Influence of ammonium on the formation of mineral-associated organic carbon by an ectomycorrhizal fungus

Tao Wang1,* , Zhaomo Tian1,2, Anders Tunlid1 & Per Persson1,2

1 Department of Biology, Microbial Ecology Group, Lund University, Ecology Building, SE-223 62 Lund, Sweden
2 Centre for Environmental and Climate Research (CEC), Lund University, Ecology Building, SE-223 62 Lund, Sweden

* Correspondence and material requests to: tao.wang@biol.lu.se (Tao Wang)

Running title: Formation of mineral-associated organic matter

Keywords: Ectomycorrhizal fungi, decomposition, soil organic matter, nitrogen addition, organic matter-mineral interaction
ABSTRACT

The interactions between dissolved organic matter (DOM) and mineral particles are critical for the stabilization of soil organic matter in terrestrial ecosystems. The processing of DOM by ectomycorrhizal fungi contributes to the formation of mineral-stabilized SOM by two contrasting pathways: extracellular transformation of DOM (ex vivo pathway) and the secretion of mineral-surface reactive metabolites (in vivo pathway). In this study, we examined how changes in nitrogen (N) availability affected the formation of mineral-associated carbon (C) from these two pathways. DOM was extracted from forest soils. The processing of this DOM by the ectomycorrhizal fungus *Paxillus involutus* was examined in laboratory-scale studies under different levels of ammonium. At low levels of ammonium (i.e. during N limited conditions), the DOM components were slightly oxidized and fungal C metabolites with iron reducing activity were secreted. Ammonium amendments decreased the amount of C metabolites and no additional oxidation of the organic matter was detected. In contrast, the hydrolytic activity and the secretion of N containing compounds increased, particularly when adding high levels of ammonium. During these conditions, N did not limit fungal growth, rather C. Though the overall production of mineral associated organic C was not affected by ammonium concentrations, the observed shifts in the activities of the *ex vivo* and *in vivo* pathways affected the composition of organic matter adsorbed onto the mineral particles. Such changes will affect the properties of the organic matter-mineral associations and thus ultimately the stabilization of SOM.
Nitrogen (N) availability plays a critical role in the cycling and storage of soil organic matter (SOM). However, large uncertainties remain in predicting the net effect of N addition on soil organic carbon (C) storage due to the complex interactions between organic matter, microbial activity, and mineral particles that determine the formation of stable SOM. Here, we attempted to disentangle the effects of ammonium on these interactions in controlled microcosm experiments including the ectomycorrhizal fungus *P. involutus* and dissolved organic matter extracted from forest soils. Increased ammonium levels affected the fungal processing of the organic material, as well as the secretion of extracellular metabolites. Though ammonium additions did not increase the net production of mineral adsorbed C, changes in the decomposition and secretion pathways altered the composition of the adsorbed organic matter. These changes may influence the properties of the organo-mineral associations, and thus the stabilization of SOM.
INTRODUCTION

Soil organic matter (SOM) stores the largest quantity of C in terrestrial ecosystems (1, 2). Microorganisms can decompose most SOM and that results in the release of CO$_2$ into the atmosphere. The remaining fraction of SOM is stabilized against microbial decomposition over centuries to millennia (2, 3). Changes in the magnitude of this stable SOM pool determines whether soils can act as a sink or a source of atmosphere CO$_2$ in response to environmental changes, and have therefore stimulated extensive research efforts (2).

Despite this research the mechanisms by which SOM is stabilized is not well understood. According to the classical view, SOM becomes stabilized via polymerization reactions that lead to the formation of recalcitrant humic substances (4, 5). However, the humification model have been increasingly questioned by findings indicating that stable SOM is not rich in humic polymers (6). Instead, it has been recently proposed that SOM becomes protected from microbial decomposition by interacting with mineral particles and by being incorporated into aggregates (7). Microorganisms contribute to the formation of such protected SOM by processing compounds in the litter material into smaller molecules (i.e. depolymerization). This is accompanied by an increased degree of oxidation of the processed products, which increases their water solubility and possibly also their reactivity toward mineral particles, and their propensity to form aggregates consisting of the assembly of discrete molecules (7). This emerging view highlights the critical importance of dissolved organic matter (DOM) in the formation of stable SOM. DOM is also considered the most reactive organic matter fraction in soils and subject to continuous microbial processing (8, 9).
Recent laboratory-scale experiments have shown that processing of DOM by both saprotrophic and ectomycorrhizal (ECM) fungi are able to enhance the formation of mineral-associated organic C, partly due to the depolymerization and oxidation of DOM (10). In addition, those fungi secrete substantial amounts (>10 % new biomass C) of metabolites during DOM processing. Some of the metabolites are mineral surface-reactive and also contribute to the enhanced formation of mineral-associated organic C (10). These findings fit into a novel conceptual framework that emphasizes the involvement of microorganisms in stabilizing SOM (11). It is proposed that microorganisms influence SOM formation by two major pathways: i) Ex vivo transformation involving the action of extracellular enzymes that transform SOM into a material which is more stable with respect to microbial decomposition; ii) In vivo turnover mechanisms that via the assimilation of organic matter – biosynthesis – growth – death, result in the release of stable microbial-derived material (11). In the context of DOM, the secretion of fungal metabolites is a main component of the in vivo turnover pathway (Fig. 1).

Besides saprotrophic fungi, ECM fungi are the major functional group of fungi in boreal forest ecosystems (12). ECM fungi obtain their energy (i.e. glucose) from their host plant and the mycelium is mainly located in deeper soil horizons that are enriched in decomposed and oxidized SOM (13). The ability of ECM fungi to decompose such SOM has been questioned due to the fact that these fungi have lost many genes encoding enzymes associated with plant litter decomposition that are present in saprotrophic fungi (14). However, recent studies have shown that ECM fungi have the capacity to decompose SOM using oxidative mechanisms (15-17). It has been suggested that these mechanisms are mainly used for mobilizing nutrients including nitrogen (N), which are entrapped in complex SOM (18).
Soils in northern forest ecosystems have experienced an unintended ammonium (NH$_4^+$) fertilization due to N deposition, and the effects of N additions on SOM decomposition have been extensively studied (19, 20). Many of these studies have shown that N deposition suppresses microbial activity and thereby decreases SOM decomposition (19, 21-23). Although the mechanisms underlying the retardation of decomposition are not entirely clear, several studies have shown that increased N levels can suppress the activity of plant litter-degrading enzymes, including phenol oxidases and peroxidases (19, 24, 25). In the case of ECM fungi, it is well known that increased NH$_4^+$ concentrations will reduce the biomass and alter the community composition of ECM fungi (26, 27). Less is known about how NH$_4^+$ affects the decomposition activity of ECM fungi. Laboratory-scale studies have shown that NH$_4^+$ amendments had only minute effects on the oxidation of SOM by the ECM fungus *Paxillus involutus* (28), whereas field studies suggest that NH$_4^+$ addition represses the oxidation decomposing activity of ECM *Cortinarius* species (17).

In this study we examined the effects of increased NH$_4^+$ levels on the processing of DOM, extracted by hot water from a forest soil, and the formation of mineral-associated organic C by *P. involutus* via two pathways; the extracellular transformation of DOM (ex vivo pathway) and the secretion of fungal metabolites (in vivo pathway) (Fig. 1). Previous experiments have shown that the decomposition of DOM by *P. involutus* is associated with the liberation and uptake of N (15). *P. involutus* assimilates N sources in a sequence, preferentially utilizing NH$_4^+$ over other N sources, such as proteins (29). Hence, our main hypothesis was that NH$_4^+$ additions will lead to decreased assimilation of organic N and thereby decreased ex vivo transformation of the DOM. SOM decomposition by *P. involutus* involves the action of hydroxyl radicals generated by Fenton chemistry, and secreted Fe(III)-reducing metabolites are needed to that drive this reaction (30).

Our second hypothesis was therefore that decreased ex vivo transformation will be associated
with a reduced secretion of metabolites, i.e., decreased activity in the in vivo pathway. Since we expect that both extracellular transformation and the secretion of fungal metabolites will be negatively affected by NH$_4^+$ amendments our third hypothesis is that also the formation of mineral-associated organic C will decrease with increasing NH$_4^+$ concentrations. In order to test these hypotheses, a firm control of the physiology of the fungus, characterization of DOM, and quantitative estimation of fungal metabolites is needed and this was accomplished in microcosm experiments where the fungus was grown on DOM amended with different levels of NH$_4^+$. We evaluated the extracellular transformation of DOM by analyzing the oxidation and depolymerization using Infrared (IR) spectroscopy and size exclusion chromatography (SEC), respectively. The secretion of secondary metabolites was estimated from the production of $^{13}$C and $^{15}$N enriched compounds in the processed DOM after labeling the fungal mycelium with stable isotopes ($^{13}$C and $^{15}$N). The formation of mineral-associated organic C was investigated by adsorption of initial and processed DOM on goethite, which is a ubiquitous soil iron mineral (31).

**RESULTS**

**NH$_4^+$ treatments.** The freshly extracted DOM contained 30 mg l$^{-1}$ of NH$_4^+$-N, whereas NO$_3^-$ was not detected. In order to study the effects of NH$_4^+$ additions, before the experiments a large part of the indigenous NH$_4^+$ was removed from the DOM by dialysis (cutoff: 1 kDa) (supplemental material, Fig. S1). After dialysis, the NH$_4^+$-N content was 4.4 mg l$^{-1}$. The total N content was 25.3 mg l$^{-1}$; thus, the organic N accounted for ca. 80% of the total N in the dialyzed DOM. NH$_4^+$-N was added at two levels to the dialyzed DOM, 15 mg l$^{-1}$ (denoted DOM+lowN) and 30 mg l$^{-1}$ (denoted DOM+highN; the concentration of NH$_4^+$-N in this medium was similar to that in the freshly extracted DOM). Two levels of NH$_4^+$-N were also added to a two-fold diluted DOM extract (i.e., 15 mg N l$^{-1}$ denoted dDOM+lowN, and 42 mg N l$^{-1}$ denoted dDOM+highN) (Fig. [7](#))
We expected to observe a greater extent of modification in the dDOM than the DOM treatments. Since previous experiments have shown that the decomposition of DOM by *P. involutus* only occurs in the presence of an energy source (28), the fungus was grown for seven days in the DOM and dDOM media supplemented with glucose. Part of the added NH$_4^+$ and glucose was labeled with $^{15}$N and $^{13}$C, respectively. Amendments with NH$_4^+$ in the DOM and dDOM significantly decreased the pH of the media at the end of the incubation period (Fig. S2), in agreement with previous reports (29). The pH values dropped by 0.4 units in the medium amended with the low NH$_4^+$ level and by up to 0.7 units in the dDOM+highN medium.

**Fungal biomass and C/N ratios.** Additions of NH$_4^+$ significantly increased the biomass of *P. involutus* in both the DOM and dDOM media ($P < 0.05$) (Fig. 2A; Table 1). There were no significant differences in the biomass of the mycelium grown in the DOM and dDOM media amended with NH$_4^+$. The C/N ratio significantly decreased in the fungal mycelium that was grown in the media supplemented with NH$_4^+$, suggesting an increased N assimilation upon NH$_4^+$ additions. The decrease was more pronounced in the medium with the high NH$_4^+$ amendment ($P < 0.05$) (Fig. 2B).

**Uptake of N and C and changes in DOM composition.** No NH$_4^+$ was detected in the media at the end of the incubation, and almost all of the added glucose was taken up by *P. involutus*, particularly in the media with high NH$_4^+$ additions (Tables S1 and S2). The chemical analysis showed that ~10 to 20% of the organic N was taken up by the fungus (Table S1). Uptake of organic N was also inferred by isotope analysis showing that the $^{15}$N atom% contents of the mycelia grown on DOM and dDOM were significantly lower than that of a mycelium grown in a synthetic mineral nutrient medium spiked with an equal $^{15}$N atom% in added NH$_4^+$ ($P < 0.05$; Fig. 2C). There was a tendency towards higher organic N uptake by the mycelium grown in the DOM.
medium as compared to dDOM, and also from the medium with the low NH$_4^+$ amendment as compared to high NH$_4^+$, but these differences were not significant ($P > 0.05$, Table S1). The fraction of the organic N assimilated by the fungus was slightly higher in dDOM than in DOM at similar levels of NH$_4^+$ amendment ($P > 0.05$; Fig. 3A). This is consistent with our expectation that organic compounds in dDOM were modified to a larger extent than those in DOM.

The $^{13}$C atom% of the mycelia grown in the DOM and dDOM media was not significantly different from a mycelium grown in a $^{13}$C-labeled mineral nutrient medium suggesting that an undetectable amount of organic C was taken up from the DOM and dDOM media (Fig. 2D). However, the contents of both organic C and total reduced sugars decreased in the processed DOM and dDOM media as compared to the corresponding initial media ($P < 0.05$; Fig. 3B and C; Table S2; Table 1). The decreases in DOM C and total reduced sugars tended to be larger in the DOM or dDOM medium amended with the high level of NH$_4^+$, even though these changes were not statistically significant ($P > 0.05$). In contrast, the concentration of phenolic compounds in the processed media increased during incubation ($P < 0.05$), and the increases were larger in the organic matter medium with low NH$_4^+$ amendment (Fig. 3D; Table S2).

**Chemical modifications of the DOM.** Size exclusion chromatography (SEC) showed that a majority of the molecules in the DOM had molecular masses between ~1.4 to 12.5 kDa (Fig. 4A, bottom panel). The changes in molecular mass distributions due to fungal processing are shown as the differences in the area-normalized SEC chromatograms between processed DOM/dDOM and the initial DOM (upper panel), where the horizontal axis indicates no differences and values above the axis indicate increases, and *vice versa*. The proportion of compounds with masses of ~2.1 to 12.5 kDa decreased after fungal processing. Concomitantly, the relative contribution of compounds of other masses (> 12.5 kDa, and < 2.1 kDa) increased. The observed changes in
molecular mass distributions were larger in the dDOM medium than in DOM, as expected. The changes were also larger in the medium with the high NH$_4^+$ amendment (Table 1), suggesting the depolymerization was enhanced at increasing levels of NH$_4^+$.

Closer inspections of the chemical modifications of the processed organic matter were accomplished by separation into hydrophilic and hydrophobic fractions using solid-phase extraction (SPE). SEC of the hydrophilic fraction showed that the proportion of small molecules (ranging from ~75 Da to 1.4 kDa) was higher in the processed media than in the initial organic matter (Fig. 4B). At the same time, the proportions of large molecules (> 12.5 kDa) decreased, which was not observed in the SEC of unfractionated DOM and dDOM (Fig. 4A). The observed changes in the molecular masses of organic compounds were more pronounced in the high NH$_4^+$ amendments and also in the dDOM medium as compared to DOM (Fig. 4B).

Glucose remained in the DOM/dDOM media interfered with the IR characterization of DOM components. Therefore, we excluded glucose in the media using SPE and collected a hydrophobic fraction which contained over 80% DOM C (10). The changes in functional groups of DOM due to fungal processing are shown as the differences in the area-normalized IR spectra between processed DOM/dDOM and the initial DOM (upper panel), where the horizontal axis indicates no differences and values above the axis indicate increases, and vice versa. Minor increases were detected in IR intensities associated with aromatic (ca 1600 and 1520 cm$^{-1}$) and phenolic (1270 cm$^{-1}$) functional groups in the processed DOM and dDOM as compared to the initial DOM (Fig. 4C upper). These increases tended to be greater at low NH$_4^+$ levels (Fig. 4C upper). Furthermore, the intensity of the band at 1710 cm$^{-1}$ originating from protonated carboxyl or carbonyl functional groups slightly increased in the processed DOM and dDOM as compared
to the initial DOM, indicating a higher oxidation state of C in the processed media (Fig. 4C upper). The changes were minor and were not correlated to the NH$_4^+$ levels (Table 1).

**Secretion of fungal compounds.** Considerable amounts of both $^{13}$C- and $^{15}$N-enriched compounds were detected in the processed DOM. The amounts of secreted compounds (mg l$^{-1}$ of media) were affected by the levels of added NH$_4^+$. The quantity of secreted total C decreased whereas that of secreted total N increased with increasing levels of NH$_4^+$ (Fig. 5A and B; Table 1). The dDOM+lowN medium had a slightly lower amount of secreted C but a similar amount of secreted N, as compared to the DOM+lowN medium, indicating that fungal C secretion, but not N secretion, was influenced by organic N concentration.

Although a previous study indicated that fungal secretion increased with fungal growth (10), we found no correlation between the amounts of secreted C and N and an increase in fungal biomass (Fig. 5C). As *P. involutus* secretes Fe(III)-reducing metabolites to drive the decomposition of DOM using Fenton chemistry (30), we related the levels of secreted C or N to the Fe(III)-reducing capacity (Fig. 5D). The concentration of secreted C, as expected, was positively correlated whereas the concentration of secreted N was negatively correlated with the level of Fe(III)-reducing activity detected in the processed DOM and dDOM.

Chemical characterization of the secreted metabolites was accomplished via ethyl acetate extraction of the processed DOM and dDOM. This procedure has previously been used for isolating metabolites secreted by *P. involutus* during organic matter decomposition (30). The ethyl acetate fraction of the processed DOM and dDOM contained slightly higher intensities of IR bands originating from aromatic and phenolic functional groups as compared to the initial DOM extract (Fig. 5E). The changes were most pronounced in the fractions recovered from the media containing the highest levels of secreted C (normalized to total C in the media). These
results suggest that the increase in aromatic and phenolic functional groups in the hydrophobic fraction of the processed DOM and dDOM (Fig. 4C) partly is due to the production of fungal metabolites. SEC of the ethyl acetate fraction revealed increased UV$_{254}$ intensities of at least three compounds after fungal processing (Fig. 5F, upper panel). The extent of this increase was related to the increase of the secreted C in the processed media.

**Formation of mineral-associated organic matter.** Iron oxides are omnipresent and play disproportionally important roles in stabilizing organic matter in soils (32). Therefore we examined the reactivity of processed organic matter towards a typical iron mineral, goethite, using batch adsorption approach. Quantitative adsorption of the initial and processed DOM and dDOM on goethite was examined in batch experiments at different concentrations of added organic C at pH 4. The adsorption of organic C was enhanced after fungal processing, especially in the dDOM medium. At the highest concentration of organic C (corresponding to complete surface saturation of DOM; Fig. 6A; Table 1), ca 8 − 9% more C was adsorbed in the processed DOM media, and 12 − 14% more C was adsorbed in the processed dDOM media than in the initial organic matter extracts. The enhancement in adsorption was partially attributed to the secretion of fungal compounds. The adsorbed organic C in the processed DOM and dDOM samples had significantly higher $^{13}$C atom% as compared to that adsorbed in the initial DOM (Fig. 6B). In contrast, the adsorption of organic N onto goethite was not higher in the DOM and dDOM processed by the fungus than in the initial DOM (Fig. 6C). However, the secreted N compounds were enriched in the adsorbed fraction (Fig. 6D). According to isotope mixing models, secreted C accounted for 2 to 5% of the total adsorbed C, depending on the contribution of secreted C to total organic C in the processed organic matter. In addition, secreted N accounted for 1 to 3% of the adsorbed N.
Approximately one third of the observed enhancement of C adsorption was explained by the fungal secretion; accordingly, the *ex vivo* transformations of indigenous organic matter by *P. involutus* contributed to two thirds of the increased C adsorption. These transformations were further analyzed by means of IR spectroscopy of the organic matter adsorbed on goethite and SEC of the un-adsorbed DOM and dDOM. The adsorbed processed DOM and dDOM possessed higher intensities of bands associated with symmetric (ca 1400 cm\(^{-1}\)) and asymmetric (ca 1580 cm\(^{-1}\)) carboxylate vibrations as compared with the adsorbed initial DOM (33) (Fig. 6E). This indicated an increase in the average oxidation state of the adsorbed DOM and dDOM after fungal processing. The intensity was slightly greater for the DOM and dDOM media amended with high than with low NH\(_4^+\) levels. Higher intensities were also detected for IR bands that originate from aromatic functional groups (1520 and 1600 cm\(^{-1}\)) of adsorbed processed DOM and dDOM, which corroborated that compounds secreted by the fungus contributed to the adsorption of the processed organic matter. The size distribution of the adsorbed organic matter (Fig. 6F lower panel) was calculated by subtracting the area-normalized SEC chromatograms of the DOM and dDOM remaining in solution after adsorption from those of the corresponding DOM and dDOM added before adsorption. This indicated preferential adsorption of molecules with sizes larger than 2.1 kDa (Fig. 6F, lower panel). The contribution of molecules, with sizes ranging from 1.4 to 6.5 kDa, to the total adsorption increased after fungal processing of both the DOM and dDOM media. The increase was greater if the media were amended with the high levels of NH\(_4^+\) (Fig. 6F, upper panel). Moreover, the contribution of molecules, larger than 12.5 kDa, to the total adsorption increased in the dDOM medium after fungal processing (Fig. 6F, upper panel).
DISCUSSION

In agreement with previous experiments, the decomposition of DOM by *P. involutus* enhanced the formation of mineral-associated organic matter, partly due to the extracellular modifications of the organic matter and partly by synthesizing mineral surface-reactive metabolites (10) (Fig. 1). We hypothesized that NH$_4^+$ amendments should lower the formation of such mineral-stabilized organic matter because increased availability of NH$_4^+$ should decrease the DOM decomposition activities that are linked to the acquisition of organic N sources. Our data 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 (Table 1). 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. The decreased acquisition of organic N at higher NH$_4^+$ levels was not accompanied with 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. Taken together, the observed shifts in DOM processing pathways did not increase the overall production of the mineral-associated organic C but the chemical composition of this fraction changed. Such compositional changes can potentially influence a broad range of physicochemical and biological processes in soils. This includes the rates of adsorption and desorption of mineral bound organic compounds (34), soil aggregate formation and destruction (35), and the structure and activity of microbial communities structure and metabolic capacity of microorganisms (36, 37). All these processes can have profound effects on SOM stabilization (6).

The addition of NH$_4^+$ to the soil organic matter extracts significantly increased the biomass of *P. involutus* (Fig. 2A), which suggests that the fungus was N limited when grown on...
the dialyzed DOM extract. Amendments with higher levels of NH$_4^+$ did not further increase the mycelial biomass indicating that under these conditions growth was limited by nutrients and/or factors other than N. It has been shown that N from NH$_4^+$ assimilated by ECM fungi is used for biosynthesis of the amide group of glutamine or the amino group of glutamate (38). Amino acid synthesis requires a supply of C, such as glucose (39). In our experiments, more than 90% of the added glucose was utilized in the DOM and dDOM media that were supplied with low levels of NH$_4^+$, and almost all of the glucose was consumed at higher NH$_4^+$ amendments (Table S2). This suggests that *P. involutus* probably was limited by C at the end of incubation when supplied with high levels of NH$_4^+$. Prolonged C limitation can induce a C starvation response in *P. involutus* involving autolysis, increased secretion of NH$_4^+$, and a decline in biomass (40). Such changes in the biomass and secretion of NH$_4^+$ were not observed, which suggests that a C-starvation response was not induced at any of the experimental conditions explored in this study. However, the observed decrease in the C:N ratios of the mycelial biomass grown on the media with high NH$_4^+$ amendments suggests that the metabolism of *P. involutus* was adjusted to the availability of C and N.

Recent time-resolved experiments using spectroscopy and transcriptomics have shown that the decomposition of DOM by *P. involutus* is a two-step mechanism involving oxidation and hydrolysis where oxidation precedes hydrolysis (41). Oxidation, which is a non-enzymatic mechanism involving the actions of hydroxyl radicals generated by Fenton chemistry is initiated when NH$_4^+$ is depleted from the DOM extract and organic N sources are assimilated (29). Subsequently, when the energy source (i.e. glucose) is limited *P. involutus* expresses a large number of hydrolytic enzymes including proteases, chitinases and glycoside hydrolases (41). The present study that experimentally manipulate the nutritional conditions provides further support
that the activity of the oxidation and hydrolytic decomposition systems in *P. involutus* are distinctively regulated in response to the availability of N and C sources. Under NH$_4^+$-limited conditions (i.e. the dialyzed DOM), components in the organic matter extracts were slightly oxidized and Fe(III)-reducing metabolites that are required for Fenton chemistry (30) were secreted. During this condition, some depolymerization of the DOM was observed. NH$_4^+$-additions decreased the amount of secreted C compounds that was correlated to the Fe(III)-reducing activity and no additional oxidation of the organic matter extract was detected. In contrast, NH$_4^+$ additions increased the depolymerization, likely due to increased activity in the hydrolytic pathway. Concomitantly the concentration of secreted N compounds increased.

Although the N containing compounds were not characterized, the fact that they increased with the degree of depolymerization suggested that they contained at least some extracellular enzymes. Higher levels of NH$_4^+$ further increased the concentration of secreted N compounds whereas the concentration of secreted C compounds decreased. Overall, our data suggest that shifts from N to C limited growth conditions will reduce the oxidation activity and increase the hydrolytic decomposition activities in *P. involutus* and that these changes are correlated with changes in the secretion of metabolites and enzymes. Similar shifts in hydrodase and oxidase activities were recently reported for saprotrophic fungi in a meta-analysis of soil extracellular enzyme activities under N fertilization (24).

The capacity of ECM fungi to metabolize soil C as an alternative C source to the photosynthate from the host plant remains controversial among ecologists (42, 43). Field studies using $^{13}$C-labelled leaf litter material in temperate forests did not indicate any incorporation of leaf litter C into the biomass (44). In contrast, other studies relying on enzyme assays of ECM root tips suggest that ECM fungi can hydrolyze SOM and that these activities are high when the
supply of host C is low (45). The increased degree of depolymerization observed in our laboratory experiments with *P. involutus* was accompanied with decreased levels of organic C in the DOM extract, which suggest that at least some of the released C was taken up by the fungus. However, analysis of the mycelial $^{13}$C-content did not show any significant levels of DOM C in the biomass (Fig. 2D). A possible explanation for this apparent discrepancy is that during low availability of energy, the assimilated C is almost exclusively used for maintenance and not for growth processes. Such non-growing states in which the viability and metabolic activity can be maintained for prolonged periods differs from starvation and has been characterized in industrially relevant fungi (46), but not yet in ECM fungi.

Recent field (47, 48) and modeling studies (49) suggest that the net effect of N additions on SOM dynamics depends on the interactions among N availability, microbial physiology, SOM decomposition, and soil minerals. In this study, we show that these interactions are complex and difficult to predict even in a laboratory-scale microcosm with a single organism, firmly controlled nutrient conditions and DOM chemistry. We show that NH$_4^+$ amendments can influence both the *ex vivo* and *in vivo* pathways by which ECM fungi transform DOM (Fig. 1). The activity in these pathways can increase, decrease or not change and the processes must be considered simultaneously when investigating the net effect of N additions on the stabilization of soil C. The complex nature of these interactions may explain why the responses of SOM dynamics to N additions in the field are highly variable or even contradictory (22, 49). Our study should encourage research in more complex soil systems to quantify the activity in the pathways underlying the microbial processing of DOM and how they affect the stabilization of SOM under N fertilization.

**MATERIALS AND METHODS**
**Fungal species and culture conditions.** *P. involutus* (Batsch) Fr. (strain ATCC 200175) was maintained on modified Fries medium (50) containing 1% agar. The composition of the Fries medium is: d-glucose (33.3 mM or 2.5 g l⁻¹), NH₄Cl (3.7 mM), MgSO₄·7H₂O (0.41 mM), KH₂PO₄ (0.22 mM), CaCl₂·2H₂O (0.18 mM), NaCl (0.34 mM), KCl (1.34 mM), H₃BO₃ (0.24 mM), ZnSO₄·7H₂O (20 μM), CuSO₄·5H₂O (5.01 μM), MnSO₄·H₂O (50.29 μM), (NH₄)₆Mo₇O₂₄·7H₂O (0.16 μM), FeCl₃·6H₂O (73.99 μM), myo-inositol (55.51 μM), thiamine·HCl (0.3 μM), biotin (0.1 μM), pyridoxine (0.59 μM), riboflavin (0.27 μM), nicotinamide (0.82 μM), p-aminobenzoic acid (0.73 μM), and Ca-pantothenate (0.46 μM), pH 4.8 (50). In the decomposition experiment, the fungus was grown in Petri dishes on a layer of glass beads immersed in a liquid medium (51). The fungus was first grown in 10 ml of the Fries medium containing ¹³C-D-glucose (ca 10 atom% ¹³C) and ¹⁵N-ammonium (ca 2.3 atom% ¹⁵N) for 10 d (at 18°C in dark). At this time, the colony reached a size of approximately 4 cm in diameter and a biomass of ca 8.5 mg (dry weight). The Fries medium was removed. The mycelium and glass beads were washed with 10 ml of sterile Milli-Q (MQ) water, and 10 ml of the Fries medium without N was added to induce a N-deprived mycelium (containing ca 10 atom% ¹³C D-glucose) (52).

After 24 h, the mycelium was washed in MQ water, and 10 ml of DOM medium was added. DOM was extracted from soil collected from the upper 10-cm soil layer of a forest site at Simlångsdalen (North: 56° 42' 2.47", West: 13° 6' 57.75"), Halmstad, Halland, Sweden, using hot water (53). The soil is classified as a Haplic Podzol according to WRB (54). The soil was mixed with MQ water (ratio 1:5, w/v), boiled for one hour and the extract was filtered through a 0.22-um sterile PES membrane (Millipore Inc., Bedford, Massachusetts). This type of membrane was used for filtration and sterilization throughout the study if not otherwise stated. In order to
remove free NH$_4^+$ and other low-molecular-weight N compounds, the DOM solution was dialyzed at 4°C against MQ water using a standard regenerated cellulose dialysis tubing (a cutoff of 1 kDa; Spectra/Por$^\circledR$ 7; Spectrum Laboratories Inc., Rancho Dominguez, California). A detailed comparison between the dialyzed and un-dialyzed samples is presented in the supplemental material (Fig. S1). The dialyzed DOM (denoted as “DOM”) was diluted twice with an equal volume of MQ water to create a diluted DOM solution (denoted as “dDOM”), which accordingly have the same C and N chemistry but a different organic matter concentration. Both the DOM and the dDOM solutions were supplemented with $^{13}$C-$\delta$-glucose (ca 10 atom% $^{13}$C) to a final concentration of 2.5 g l$^{-1}$. Moreover, by controlling the availability of C, the mycelium was not subjected to complete glucose depletion that would induce autolysis and an extensive release of cellular compounds from the mycelium (40). Other nutrients present in the Fries medium except for N were also added. Finally, the substrates were amended with ca 2.3 atom% $^{15}$N-ammonium to various N concentrations as shown in Table S1 and Fig. 1B.

The fungus was incubated in the organic matter media for 7 d. At the end of the incubation, the fungal mycelium was collected and lyophilized to determine the fungal biomass. The DOM and dDOM media were filtered (<0.22 um) and stored at -20 °C for further chemical analyses.

**Chemical analyses of fungal mycelium, DOM and dDOM.** Total C, N, $^{13}$C atom% and $^{15}$N atom% in freeze-dried fungal mycelium were analyzed using an elemental analysis-isotope ratio mass spectrometer (EA-IRMS) that couples an elemental analyzer (Flash 2000) via a ConFlo IV universal interface unit to a continuous-flow IRMS (Delta V Advantage; Thermo Scientific, Waltham, Massachusetts). Isotope mixing models were used to estimate the fungal uptake of C and N from the DOM (the principle of the calculation is shown in the following section).
Media before and after fungal processing were analyzed for the concentrations of total organic C (TOC), total N, ammonium, nitrate, D-glucose, total reduced sugar and phenolics and the specific UV absorbance at 254 nm (SUVA$_{254}$) (55). The total organic C (TOC) concentration was measured using an organic C analyser [TOC-V(CPH), Shimadzu, Kyoto, Japan]. The total N content was measured using the same apparatus equipped with a TNM-1 detector (Shimadzu, Kyoto, Japan). The ammonium and nitrate concentrations were analyzed using a flow injection analysis system (FIAstar 5000, FOSS, Hillerød, Denmark). D-glucose concentration was determined by a glucose kit (D-glucose HK assay kit, K-GLUHK-220A, Megazyme, Wicklow, Ireland). The total reduced sugar content was analyzed by the phenol-sulphuric acid method (56) using D-glucose as a standard. The phenolic content was determined utilizing the Folin-Ciocalteu method, as modified by Ainsworth and Gillespie (57), with tannic acid as a standard. The absorbance at 254 nm (UV$_{254}$) was recorded using a UV/visible spectrophotometer (Ultrospec 3000; Pharmacia Biotech, Uppsala, Sweden), and SUVA$_{254}$ was calculated by dividing UV$_{254}$ by TOC (55).

To remove interferences of glucose in the spectroscopic analyses, DOM was fractionated into two fractions by solid phase extraction (SPE) using a hydrophilic-lipophilic balanced cartridge (3 cc vac cartridge, 60 mg sorbent of a combination of the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone polymers; Oasis® HLB, Waters, USA). The hydrophilic fraction, containing glucose, was not retained on the cartridge (at pH 2). The hydrophobic fraction was glucose-free and collected by eluting the cartridge with 0.01 M NaOH (in 50% methanol solution).

The DOM and dDOM in the hydrophobic fraction (acidified to pH 2) were characterized using attenuated total reflectance (ATR) Fourier-transform IR (FTIR) spectroscopy (VERTEX...
80v; Bruker, Germany). Briefly, an aliquot of an acidified sample (10 µl) was dried under N\textsubscript{2} purging to make a film on a multiple reflection diamond crystal assembled in the MIRacle ATR Accessory (Pike Technologies, Madison, Wisconsin). Each IR spectrum was recorded on the film by repeating 128 scans at a resolution of 4 cm\textsuperscript{-1}. The background spectrum was collected under identical conditions but without the organic film. IR spectra were cut from 1800 cm\textsuperscript{-1} to 850 cm\textsuperscript{-1} and normalized to the area below the curves.

The molecular weight distributions of DOM and dDOM (the unfractionated samples) and their hydrophilic fractions from SPE (neutralized with 0.01 M NaOH solution) were analyzed by size exclusion chromatography (SEC) using a Superdex Peptide column (PC3.2/30 with an optimal separation range from 100 to 7000 Da; GE Healthcare Life Sciences, UK) attached to an HPLC system (Ultimate 3000; Thermo Scientific, Waltham, Massachusetts). Phosphate buffer (0.02 M phosphate and 0.25 M NaCl; pH 7.2) was chosen as the mobile phase with a flow rate of 50 µL min\textsuperscript{-1}. Data were recorded by a UV detector at 214 nm, 254 nm and 280 nm. Cytochrome c (molecular weight: 12.5 kDa), aprotinin (6.5 kDa), gastrin I (2.1 kDa), substance P (1.4 kDa) and glycine (75 Da) were used as molecular-size standards. Each SEC chromatogram was area normalized to the total area below the curve.

**Quantification and characterization of fungal metabolites.** The labeling of fungal biomass with \textsuperscript{13}C and \textsuperscript{15}N allowed the estimation of C and N secretions during DOM processing by *P. involutus*, using isotope mixing models. The hydrophobic fraction of DOM and dDOM obtained from SPE (\textsuperscript{13}C glucose free) was dried under an N\textsubscript{2} stream, re-dissolved in MQ water and freeze-dried for the determination of \textsuperscript{13}C atom\% in this fraction by EA-IRMS as described above. The unfractionated processed media (no \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} remained after the incubation) were freeze-dried for the measurement of \textsuperscript{15}N atom\% by EA-IRMS.
\[ 13C \text{ in the processed DOM and dDOM media contained three sources: secreted } \]

compounds, the remaining glucose, and DOM. In the hydrophobic DOM or dDOM fractions
obtained after SPE, the glucose was removed. Therefore, the relative abundance of \(^{13}C\) (atom\%) in the hydrophobic fraction \((A_{medium}^{13} C)\) could be expressed as:

\[ A_{\text{medium}}^{13} C = f_{\text{fungi}} A_{\text{fungi}}^{13} C + f_{\text{OM}} A_{\text{OM}}^{13} C, \quad (1) \]

using the following constraint:

\[ 1 = f_{\text{fungi}} + f_{\text{OM}}, \quad (2) \]

where \(A_{\text{fungi}}^{13} C\) is the abundance of \(^{13}C\) (atom\%) in the fungal metabolites; \(A_{\text{OM}}^{13} C\) is the abundance of \(^{13}C\) (atom\%) in the DOM or dDOM which is equal to natural abundance (NA) of \(^{13}C\); \(f_{\text{fungi}}\) and \(f_{\text{OM}}\) are the C fractions of the secreted metabolites and DOM/dDOM in the hydrophobic fraction, respectively.

After combining equations (1) and (2), solutions could be obtained as follows:

\[ f_{\text{fungi}} = \frac{A_{\text{medium}}^{13} C - A_{\text{OM}}^{13} C}{A_{\text{fungi}}^{13} C - A_{\text{OM}}^{13} C}, \quad (3) \]

\[ f_{\text{OM}} = \frac{A_{\text{fungi}}^{13} C - A_{\text{medium}}^{13} C}{A_{\text{fungi}}^{13} C - A_{\text{OM}}^{13} C}, \quad (4) \]

Similarly, the fraction of secreted N \((f_{\text{fungi}}^{\text{N}})\) in the processed DOM/dDOM could be solved as follows, given the fact that a negligible amount of added ammonium remained after fungal processing.

\[ f_{\text{fungi}}^{\text{N}} = \frac{A_{\text{medium}}^{15} N - A_{\text{OM}}^{15} N}{A_{\text{fungi}}^{15} N - A_{\text{fungi}}^{15} N}, \quad (5) \]
where At%$^{15}$N$_{fungi}$ is the abundance of $^{15}$N (atom%) in the fungal metabolites; At%$^{15}$N$_{medium}$ is that in the processed media; and At%$^{15}$N$_{NA}$ is the abundance of $^{15}$N (atom%) in the DOM/dDOM which is equal to the natural abundance (NA) of $^{15}$N.

The concentration of secreted C in the hydrophobic fraction of the processed DOM/dDOM ($mC_{secreted, hydrophobic}$) can be estimated by equation (6).

$$mC_{secreted, hydrophobic} = fC_{fungi} \times mC_{hydrophobic} \times$$  

(6)

where $mC_{hydrophobic}$ is the concentration of C in the hydrophobic fraction of the processed DOM/dDOM. Since SPE fractionation did not recover 100% of the secreted metabolites, a conversion factor of 0.44 was used to calculate the concentration of secreted C in the processed DOM ($mC_{secreted}$) according to our previous study (10):

$$mC_{secreted} = fC_{fungi} \times mC_{hydrophobic} / 0.44 .$$  

(7)

Likewise, the concentration of secreted N in the decomposed OM medium ($mN_{secreted}$) was estimated according to:

$$mN_{secreted} = fN_{fungi} \times mN_{decomposedOM} \times$$  

(8)

where $mM_{decomposedOM}$ is the concentration of N in the processed DOM.

The Fe(III)-reducing capacity of DOM was determined using a modified ferrozine assay (58). Briefly, samples from the initial and processed DOM and dDOM media were first incubated with an equal volume of freshly prepared 1.0 mM FeCl$_3$ in 0.1 M acetate buffer (pH 4.4) for 30 min. Then an absorbance reading was recorded at 562 nm on a spectrophotometer after reacting with the ferrozine reagent for 1 h. The Fe(III)-reducing capacity of fungal secreted
compounds was estimated as the difference in the readings of the processed and the initial DOM and dDOM.

Metabolites were extracted from the culture filtrates using ethyl acetate (EtOAc) as described by F. Shah et al. (30). The EtOAc solution of the metabolites was dried under a stream of N₂. In total six replicate samples from the same NH₄⁺ treatment were prepared. Three replicates were dissolved in methanol, and then transferred to and dried on the ATR crystal for IR analysis. Three replicates were dissolved in MQ water and were subjected to SEC.

**Adsorption of DOM on goethite.** The adsorption experiments were performed using a batch approach. Goethite was synthesized as described elsewhere (59). The goethite had a needle shape with an estimated width of 10 to 20 nm and an estimated length of one to several hundred nm (60). The specific surface area was estimated to be 62 m² g⁻¹ using the N₂ BET method (61). Three organic matter concentrations were evaluated: 0.25 ml, 1.5 ml, and 2 ml for initial and processed DOM media; and 0.5 ml, 3 ml, and 4 ml for the initial and processed dDOM media (refer to Supplemental Table S2 for DOM and dDOM concentrations). Each medium was mixed with 6 ml of goethite suspension (1.17 g l⁻¹ in 0.13 M NaCl), and MQ water was added to a final volume of 10 ml. A control treatment was set up by adding 4 ml MQ water into the goethite suspension. The final adsorption suspension was adjusted to and maintained at pH 4. The adsorption was conducted for 24 h; then, the suspensions were centrifuged, and the supernatants filtered and collected for UV₂₅₄ and SEC analyses. After centrifugation the goethite particles were rinsed twice with MQ water (pH 4) to remove trace amounts of loosely bound glucose (and negligible amounts of adsorbed DOM) and excess Cl⁻ ions, which otherwise may interfere with isotope analysis. One part of the goethite samples was freeze-dried for total C, total N, ¹³C atom% and ¹⁵N atom% analyses using the EA-IRMS instrument (as described above). Contributions of
secreted C and N to total adsorption were estimated using isotope mixing models (as described above). Another part of the goethite samples was dried, mixed with KBr powder and analysed using diffuse reflectance Fourier transform infrared (DRIFT-IR) spectroscopy, according to the method described in F. Rineau et al. (51). The IR spectra of adsorbed DOM were presented as the difference between spectra of the DOM-goethite complex and goethite, both of which were normalized according to the area below the goethite bands (970 – 720 cm\(^{-1}\)). The molecular size distribution of adsorbed DOM was determined by the difference in area-normalized SEC chromatograms of added DOM and DOM remaining in solution after adsorption. As the initial DOM and dDOM contained identical organic C and N chemistry, we combined all initial DOM/dDOM into a group, namely, “0d DOM”.

**Data analysis.** Means were compared using one-way ANOVA, using SPSS software (version 18; SPSS Inc., Chicago, Illinois). Tukey’s HSD test was used to analyze the differences between groups at a significance level of \( P = 0.05 \). The data generally met the homogeneous variance assumption (tested by Levene’s test), and hence, no additional transformations were performed unless otherwise stated.

**ACKNOWLEDGEMENTS**

The work was supported by grants from the Knut and Alice Wallenberg Foundation (Nr: 2013.0073) and the Swedish Research Council (Dnr: 2016-04561). Samples were analyzed for their isotopic composition by Jürgen Kuhn at the Stable Isotope Service Lab, Department of Biology, Lund University, Sweden. We also acknowledge technical support from Sofia Mebrahtu Wisén.

**REFERENCES**
1. Cotrufo MF, Soong JL, Horton AJ, Campbell EE, Haddix ML, Wall DH, Parton WJ. 2015. Formation of soil organic matter via biochemical and physical pathways of litter mass loss. Nat Geosci 8:776-779.

2. Sokol NW, Sanderman J, Bradford MA. 2019. Pathways of mineral-associated soil organic matter formation: Integrating the role of plant carbon source, chemistry, and point of entry. Global Change Biol 25:12-24.

3. Dungait JA, Hopkins DW, Gregory AS, Whitmore AP. 2012. Soil organic matter turnover is governed by accessibility not recalcitrance. Global Change Biol 18:1781-1796.

4. Schaeffer A, Nannipieri P, Kästner M, Schmidt B, Botterweck J. 2015. From humic substances to soil organic matter--microbial contributions. In honour of Konrad Haider and James P. Martin for their outstanding research contribution to soil science. J Soils Sed 15:1865-1881.

5. Schnitzer M, Monreal CM. 2011. Quo vadis soil organic matter research? A biological link to the chemistry of humification. Adv Agron 113:139-213.

6. Schmidt MW, Torn MS, Abiven S, Dittmar T, Guggenberger G, Janssens IA, Kleber M, Kögel-Knabner I, Lehmann J, Manning DA. 2011. Persistence of soil organic matter as an ecosystem property. Nature 478:49-56.

7. Lehmann J, Kleber M. 2015. The contentious nature of soil organic matter. Nature 528:60-68.

8. Sparling G, Vojvodić-Vuković M, Schipper L. 1998. Hot-water-soluble C as a simple measure of labile soil organic matter: the relationship with microbial biomass C. Soil Biol Biochem 30:1469-1472.

9. Landgraf D, Leinweber P, Makeschin F. 2006. Cold and hot water–extractable organic matter as indicators of litter decomposition in forest soils. J Plant Nutr Soil Sci 169:76-82.
10. Wang T, Tian Z, Bengtson P, Tunlid A, Persson P. 2017. Mineral surface-reactive metabolites secreted during fungal decomposition contribute to the formation of soil organic matter. Environ Microbiol 19:5117-5129.

11. Liang C, Schimel JP, Jastrow JD. 2017. The importance of anabolism in microbial control over soil carbon storage. Nat Microbiol 2:nmicrobiol2017105.

12. Lindahl BD, Clemmensen KE. 2016. Fungal ecology in boreal forest ecosystems, p 387-404. In Martin F (ed), Molecular Mycorrhizal Symbiosis. John Wiley & Sons, Inc., Hoboken, New Jersey.

13. Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD. 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol 173:611-620.

14. Kohler A, Kuo A, Nagy LG, Morin E, Barry KW, Buscot F, Canbäck B, Choi C, Cichocki N, Clum A. 2015. Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nat Genet 47:410-415.

15. Shah F, Nicolás C, Bentzer J, Ellström M, Smits M, Rineau F, Canbäck B, Floudas D, Carleer R, Lackner G. 2016. Ectomycorrhizal fungi decompose soil organic matter using oxidative mechanisms adapted from saprotrophic ancestors. New Phytol 209:1705-1719.

16. Phillips LA, Ward V, Jones MD. 2014. Ectomycorrhizal fungi contribute to soil organic matter cycling in sub-boreal forests. ISME J 8:699-713.

17. Bödeker I, Clemmensen KE, Boer W, Martin F, Olson Å, Lindahl BD. 2014. Ectomycorrhizal Cortinarius species participate in enzymatic oxidation of humus in northern forest ecosystems. New Phytol 203:245-256.

18. Lindahl BD, Tunlid A. 2015. Ectomycorrhizal fungi—potential organic matter decomposers, yet not saprotrophs. New Phytol 205:1443-1447.
19. Janssens IA, Dieleman W, Luyssaert S, Subke J-A, Reichstein M, Ceulemans R, Ciais P, Dolman AJ, Grace J, Matteucci G. 2010. Reduction of forest soil respiration in response to nitrogen deposition. Nat Geosci 3:315-322.

20. Aber JD, Nadelhoffer KJ, Steudler P, Melillo JM. 1989. Nitrogen saturation in northern forest ecosystems. Bioscience 39:378-286.

21. Berg B, Matzner E. 1997. Effect of N deposition on decomposition of plant litter and soil organic matter in forest systems. Environ Rev 5:1-25.

22. Fog K. 1988. The effect of added nitrogen on the rate of decomposition of organic matter. Biol Rev 63:433-462.

23. Knorr M, Frey S, Curtis P. 2005. Nitrogen additions and litter decomposition: A meta-analysis. Ecology 86:3252-3257.

24. Jian S, Li J, Chen J, Wang G, Mayes MA, Dzantor KE, Hui D, Luo Y. 2016. Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: A meta-analysis. Soil Biol Biochem 101:32-43.

25. Sinsabaugh RL. 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biol Biochem 42:391-404.

26. Lilleskov E, Hobbie E, Horton T. 2011. Conservation of ectomycorrhizal fungi: exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. Fungal Ecol 4:174-183.

27. Treseder KK. 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. New Phytol 164:347-355.
28. Rineau F, Shah F, Smits M, Persson P, Johansson T, Carleer R, Troein C, Tunlid A. 2013. Carbon availability triggers the decomposition of plant litter and assimilation of nitrogen by an ectomycorrhizal fungus. ISME J 7:2010-2022.

29. Op De Beeck M, Troein C, Peterson C, Persson P, Tunlid A. 2018. Fenton reaction facilitates organic nitrogen acquisition by an ectomycorrhizal fungus. New Phytol 218:335-343.

29. Shah F, Schwenk D, Nicolás C, Persson P, Hoffmeister D, Tunlid A. 2015. Involutin is an Fe\(^{3+}\) reductant secreted by the ectomycorrhizal fungus *Paxillus involutus* during Fenton-based decomposition of organic matter. Appl Environ Microbiol 81:8427-8433.

30. Kubicki JD, Paul KW, Kabalan L, Zhu Q, Mrozik MK, Aryanpour M, Pierre-Louis A-M, Strongin DR. 2012. ATR–FTIR and density functional theory study of the structures, energetics, and vibrational spectra of phosphate adsorbed onto goethite. Langmuir 28:14573-14587.

31. Kögel-Knabner I, Guggenberger G, Kleber M, Kandeler E, Kalbitz K, Scheu S, Eusterhues K, Leinweber P. 2008. Organo-mineral associations in temperate soils: Integrating biology, mineralogy, and organic matter chemistry. J Plant Nutr Soil Sci 171:61-82.

32. Hay MB, Myneni SC. 2007. Structural environments of carboxyl groups in natural organic molecules from terrestrial systems. Part 1: Infrared spectroscopy. Geochim Cosmochim Acta 71:3518-3532.

33. Gu B, Schmitt J, Chen Z, Liang L, McCarthy JF. 1995. Adsorption and desorption of different organic matter fractions on iron oxide. Geochim Cosmochim Acta 59:219-229.
35. Oades JM. 1984. Soil organic matter and structural stability: mechanisms and implications for management. Plant Soil 76:319-337.

36. Gupta VV, Germida JJ. 2015. Soil aggregation: Influence on microbial biomass and implications for biological processes. Soil Biol Biochem 80:A3-A9.

37. Smith A, Marín-Spiotta E, de Graaff M, Balser T. 2014. Microbial community structure varies across soil organic matter aggregate pools during tropical land cover change. Soil Biol Biochem 77:292-303.

38. Javelle A, Morel M, Rodriguez - Pastrana BR, Botton B, Andre B, Marini AM, Brun A, Chalot M. 2003. Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium - metabolizing enzymes (GS, NADP - GDH) in the ectomycorrhizal fungus Hebeloma cylindrosporum. Mol Microbiol 47:411-430.

39. Martin F, Ramstedt M, Söderhäll K, Canet D. 1988. Carbohydrate and amino acid metabolism in the ectomycorrhizal ascomycete Sphaerospora brunnea during glucose utilization: a $^{13}$C NMR study. Plant Physiol 86:935-940.

40. Ellström M, Shah F, Johansson T, Ahrén D, Persson P, Tunlid A. 2015. The carbon starvation response of the ectomycorrhizal fungus Paxillus involutus. FEMS Microbiol Ecol 91:fiv027.

41. Nicolás C, Martin-Bertelsen T, Floudas D, Bentzer J, Smits M, Johansson T, Troein C, Persson P, Tunlid A. 2018. The soil organic matter decomposition mechanisms in ectomycorrhizal fungi are tuned for liberating soil organic nitrogen. ISME J doi:10.1038/s41396-018-0331-6.

42. Talbot J, Allison S, Treseder K. 2008. Decomposers in disguise: mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change. Funct Ecol 22:955-963.
43. Baldrian P. 2009. Ectomycorrhizal fungi and their enzymes in soils: is there enough evidence for their role as facultative soil saprotrophs? Oecologia 161:657-660.

44. Treseder KK, Torn MS, Masiello CA. 2006. An ecosystem-scale radiocarbon tracer to test use of litter carbon by ectomycorrhizal fungi. Soil Biol Biochem 38:1077-1082.

45. Courty P-E, Franc A, Garbaye J. 2010. Temporal and functional pattern of secreted enzyme activities in an ectomycorrhizal community. Soil Biol Biochem 42:2022-2025.

46. Erkan O, Bisschops MMM, Overkamp W, Jørgensen TR, Ram AF, Smid EJ, Pronk JT, Kuipers OP, Daran-Lapujade P, Kleerebezem M. 2015. Physiological and transcriptional responses of different industrial microbes at near-zero specific growth rates. Appl Environ Microbiol 81:5662-5670.

47. Zak DR, Freedman ZB, Upchurch RA, Steffens M, Kögel‐Knabner I. 2017. Anthropogenic N deposition increases soil organic matter accumulation without altering its biochemical composition. Global Change Biol 23:933-944.

48. Neff JC, Townsend AR, Gleixner G, Lehman SJ, Turnbull J, Bowman WD. 2002. Variable effects of nitrogen additions on the stability and turnover of soil carbon. Nature 419:915-917.

49. Averill C, Waring B. 2018. Nitrogen limitation of decomposition and decay: How can it occur? Global Change Biol 24:1417-1427.

50. Fries N. 1978. Basidiospore germination in some mycorrhiza-forming hymenomycetes. Trans Br Mycol Soc 70:319-324.

51. Rineau F, Roth D, Shah F, Smits M, Johansson T, Canbäck B, Olsen PB, Persson P, Grell MN, Lindquist E. 2012. The ectomycorrhizal fungus *Paxillus involutus* converts organic
matter in plant litter using a trimmed brown-rot mechanism involving Fenton chemistry.

52. Shah F, Rineau F, Canbäck B, Johansson T, Tunlid A. 2013. The molecular components of the extracellular protein-degradation pathways of the ectomycorrhizal fungus *Paxillus involutus*. New Phytol 200:875-887.

53. Davidson EA, Galloway LF, Strand MK. 1987. Assessing available carbon: comparison of techniques across selected forest soils. Commun Soil Sci Plant Anal 18:45-64.

54. IUSS Working Group WRB. 2006. World reference base for soil resources. FAO, Rome, Italy.

55. Weishaar JL, Aiken GR, Bergamaschi BA, Fram MS, Fujii R, Mopper K. 2003. Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon. Environ Sci Technol 37:4702-4708.

56. DuBois M, Gilles KA, Hamilton JK, Rebers P, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-356.

57. Ainsworth EA, Gillespie KM. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc 2:875-877.

58. Goodell B, Daniel G, Jellison J, Qian Y. 2006. Iron-reducing capacity of low-molecular-weight compounds produced in wood by fungi. Holzforschung 60:630.

59. Krumina L, Kenney JPL, Loring JS, Persson P. 2016. Desorption mechanisms of phosphate from ferrihydrite and goethite surfaces. Chem Geol 427:54-64.

60. Lyngsie G, Krumina L, Tunlid A, Persson P. 2018. Generation of hydroxyl radicals from reactions between a dimethoxyhydroquinone and iron oxide nanoparticles. Sci Rep 8:10834.
61. Schenck C, Dillard J, Murray J. 1983. Surface analysis and the adsorption of Co (II) on goethite. J Colloid Interface Sci 95:398-409.
FIGURE LEGENDS

FIG 1 A conceptual model describing the formation of mineral-associated organic matter by fungi and the nitrogen treatments used in this study. (A) Fungal processing of dissolved organic matter (DOM) can contribute to the formation of mineral-associated organic C by two different pathways: extracellular transformation (ex vivo), including oxidation and depolymerization of compounds in the DOM; and the secretion of fungal metabolites (in vivo). Both modifications can enhance the retention of the processed DOM on mineral particles (10). The relative importance of each mechanism is indicated by solid arrow thickness. Dotted arrows indicate desorption process. (B) Nitrogen treatments used in this study.

FIG 2 Biomass, C/N ratio and uptake of C and N by *P. involutus* grown for 7 d on DOM or diluted DOM (dDOM) medium amended with different levels of NH$_4^+$ Data are presented as means and error bars indicate one standard deviation. Open bars show the data for the fungus grown for 0 d (“Fries (0 d)”) and 7 d (“Fries (7 d)” on Fries medium. (A) Biomass (*n* = 10); (B) C/N ratios of the mycelium (*n* = 5); (C) $^{15}$N atom% of the mycelium (*n* = 5); (D) $^{13}$C atom% of the mycelium (*n* = 5). Different lowercase letters above bars in each panel denote significant differences according to Tukey’s HSD test (*P* < 0.05).

FIG 3 Changes in the chemical composition of the DOM processed by *P. involutus* at different NH$_4^+$ levels. Shown are changes (Δ) related to the initial contents of (A) Organic N, (B) Total reduced sugars, (C) DOM C, and (D) Phenolic compounds. Data are represented as means (*n* ≥ 4), and error bars denote one standard deviation. Error bars in plot (D) are within the symbols. Absolute values for organic N, reduced sugars, total organic C, and phenolic compounds are shown in Tables S1 and S2.
FIG 4 Changes in the molecular size and functional group chemistry of the DOM processed by *P. involutus* at different NH$_4^+$ levels. Fungal-associated changes in the area-normalized size exclusion chromatograms of the processed DOM (A) and its hydrophilic fraction recovered by solid phase extraction (B). The lower panels present the area-normalized chromatograms, and the upper panels the differences between processed DOM and initial DOM. Note that the upper and lower panels have different scales (arbitrary units). The molecular sizes of a series of peptide standards are indicated at the top of the upper panel. The elution profiles were recorded by a UV detector at 254 nm. All of the curves are shown as an average chromatogram of three repeated measurements. (C) IR spectral changes of the hydrophobic fractions of the processed DOM. In the lower panel are averages of area-normalized spectra from triplicate measurements. The upper panel shows the differences between area-normalized spectra of the processed and the initial DOM.

FIG 5 Quantification and characterization of secreted compounds into the DOM and dDOM media during processing by *P. involutus* at different NH$_4^+$ levels. Shown are the mean values ± one standard deviation (*n*=3). Quantification of total secreted C (A) and total secreted N (B) after 7 d of fungal processing. Different lowercase letters above the bars denote significant differences according to Tukey’s HSD test (*P* < 0.05). (C) Correlations between total secreted C or secreted N (for secreted N, a factor of 10 was multiplied in order to visualize) and mycelial biomass C produced during 7 d of DOM processing by *P. involutus*. (D) Correlations between the amounts of secreted C or secreted N (by multiplying a factor of 10) and the levels of Fe(III)-reducing activity. (E) IR spectral changes of an ethyl acetate phase of the processed DOM. The lower panel displays area-normalized spectra of the ethyl acetate extract. The upper panel shows the differences between area-normalized spectra of the processed and initial DOM. (F) Fungal-
associated changes in the size exclusion chromatograms of the ethyl acetate phase of the processed DOM. The lower and upper panels present the area-normalized chromatograms and the differential chromatograms, respectively. The upward arrows indicate intensities increased likely relating to fungal metabolites.

**FIG 6** Adsorption of DOM and dDOM to goethite before and after fungal processing at different NH₄⁺ levels. Data are presented as means and error bars indicate one standard deviation. $n = 3$, except initial DOM $n = 5$, unless otherwise stated. (A) Adsorbed C on goethite surface as a function of added DOM C. The insert shows a zoom of data points of processed DOM and dDOM at added DOM C concentrations higher than 75 mg l⁻¹. (B) $^{13}$C atom% of adsorbed C as a function of added DOM C. (C) Adsorbed N on the goethite surface as a function of added DOM C. (D) $^{15}$N atom% of adsorbed N as a function of added DOM C. (E) IR spectra of adsorbed DOM. Lower panel: area-normalized spectra ($n = 2$) and upper panel: the differences in spectra between the processed and initial DOM. (F) Changes in normalized size exclusion chromatograms of the adsorbed organic matter on goethite. Lower panel: area-normalized SEC of adsorbed DOM. Upper panel: the differences in SEC chromatograms between the processed and initial organic matter. Molecular weights of a series of peptide standards are shown at the top of the upper panel.
TABLE 1 Summary of the effects of increased NH$_4$+ levels on the processing and the formation of mineral-associated organic C during the processing of dissolved organic matter (DOM) by *P. involutus.* ↑ and ↑↑ denote a decrease or an increase of the measured parameters between the processed organic matter (incubated for 7 d) and the initial DOM. Within a row, the number of arrows indicates the magnitude of these changes. Arrow(s) in parentheses indicates that the values are not significantly (*P > 0.05*) different when comparing NH$_4$+ amended DOM (or dDOM) and non-amended DOM that was incubated for 7 d.

| Analysis                                      | DOM                  | DOM                      | dDOM                      |
|----------------------------------------------|----------------------|--------------------------|---------------------------|
|                                              | +lowN | +high N | +lowN | +high N |
| Formation of mineral-associated C            |        |        |        |         |
| Total organic C                              | ↑      | ↑      | (↑↑)  | (↑)    |
| Fungal C                                     | ↑↑↑    | ↑      | ↑↑↑    | ↑↑     |
| Ex vivo transformation$^a$                    |        |        |        |         |
| Depolymerization                             | ↑      | ↑↑     | ↑↑     | ↑↑     |
| Oxidation                                    | ↑$^b$  | (↑)$^c$| (↑)$^c$| (↑)$^c$|
| In vivo turnover                              |        |        |        |         |
| N secretion                                  | ↑      | ↑↑     | ↑↑     | ↑↑     |
| C secretion                                  | ↑↑↑    | ↑      | ↑↑     | ↑      |
| Chemical composition changes of the DOM$^c$  |        |        |        |         |
| Organic N                                    | ↓↓↓    | (↓↓)   | ↓↓↓    | (↓↓)   |
| Organic C                                    | ↓      | (↓↓)   | (↓↓↓)  | (↓↓↓)  |
| Reduced sugars                               | ↓      | (↓↓)   | (↓↓↓)  | (↓↓↓)  |
| Phenolics                                    | ↑↑↑    | ↑↑     | (↑↑↑↑) | ↑      |
| Fungal growth                                | ↑      | ↑↑     | ↑      | ↑      |

$^a$The extent of *ex vivo* transformation during fungal processing was normalized to the total organic matter.

$^b$Oxidation was not significant between the processed and the initial DOM (*P > 0.05*).

$^c$A change was calculated as the difference between values of the processed and the initial DOM, normalized to the value of the initial DOM.
FIG 1

A

Initial DOM
• Sugars
• Phenolics
• Organic N (e.g. protein)

Processed DOM
• Sugars
• Phenolics
• Organic N
• Secondary metabolites

Ex vivo transformations

In vivo (i.e. secretion)

Size
Oxidation

Organic matter-mineral associations

B

N treatments

N concentration (mg l⁻¹)

Added NH₄⁺-N
DOM NH₄⁺-N
DOM organic N

DOM
DOM+lowN
DOM+highN
dDOM+lowN
dDOM+highN
FIG 2

A

B

C

D

Biomass (mg dish⁻¹)

Biomass C/N

Biomass ¹⁵N/¹⁴N (atom %)

Biomass ¹³C/¹²C (atom %)

DOM

DOM+lowN

dDOM+lowN

dDOM+highN

Fries (0d)

Fries (7d)
FIG 3

A

NH₄⁺-N (mg l⁻¹)

Δ(Organic N)

-30%
-20%
-10%
0%
0 10 20 30 40

B

NH₄⁺-N (mg l⁻¹)

Δ(Reduced sugars)

-40%
-30%
-20%
-10%
0%
0 10 20 30 40

C

NH₄⁺-N (mg l⁻¹)

Δ(DOM C)

-25%
-15%
-5%
0%
0 10 20 30 40

D

Δ(Phenolics)

-40%
-30%
-20%
-10%
0%
0 10 20 30 40

Δ(Organic N)

NH₄⁺-N (mg l⁻¹)

DOM
DOM+lowN
DOM+highN
dDOM+lowN
dDOM+highN
FIG 4

A DOM and dDOM

B Hydrophilic fraction

C Hydrophobic fraction

Retention time (min)

Normalized UV 254 (AU)

Relative UV 254 difference (AU)

DOM and dDOM Hydrophilic fraction

Hydrophobic fraction
**FIG 6**

A) Adsorbed C on goethite (mg m\(^{-2}\))

```
Added DOM C in solution (mg L\(^{-1}\))
0.005 0.01 0.015 0.02 0.025
Adsorbed C on goethite (mg m\(^{-2}\))
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
```

B) %C on goethite (atom%)

```
Added DOM C in solution (mg L\(^{-1}\))
1 1.1 1.2 1.3 1.4 1.5 1.6
%=C on goethite (atom%)
0.35 0.37 0.39 0.41 0.43 0.45
```

C) Adsorbed N on goethite (mg m\(^{-2}\))

```
Added DOM C in solution (mg L\(^{-1}\))
0 0.005 0.01 0.015 0.02 0.025
Adsorbed N on goethite (mg m\(^{-2}\))
0 0.01 0.02 0.03 0.04 0.05
```

D) %N on goethite (atom%)

```
Added DOM C in solution (mg L\(^{-1}\))
0 50 100 150
%=N on goethite (atom%)
0.35 0.37 0.39 0.41 0.43 0.45
```

E) IR Abs (AU) vs Wavenumber (cm\(^{-1}\))

```
Wavenumber (cm\(^{-1}\))
1750 1600 1450 1300 1150 1000
IR Abs (AU)
0 0.05 0.1 0.15 0.2 0.25
```

```
Asymmetric COO
Symmetric COO
Phenolic C=C
```

F) UV\(_{254}\) Difference vs Retention time (min)

```
Retention time (min)
15 20 25 30 35 40 45 50 55 60 65 70
UV\(_{254}\) Difference
```

```
Void 12.5 kDa 6.5 kDa 2.1 kDa 1.4 kDa 75 Da
```

```
Adsoorbed UV\(_{254}\)
DOM DOM+lowN DOM+highN dDOM+lowN dDOM+highN
```

```
Phenolic C=C
0.7 0.75 75 125
```

```
Retention time (min)
```

```
0 50 100 150
```
A

**Initial DOM**
- Sugars
- Phenolics
- Organic N (e.g. protein)

**Processed DOM**
- Sugars
- Phenolics
- Organic N
- Secondary metabolites

*In vivo (i.e. secretion)*

*Ex vivo transformations*

Size

Oxidation

**Organic matter-mineral associations**

B

**N treatments**

| N concentration (mg l⁻¹) | Added NH₄⁺-N | DOM NH₄⁺-N | DOM organic N |
|--------------------------|--------------|-------------|----------------|
| DOM                      |              |             |                |
| DOM+lowN                 |              |             |                |
| DOM+highN                |              |             |                |
| dDOM+lowN                |              |             |                |
| dDOM+highN               |              |             |                |
