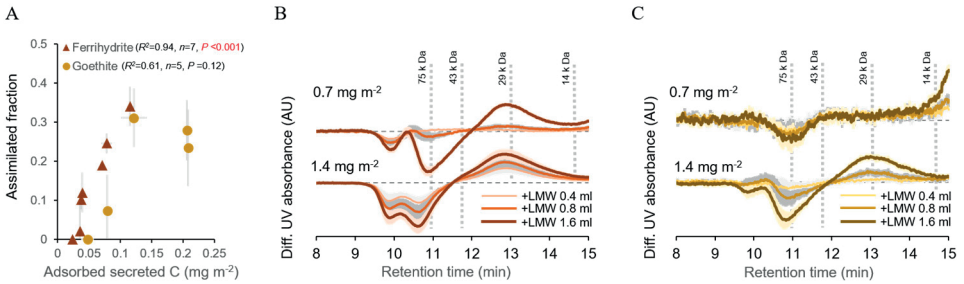

Fig. 8 Increased assimilation of N from iron oxide-associated BSA by the secretion of metabolites.
(A) Correlation between N assimilated from iron oxide-associated BSA and adsorption of secreted fungal C. Increased proteolysis of ferrihydrite-associated BSA (B) and goethite-associated BSA (C) with increasing amounts of the deactivated low-molecular-weight (LMW) organic compounds (i.e. metabolites). Figure from Paper V.

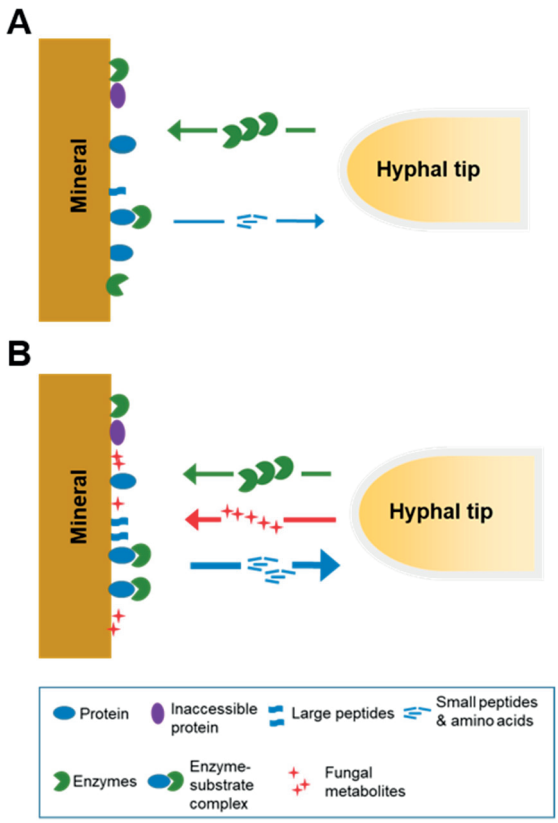

Fig. 9 A schematic representation of mechanisms that control the assimilation of iron oxide mineral-associated proteins by the ECM fungus *P. involutus*.
(A) Proteolysis of iron oxide mineral-associated proteins by extracellular proteases secreted by the fungus. (B) The proteolysis is facilitated by fungal secretion of surface-reactive metabolites. Such metabolites can prevent the adsorption of proteases on the mineral surface, and thereby promote the formation of enzyme-substrate complexes. Figure from Paper V.

Environmental perspectives

Traditionally, SOM persistence has been mainly attributed to the chemical recalcitrance of OM, which assumes that certain molecular structures of SOM (e.g. aromatic and/or long aliphatic chains) endow SOM with resistance to microbial decomposition. However, recent advances in SOM research suggest that it are the interactions between the chemical components of SOM and the biological, chemical and physical aspects of natural environments that determine the fate of SOM; i.e., whether it is respired or stored for longer periods of time (Schmidt et al. 2011). Soil is the largest terrestrial pool of C, containing nearly three times more C than vegetation and the atmosphere combined (Lehmann and Kleber 2015). It has been estimated that approximately one-quarter of soil organic C is associated with reactive Fe- and Al-bearing soil minerals. These minerals exhibit a strong adsorption affinity for OM and thus play a dominant role in the storage of SOM (Kramer and Chadwick 2018). The amount, persistence and sensitivity to climate change of mineral-associated C vary substantially across climates where OM sources and properties of soil minerals are different (Kramer and Chadwick 2018). This indicates that there is considerable variation in C stabilization capacity of soils. Mechanisms involved in the formation of MAOM within Earth's soils may vary substantially as well. Results in this thesis illustrate that this large variability in MAOM composition and properties are largely dependent on the chemical composition of source DOM composition. Furthermore, the composition of OM is continuously being altered by microbial decomposition through both *ex vivo* and *in vivo* pathways. Depending on the availability of N, the activity linked to the acquisition of organic N sources in these pathways can increase, decrease or remain unchanged, in turn, affecting the composition and properties of MAOM.

It is unclear if this complexity and heterogeneity of organo-mineral interactions will also lead to a large variation in microbial accessibility of MAOM, i.e. whether heterogeneous MAOM also exhibits a variety of levels of stability against microbial decomposition. Our results indicate that microbes may directly assimilate C and N from MAOM without the need for desorption and that the assimilation of these nutrients can be promoted through decreased enzyme adsorption to mineral surfaces and/or increased microbial activity. This implies that MAOM might be more dynamic than previously thought. Our experimental evidence also offers a mechanistic understanding of how mineral-associated

proteins can be utilized by ECM fungi in boreal forests, considering the strong adsorption of proteins to minerals.

The changing perception of the persistence of SOM has fostered the development of process-based SOM models which put an emphasis on the environmental processes governing soil C cycling. For example, organo-mineral interactions have been explicitly represented using a MAOM pool in models (Averill and Waring 2018) which has some advantages to evaluate the influences of mineral matrix-involved processes on soil C fluxes. Nonetheless, simply defining a MAOM pool as a microbially-inaccessible C pool (i.e., defining this pool as only determined by the balance between C adsorption and desorption rates (Robertson et al. 2019)) is insufficient to reflect the true heterogeneity of the MAOM pool. Firstly, the MAOM pool may be more dynamic than can be simulated in models. Taking the adsorption–desorption processes as an example, our results show that the composition of OM and initially adsorbed OM can largely affect the adsorption and desorption of MAOM formation. Secondly, C fluxes between the MAOM pool and microbial pool need to be assessed in each specific environmental context, such as in a boreal forest ecosystems. Acknowledging the complexity of MAOM-related processes and the fact that the reaction mechanisms involved in MAOM formation are still poorly understood, we argue that future models should take into account more the dynamic nature and heterogeneity of MAOM. This will enable models to better predict the contribution of MAOM in soil C dynamics and, eventually, its response to global environmental changes.

The environment-driven SOM persistence also sheds light on the degradation of OM in aquatic ecosystems. Despite large terrestrial inputs into rivers and oceans, only a small portion of the total OM pool in these systems is of terrestrial origin, based on the analyses of the biomarkers of terrestrial-derived compounds, such as lignin (Hedges et al. 1997). This indicates the terrestrial-derived C is readily mineralized by microorganisms across the soil–water interface. While the abundant soil mineral surfaces, the complex structure of the soil matrix and the lack of light in soil lead to the slow OM decomposition rates, the release of these constraints in aquatic ecosystems facilitates the microbial- and photo- degradation of OM (Kellerman et al. 2015). This agrees with the observation of an increased mineralization rate of OM during its transportation from soils to inland water where the protection offered by the soil matrix and minerals is lost (Marín-Spiotta et al. 2014).

Organo-mineral interactions also strongly influence the behavior of a broad range of nutrient elements, such as phosphorus (P) and sulphur (S) and contaminants, such as arsenic (As) and chromium (Cr) (Borch et al. 2010). For example, **Paper I** shows that the adsorption rate of phosphate onto mineral surfaces was influenced by the properties of MAOM. Moreover, the release and bioavailability of these

elements can be increased by mineral dissolution (Hinsinger 2001). Data in **paper V** illustrates that the reductive dissolution of iron oxides was decreased at an increasing protein surface concentration, suggesting that adsorbed OM might have an impact on the redox properties of iron minerals, in turn determining iron mineral dissolution and the bioavailability of co-adsorbed nutrients and pollutants. The redox reactions associated with the MAOM also mediate the oxidation or reduction of contaminants, thereby alternating their mobility and toxicity (Borch et al. 2010). For example, it has been shown that ferrihydrite-associated humic acid hindered the oxidation of As(III) on ferrihydrite (Xue et al. 2019). Moreover, it has been demonstrated that the redox reactions between redox-active SOM components, such as hydroquinone species and iron oxide minerals, can generate hydroxyl radicals (Krumina et al. 2017) that can oxidize soil organic contaminants into less toxic compounds.

Synthesis and outlook

In this thesis, the formation properties and persistence of MAOM were investigated in respect to adsorption-desorption properties and enzymatic/fungal availability of iron oxide mineral-associated OM. The effects of environmental factors on these processes were also tentatively evaluated. These studies revealed that MAOM is highly variable in terms of chemical composition, desorption rate and availability to enzymes and fungi. The composition of MAOM depends on the chemical composition of source DOM and the order in which DOM is exposed to mineral surfaces **(Paper I)**. It can be further influenced by the fungal processing of DOM, either via modifications of sizes and chemical structures of DOM, or by the secretion of fungal metabolites **(Paper II)**. Accordingly, factors affecting the fungal processing of DOM, such as the ammonium concentration, also have an impact on the composition of MAOM **(Paper III)**. MAOM could be more dynamic than previously thought, as supported by the findings that the iron oxide mineral-associated proteins can be hydrolysed by a fungal enzyme **(Paper IV)** and N contained in iron oxide mineral-associated proteins can be assimilated by a common ectomycorrhizal fungus **(Paper V)**. Another novel finding associated with the bioavailability of iron oxide mineral-associated proteins is that the proteolysis of proteins occurs directly at the mineral surfaces without a prior desorption step of the substrate protein **(Paper V)**. This supports the idea that the enzyme-substrate (ES) complexes crucial for proteolysis are formed at the mineral surfaces. Any factor influencing the formation of such ES complexes can have a profound effect on the proteolysis of mineral-associated proteins. As a result, the bioavailability of iron oxide mineral-associated proteins depends on the protein surface coverage, co-adsorption of competitive ligands, and fungal secretion of mineral-surface reactive metabolites **(Paper IV and V)**.

In conclusion, this thesis provides new insights into the formation and bioavailability of MAOM. Still many questions remain unsolved and warrant future studies.

- The structural architecture of MAOM may have important implications for its stability against microbial decomposition (Kleber et al. 2007). Although our studies provided insights into the temporal composition of MAOM, it is difficult to pinpoint whether and how the kinetic properties of MAOM are related to its spatial structure. If there is a link between the

two, we may be able to deduce how the structural architecture of MAOM is formed in-situ and evolved with time.

- We demonstrated that the adsorption of DOM to iron oxide minerals was enhanced after it has been processed by fungi. Yet, we found that this enhanced adsorption to mineral surfaces did not necessarily imply that the kinetic stability of MAOM also increased. Instead, the introduction of specific structural or functional group features to DOM during decomposition might be more important for the kinetic stability of MAOM. Therefore, a clearer link between the chemical properties of the microbial-modified DOM, the enhanced adsorption and biological stability needs to be established to better understand the role of microorganisms in the formation of stable SOM.
- Our study revealed that an ECM fungus, *P. involutus*, can assimilate N from iron oxide mineral-associated proteins directly. The metabolites secreted by this fungus enhanced the assimilation of N. Our study raises a number of questions: (i) How common are metabolite-enzyme-based decomposition mechanisms in microorganisms? (ii) How are the production of enzymes and metabolites co-ordinated and tuned to be efficient at water-mineral interfaces in real soils? (iii) Whether similar mechanisms facilitate acquisition of other nutrients associated with mineral surfaces, such as organic phosphorus (P)? By addressing these questions, the key microbial players and processes controlling SOM decomposition will be identified and explicitly incorporated into process-based models to better predict C and nutrients cycling.

Answers to these questions will not be easy to come to. We acknowledge that the reactions occurring in natural soils are far more complex than our simplified study system. However, insights into the biotic and abiotic mechanisms controlling SOM dynamics can be only obtained in experiments where the activity of microbial decomposers is carefully controlled, and the chemical transformations of SOM constituents and their properties can be examined in detail. Future research to develop long-term, controllable field experiments that can investigate mechanisms in-situ are vital to bridge the gap between fine-scale mechanistic lab-based research and field-scale research.

Main materials and methodologies

A schematic figure describing main experimental approaches that have been used in this thesis is shown in **Fig. 10**.

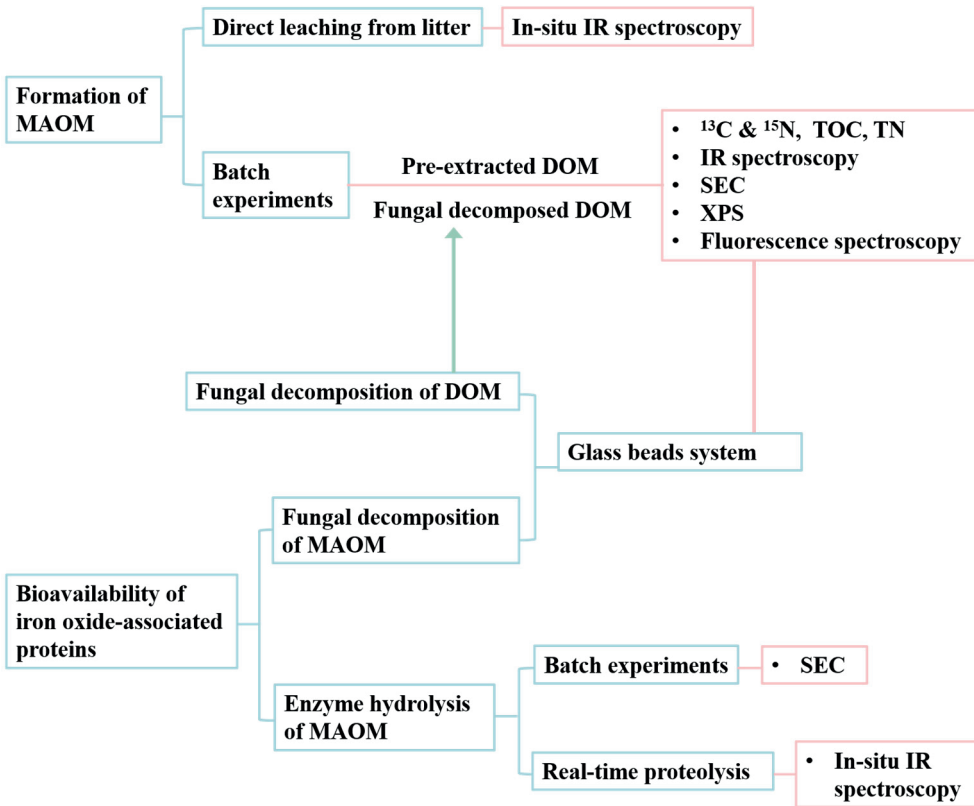

Fig. 10 Main experimental approaches in this thesis.

A brief description of the materials and methods are given below. More details can be found in each manuscript or paper.

Goethite and ferrihydrite synthesis and characterization

Goethite was synthesized according to the method described by Hiemstra et al. (1989). 6-line ferrihydrite was synthesized following the method of Schwertmann and Cornell (2008). Both ferrihydrite and goethite mineral phases were confirmed by X-ray diffraction (XRD) (Lyngsie et al. 2018). The specific surface area (SSA) of goethite was determined to be ca. 50-60 m^2 g^{-1} for different batches by N_2 Brunauer− Emmett−Teller (BET) (Brunauer et al. 1938). The SSA of ferrihydrite was estimated to be ca. 300 m^2 g^{-1} (Krumina et al. 2017).

Dissolved organic matter extraction

DOM was extracted from the forest litter collected from the upper 10 cm of the soil layer of a 61-yr-old Norway spruce stand in central Sweden. Two different extracts were prepared: a cold water extract (denoted FC) and a hot water extract (denoted FH) following the extraction method of Davidson et al. (1987). FC was obtained by shaking 120 g of sieved and mixed litter with 600 ml MilliQ water (i.e. a 1:5 w/v ratio) at room temperature for 24 h. FH was also obtained from a 1:5 w/v ratio between litter and MilliQ water but was boiled for 1 h. The supernatant was collected and subjected to a sequential filtration procedure using glass fibre filters (Grade GF/D; GF/A; GF/F; Whatman, Maidstone, UK) and a 0.22 μm sterile PES Membrane filter (Millipore Inc., Bedford, MA).

In paper I, MAOM was also produced by the in-situ leaching of organic litter from a nylon mesh bag (mesh size 50 μm) in the presence of mineral particles. This method includes the component of adsorption affinity to water solubility of OM as a driving force during the extraction of DOM.

In-situ IR adsorption-desorption experiments

We applied Simultaneous Infrared and Potentiometric Titration (SIPT) **(Fig. 11)** developed by Loring et al. (2009) to monitor adsorption and desorption processes at interfaces between aqueous solutions and mineral particles in-situ and in real-time. The measurements were based on the attenuated total reflectance (ATR)

sampling technique. The experimental setup consisted of an FTIR spectrometer (VERTEX 80v; Bruker, Ettlingen, Germany) and a single-reflection 45° ATR accessory (FastIR, Harrick Scientific, NY). The ATR crystal (composed of ZnSe) formed the bottom of a titration vessel and the vessel was attached to the ATR accessory, which was mounted in the sample compartment. A thin film of goethite or ferrihydrite was dried on the ATR crystal. Adsorption of OM to the minerals was monitored by collecting IR spectra over time after adding the OM into the titration vessel. A propeller stirrer ensured the homogeneity of the solution or suspension in the vessel. The pH of the reaction was controlled by an automatic burette system.

Desorption was accomplished by removing the contents from the titration vessel but leaving the goethite film on the ATR crystal. This step was followed by carefully adding an OM-free suspension or solution using a serological pipette. The only OM in the system was thus the OM that was pre-adsorbed on the goethite or ferrihydrite film. The OM-free suspension or solution created a thermodynamic driving force for desorption (Krumina et al., 2016).

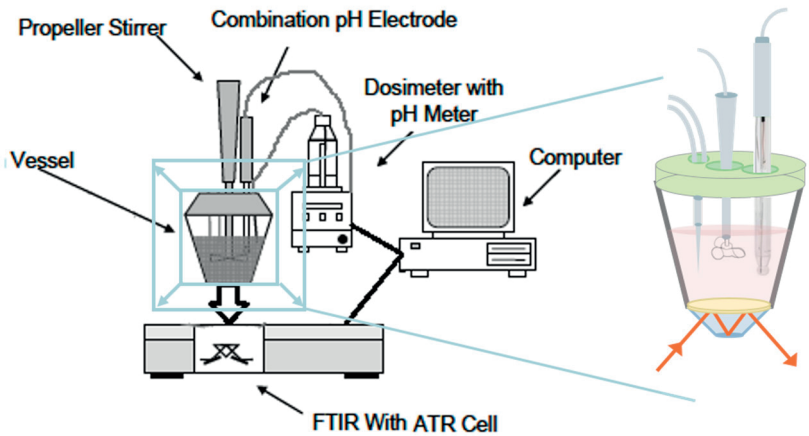

Fig. 11 Simultaneous Infrared and Potentiometric Titration (SIPT) setup.
Modified from Loring et al. (2009).

IR spectra collected over time were first pre-processed in OPUS software (v7.2, Bruker, Billerica, MA), and then analysed by means of the multivariate curve resolution-alternating least squares (MCR-ALS) formalism (Tauler and de Juan 2006) as implemented in the Matlab program PLS Toolbox v. 8.6 (Eigenvector Research, Inc). This way, variation in raw spectral data set can be expressed as a linear additive model of pure responses and contributions. In the context of our study, the responses and contributions were spectra and concentrations,

respectively. The basic equation associated with the MCR-ALS method is D= CS^T+E, where D ($r \times c$) is a matrix of the raw spectral data set. S^T ($n \times c$) is the matrix containing the spectra of pure components that explain the data variation in the column direction of the raw spectral matrix D, and C ($r \times n$) is the matrix containing the concentration profile of pure components that explains the data variation in the row direction of the raw spectral matrix D. E ($r \times c$) is the matrix that represents the residual variation. The variables r and c represent the number of rows and the number of columns of the raw spectral matrix, respectively, and n is the number of pure components.

MCR-ALS uses an approach to iteratively find the optimal solutions for C and S^T (Tauler and de Juan 2006). The procedure starts with the determination of the number of components. Then, the initial estimates of C or S^T will be calculated. Using the initial estimates, C and S^T will be optimized using least squares under constraints in each iterative cycle until convergence is achieved. Our modelling was performed with the constraint to obtain maximum contrast in the spectral dimension, and non-negative constraints were applied for both the spectra and the contributions. The general procedure is illustrated in **Fig. 12.** The ability of MCR-ALS to resolve spectral components and their corresponding contribution profiles depends on the complexity of the system and the extent of spectral and concentration overlap between each component. A pure component spectrum can only be obtained if the contribution profile is not completely embedded under the contribution profiles of other more dominating components (Tauler and de Juan 2006).

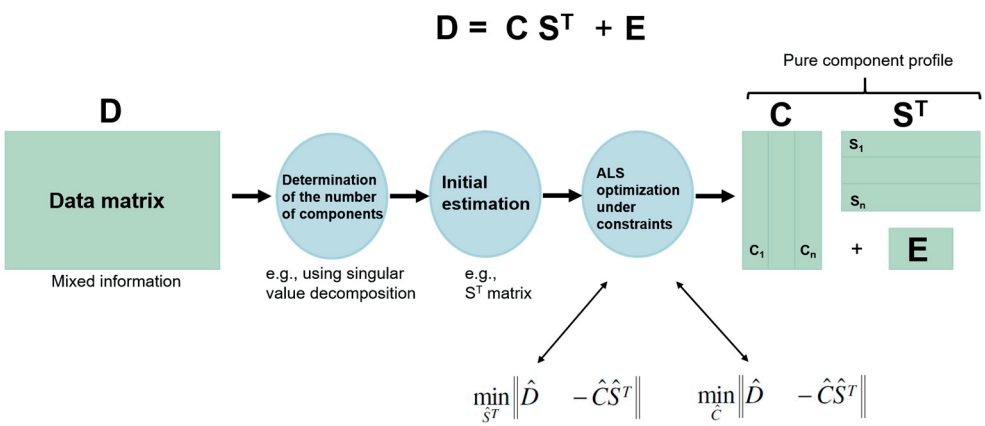

Fig. 12 Principal of MCR-ALS.

Batch adsorption experiments

Batch adsorption was conducted at room temperature (ca. 20 °C) in the dark by mixing an aliquot of ferrihdyrite or goethite suspensions with an aliquot of OM followed by shaking on an end-over-end shaker (PTR-35, Grant Instruments, Cambridge, UK). OM and experimental conditions vary in different studies and details can be found in each paper or manuscript.

Fungal species and decomposition experiments

Two fungal species were used in the study: *P. involutus* (Batsch) Fr. (strain ATCC 200175) and *H. pinastri* (Fr.) Jarosch & Besl. Both species are basidiomycetes and belong to the clade Boletales (Kohler et al., 2015). The ECM fungus, *P. involutus*, is widespread in the northern hemisphere and forms ectomycorrhizal symbioses with numerous species of coniferous and deciduous trees (Wallander and Söderström, 1999). The saprotrophic fungus, *H. pinastri*, is a brown-rot wood decaying fungus (Binder and Hibbett, 2006).

For the fungal decomposition experiment, both fungi were grown in Petri dishes on a layer of glass beads immersed in a liquid medium. To generate the fungal biomass, the fungi were first grown in Fries medium (Rineau et al. 2012) **(Fig. 13, stage 1, Biomass development)**. The Fries medium was then removed, and the mycelium and glass beads were washed with sterile Milli-Q (MQ) water. Fries medium without N was subsequently added to induce an N-deprived mycelium (Shah et al. 2013). After 24 h, the mycelium was washed with MQ water again, and the substrates were added for fungal incubation **(Fig. 13, stage 2, Decomposition)**. In our study, either FH or iron oxide mineral-associated protein (BSA) was used as the substrate. At the end of the incubation periods, the mycelia and substrates were collected for further chemical analyses.

^{13}C and ^{15}N labelling experiments

In order to distinguish organic compounds produced by the fungi from soil DOM or BSA, and to estimate C and N assimilations from DOM or BSA, fungal mycelia were labelled with ^{13}C and ^{15}N at both stage 1 and 2 when studying the decomposition of DOM, or only at stage 1 in the case of iron oxide mineral-associated BSA **(Fig. 13)**. To be more specific, to label the fungal mycelium with ^{13}C and ^{15}N at stage 1, the fungus was grown in Fries medium containing ^{13}C-glucose (ca. 10 atom% ^{13}C) and ^{15}N-NH_4^+ (ca. 2.3 atom% ^{15}N). To label the fungal

mycelium with ^{13}C and ^{15}N at stage 2, the substrate FH solution was supplemented with ^{13}C-glucose (ca. 10 atom% ^{13}C) and ^{15}N-NH_4^+ (ca. 2.3 atom% ^{15}N).

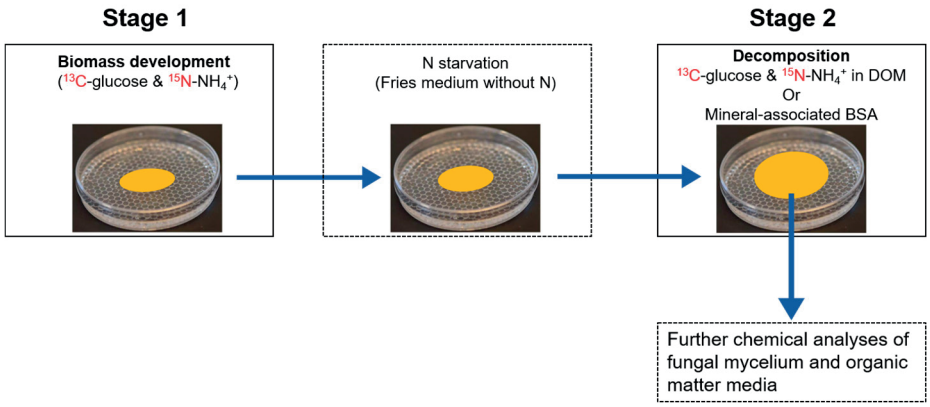

Fig. 13 Experimental setup for the fungal decomposition experiments.
Fungal biomass was generated during stage 1, by growing the mycelium in a mineral nutrient medium. The mycelium was subsequently starved for N for 24 h and washed with water, before the substrate was added (stage 2).

The assimilation of C and N from DOM or BSA by the fungi was calculated using isotope mixing models (Fry 2006). The amount of metabolic C and N secreted by the fungi was estimated using a similar principle. An example of fungal N assimilation from mineral-associated BSA **(Paper V)** is given to illustrate the procedure of calculation. Details of other isotope calculations can be found in each paper or manuscript.

Nitrogen in the fungal mycelium originated from two sources: BSA and ^{15}N-labled NH_4^+. Therefore, the abundance of ^{15}N in the mycelium ($At\%^{15}N_{fungus}$) at the end of incubation can be expressed as:

$$At\%^{15}N_{fungus} = f^{15}N_{ammonium} * At\%^{15}N_{ammonium} + f^{15}N_{BSA} * At\%^{15}N_{NA} \quad (1),$$

which is associated with the following constraint:

$$fN_{ammonium} + fN_{BSA} = 1 \quad (2),$$

where $At\%^{15}N_{ammonium}$ is the abundance of ^{15}N (atom%) in NH_4^+ that was present in the medium. $At\%^{15}N_{NA}$ is the abundance of ^{15}N (atom%) in BSA, equal to the natural abundance *(NA)* of ^{15}N. $fN_{ammonium}$ and fN_{BSA} are the fractions of fungal N originating from NH_4^+ and BSA, respectively.

After combining equations (1) and (2), the following are obtained:

$$fN_{ammonium} = \frac{At\%^{15}N_{fungus} - At\%^{15}N_{NA}}{At\%^{15}N_{ammonium} - At\%^{15}N_{NA}} \quad (3),$$

$$fN_{BSA} = \frac{At\%^{15}N_{ammonium} - At\%^{15}N_{fungus}}{At\%^{15}N_{ammonium} - At\%^{15}N_{NA}} \quad (4).$$

The amount of BSA N assimilated by the fungus ($mN_{BSA\_assimilated}$) can be calculated as:

$$mN_{BSA\_assimilated} = fN_{BSA} * mN_{fungus} \quad (5),$$

where mN_{fungus} is the total amount of N in the fungal biomass collected after the incubation.

Size exclusion chromatography (SEC)

SEC was performed on a high performance liquid chromatography system (Ultimate 3000; Thermo Scientific, Waltham, Massachusetts). This technique was used to examine how size distribution of DOM or BSA changes as a result of fungal/enzyme degradation or mineral interaction. Separation during SEC is based on the differences in sizes of biomolecules. Biomolecules with different sizes are retained more or less as they pass through a chromatography column packed with porous beads. Therefore larger molecules flow through the column more quickly than smaller molecules, so that larger molecule have shorter retention times than smaller molecules (Hong et al. 2012) **(Fig. 14)**.

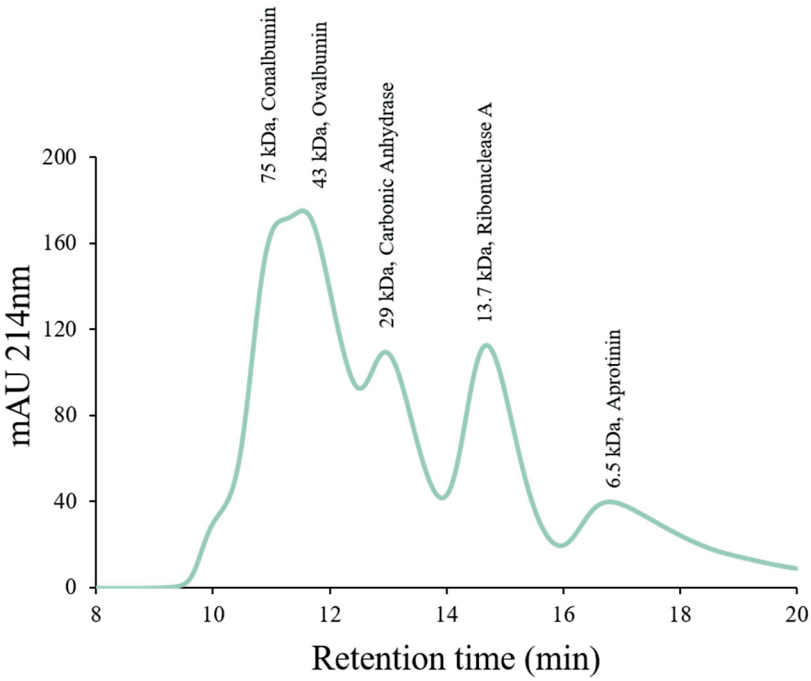

Fig. 14 Principal of SEC.
SEC of protein standards. Column: Superdex 75 column PC 3.2/30; Optimal separation range: 3-70 kDa; Mobile phase: 0.05 M phosphate in 0.15 M NaCl, pH 8.5; Flow rate: 0.1 ml min^{-1}.

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List of papers

I. Tian, Z., Wang, T., Tunlid, A. and Persson, P. Adsorption and desorption properties of mineral-associated organic matter formed by leaching from forest litter. Submitted.

II. Wang, T., Tian, Z., Bengtson, P., Tunlid, A. and Persson, P. (2017). Mineral surface-reactive metabolites secreted during fungal decomposition contribute to the formation of soil organic matter. Environ Microbiol, 19:5117-5129.

III. Wang, T., Tian, Z., Tunlid, A. and Persson, P. (2019). Influence of ammonium on formation of mineral-associated organic carbon by an ectomycorrhizal fungus. Appl. Environ. Microbiol., 85(10), e03007-18.

IV. Tian, Z., Wang, T., Tunlid, A. and Persson, P. Proteolysis of iron oxide-associated bovine serum albumin (BSA). Submitted.

V. Wang, T., Tian, Z., Tunlid, A., Persson, P. Nitrogen acquisition from mineral-associated proteins by an ectomycorrhizal fungus. Submitted.

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