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Research

Rapid report

The below-ground perspective of forest plants: soil provides mainly organic nitrogen for plants and mycorrhizal fungi

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Summary

- Nitrogen (N) availability has a major impact on a wide range of biogeochemical processes in terrestrial ecosystems. Changes in N availability modify the capacity of plants to sequester carbon (C), but despite the crucial importance for our understanding of terrestrial ecosystems, the relative contribution of different N forms to plant N nutrition in the field is not known. Until now, reliably assessing the highly dynamic pool of plant-available N in soil microsites was virtually impossible, because of the lack of adequate sampling techniques.
- For the first time we have applied a novel microdialysis technique for disturbance-free monitoring of diffusive fluxes of inorganic and organic N in 15 contrasting boreal forest soils *in situ*.
- We found that amino acids accounted for 80% of the soil N supply, while ammonium and nitrate contributed only 10% each. In contrast to common soil extractions, microdialysis revealed that the majority of amino acids are available for plant and mycorrhizal uptake.
- Our results suggest that the N supply of boreal forest soils is dominated by organic N as a major component of plant-available N and thus as a regulator of growth and C sequestration.

Introduction

The close link between nitrogen (N) and other elemental cycles, especially the global carbon (C) cycle (Gruber & Galloway, 2008), emphasizes the necessity for ecosystem models to incorporate the impact of changing soil N availabilities on the terrestrial C sink (Bonan, 2008; Janssens *et al.*, 2010). In most cases, the predictions of the impact of global change on ecosystem functioning that are made by models linking only C and climate have been shown to differ significantly from the results obtained from those that also include N availabilities (Kielland, 1994). However, these models consider only mineral N as a plant-relevant N source, neglecting organic N. Recent evidence strongly suggests that plants are capable of taking up organic N forms, particularly amino acids (Kielland, 1994; Näsholm *et al.*, 1998, 2009), and in N-limited ecosystems, such as boreal forests, organic N is thus a potential key player in the N cycle. Acquisition of N by plant roots and by mycorrhizal fungi is a complex process involving transport both in the soil and across root or hyphal membranes (Leadley *et al.*, 1997; Tinker & Nye, 2000). Several studies indicate that soil supply rates, not root or hyphal uptake rates, limit plant N acquisition (Clarkson & Hanson, 1980; Leadley *et al.*, 1997). The two major mechanisms supplying N to root and fungal surfaces are mass flow and diffusion, and any nutrient can be supplied by either mechanism if the conditions are appropriate (Tinker & Nye, 2000; Comerford, 2005). While the relative contribution of each of these processes is hard to assess, diffusion becomes increasingly important when mass flow cannot meet the N demand of plants (Clarke & Barley, 1968; Comerford, 2005). In most N-poor ecosystems, soil N supply to plant roots is driven by the diffusional gradient that develops around roots or hyphae as a result of active absorption of N compounds by these organs (Nye, 1979). Thus, any analysis of plant N nutrition and of the relative contributions of inorganic and organic N compounds should be based on estimates of diffusive fluxes of individual N compounds (Nye, 1979; Shaver & Chapin, 1991; Leadley *et al.*,

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1997). Owing to the lack of an adequate soil sampling technique that can provide a characterization of soil N supply relevant for plant and mycorrhizal N acquisition, however, our current understanding of soil N availability is based mainly on N concentrations in the soil solution, while soil N supply rates are still major unknowns in terrestrial ecosystem ecology and biogeochemistry, a deficiency already pointed out decades ago (Neubauer, 1937).

Recently, a novel technique based on passive microdialysis was presented as a possible tool to monitor N dynamics in soils (Inselsbacher & Näsholm, 2011; Inselsbacher *et al.*, 2011). This noninvasive technique samples dissolved compounds from the soil solution through an induced diffusive flux driven by the concentration gradient between the perfusate and the soil solution (Miro & Frenzel, 2004, 2005). Thus, this technique corresponds better with, and should hence give a more accurate representation of, soil N supply than traditional soil sampling techniques (Inselsbacher *et al.*, 2011).

We hypothesized that *in situ* monitoring of soil N fluxes using the recently established microdialysis technique would provide novel insights into the N dynamics of boreal forest soils. Specifically, we estimated the relative shares of inorganic and organic N for total N diffusive fluxes and investigated the impact of N additions to boreal forests on soil N supply rates of individual N forms for root and mycorrhizal uptake.

Materials and Methods

The microdialysis system consisted of two syringe infusion pumps (CMA 100 and CMA 400) equipped with a total of seven microsyringes (5 ml, Hamilton, Bonaduz, Switzerland). We used seven microdialysis probes (CMA 20) with a polyarylethersulphone membrane (10 mm long, 500 μm outer diameter and 400 μm inner diameter) with a 20 kDa molecular weight cutoff. We used two refrigerated microfraction collectors (CMA 140 and CMA 470) and collected the samples in 300 μl glass vials. All equipment is commercially available at CMA Microdialysis AB (Solna, Sweden).

The microdialysis probes were calibrated before, in between and after sampling events. In detail, we placed the probes in a solution containing ammonium, nitrate and 18 amino acids (AAS 18 standard solution, plus additional glutamine, asparagine; Sigma Aldrich). The solution was stirred with a magnetic stirrer throughout the calibration period to prevent the formation of a depletion zone around the probe surface (Inselsbacher *et al.*, 2011). We used high-purity deionized water as perfusate at a constant flow rate of 5.0 μl min^{-1} and collected dialysate samples at 30 min intervals over a total period of 2.5 h. The relative recovery was calculated as described previously (Inselsbacher *et al.*, 2011) based on general microdialysis probe calibration (Bungay *et al.*, 1990; Torto *et al.*, 2001; Nandi & Lunte, 2009). Diffusive fluxes from the soil over the probe membrane to the microdialysis sampler were estimated by calculating the total amount of each N compound diffusing over the membrane surface (15.9 mm^2) during each sampling period (Inselsbacher & Näsholm, 2011) and were then expressed as nmol cm^{-2} h^{-1}. We found that the relative recovery of each individual N form remained stable for each microdialysis probe (Supporting Information, Fig. S1) as well as the volume of each sample (150 μl at 5 μl min^{-1} flow rate, 30 min sampling time). This proved that the performance and quality of the membranes remained stable throughout the experiment, as any damage or fouling of the membrane would have led to a change in these two parameters. In order to protect the sensitive membranes from any damage caused by the rough surface of the soil matrix, however, it is necessary to use special membrane introducers during the installation of the probes into the soil.

Site description and N addition experiment

The experiments were performed at 15 boreal forest sites in northern Sweden in September 2010. The podsolic soils studied are representative of the most common soil type in boreal forests, the tree layer is dominated by *Pinus sylvestris* and *Picea abies* and the understorey by ericaceous shrubs such as *Vaccinium myrtillus* and *Vaccinium vitis-idaea*. A detailed description of each site is given in Table S1. The effects of increased inorganic N inputs on soil N fluxes were performed at the Åheden research area within the Svartberget Experimental Forest (64°14′N, 19°46′E), which has been described previously (Gundale *et al.*, 2011). The experimental site is a *c.* 60-yr-old Scots pine (*Pinus sylvestris* L.) forest with an annual mean air temperature of +1.0°C and an annual mean precipitation of *c.* 600 mm (1980–1999). Total wet and dry N deposition in the study area is *c.* 2 kg N ha^{-1} yr^{-1}. In 2003, a large-scale N-addition experiment consisting of six replicate square plots (1000 m^2) was established in a randomized block design. Experimental N additions (0, 6 and 50 kg N ha^{-1} yr^{-1}) were initiated in 2004 and were thereafter made annually at the onset of the growth period by adding granules of NH_4NO_3 (diameter 1 mm) by hand.

Microdialysis sampling

Within each study site, three subplots were chosen randomly for sampling and understorey growth was carefully lifted to access the upper soil layer. Within each subplot, microdialysis probes were inserted vertically at seven positions to *c.* 1.5 cm beneath the soil surface. High-purity deionized water was used as perfusate, the flow rate was set to 5.0 μl min^{-1} and the sampling time was set to 30 min during a total sampling time of 2.5 h. Samples were kept on ice until used for chemical analyses (within 2 wk) as described in the following sections.

Soil sampling and extraction Following microdialysis sampling, soils were collected from the surface (0–2 cm) of the same points, ensuring comparability of the two different approaches. Soils were immediately sieved (< 2 mm) and homogenized, and aliquots (4 g FW) were extracted with 30 ml of either high-purity deionized water or KCl (1 M), shaken for 90 min, and filtered through ashless Whatman filter paper. Samples were then immediately analysed for NH_4^+, NO_3^- and amino acid concentrations as described in the following section.

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Chemical analyses of amino acids, ammonium and nitrate Samples from microdialysis sampling and from soil extractions were analysed as described previously (Inselsbacher *et al.*, 2011). Briefly, amino acids and ammonium were analysed by reversed-phase liquid chromatography using a Waters (Milford, USA) Ultra High Performance Liquid Chromatography (UPLC) system with a Waters Tunable UV (TUV) detector. Samples were derivatized with a Waters AccQ-Tag Ultra Derivatization kit for amino acid analysis. Nitrate was analysed by the vanadium (III) chloride (VCl_3) and Griess method as described previously (Miranda *et al.*, 2001).

Calculations and statistical analysis Statistical analyses were carried out using one-way ANOVA followed by Tukey's honestly significant difference post-hoc test using Statgraphics 5.0 (Statistical Graphics Inc., Rockville, MD, USA). When necessary, data were either square-root- or $\log_{10}$-transformed before analysis to meet the assumptions of ANOVA after testing normality using a Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test.

Results and Discussion

The importance of each individual N compound for plant and mycorrhizal nutrition is determined by the diffusive fluxes of N through the soil to the root surface and not by soil solution concentrations (Nye, 1979; Shaver & Chapin, 1991; Leadley *et al.*, 1997). Here we present a novel approach based on microdialysis and show that this technique can be used to assess plant-available N sources directly in the field. Our results show that the diffusive flux of N compounds in boreal forest soils is dominated by amino acids, which contributed 74–89% to the total N flux, while ammonium and nitrate amounted to only 5–15 and 5–11%, respectively (Figs 1a, S2). This dominance of amino acids was evident for all forest sites studied, including those that had been supplied with inorganic N fertilizer (Figs 2a, S2). Among the 18 individual amino acids analysed, all but three (histidine, arginine and methionine) were detected in the dialysates of 10 control (nonfertilized) soils (Fig. 3). Histidine was also found in two nonfertilized plots and in three fertilized plots (Table S2). Amino acid flux was dominated by glycine and glutamine and it was noticeable that the flux rates of both these amino acids were higher than that of ammonium, while rates of nitrate flux were only 70% of those of ammonium (Fig. 3). Compared with N flux rates, concentrations of free N estimated by water extractions of soils were dominated by ammonium, which contributed as much as 79% to the total soil solution N, while amino acids and nitrate only amounted to 11 and 10%, respectively (Figs 1b, 2b). Moreover, only nine individual amino acids were found in the water extracts (Fig. 3). Nitrogen bound to soil particles estimated by KCl extraction was also dominated by ammonium but with a larger proportion of bound amino acids contributing to the amino acid fraction and therefore to the total extracted N (Figs 2c, 3). Our results show that, in the field, plant roots and mycorrhizal fungi are exposed to almost the full range of protein amino acids (15 of 18 analysed) and may potentially use all of them for N nutrition. These findings contradict our results from soil extractions and also the current assumption that amino acids are largely bound to soil particles and, therefore, accessible for plant and mycorrhizal uptake only after exchange with other ions, such as those commonly found in root exudates. Studies of N uptake capacities of boreal forest plants (Persson & Näsholm, 2001) show that both mycorrhizal and nonmycorhizal roots may absorb the full range of protein amino acids. Moreover, recent investigations of uptake mechanisms of amino acids by plant roots and mycorrhizal fungi suggest that only a few transporters are responsible for mediating the uptake of the full range of protein amino acids, suggesting that roots and mycorrhizal hyphae cannot discriminate between uptake of individual amino

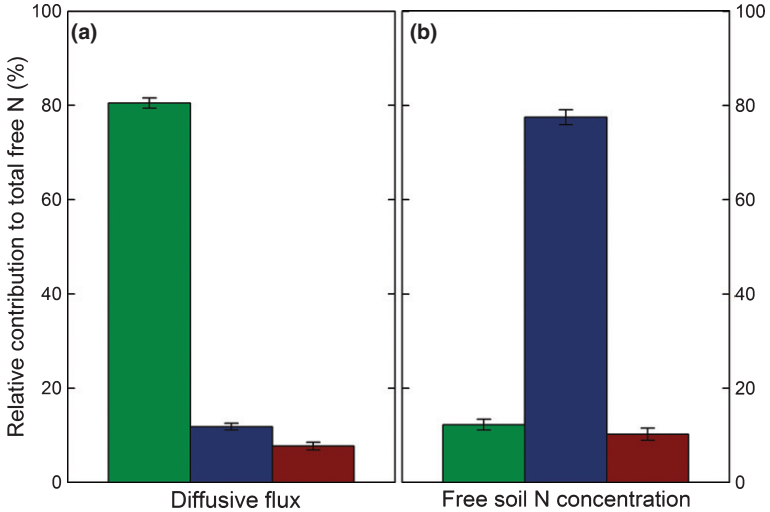

Fig. 1 Relative contribution of diffusive fluxes (a) and free soil nitrogen (N) concentrations (b) of total amino acids (green bars), ammonium (blue bars) and nitrate (red bars) to total plant-available N. Values used for calculation were flux rates of individual N forms (nmol N cm^{-2} h^{-1}) estimated by *in situ* microdialysis or concentrations of free individual N forms (μmol N kg^{-1} soil DW) in soil water extracts. Bars represent means ± SE of 12 forest sites (*n* for dialysis = 240, *n* for extracts = 108). Results for individual sites are presented in Supporting Information, Fig. S1.

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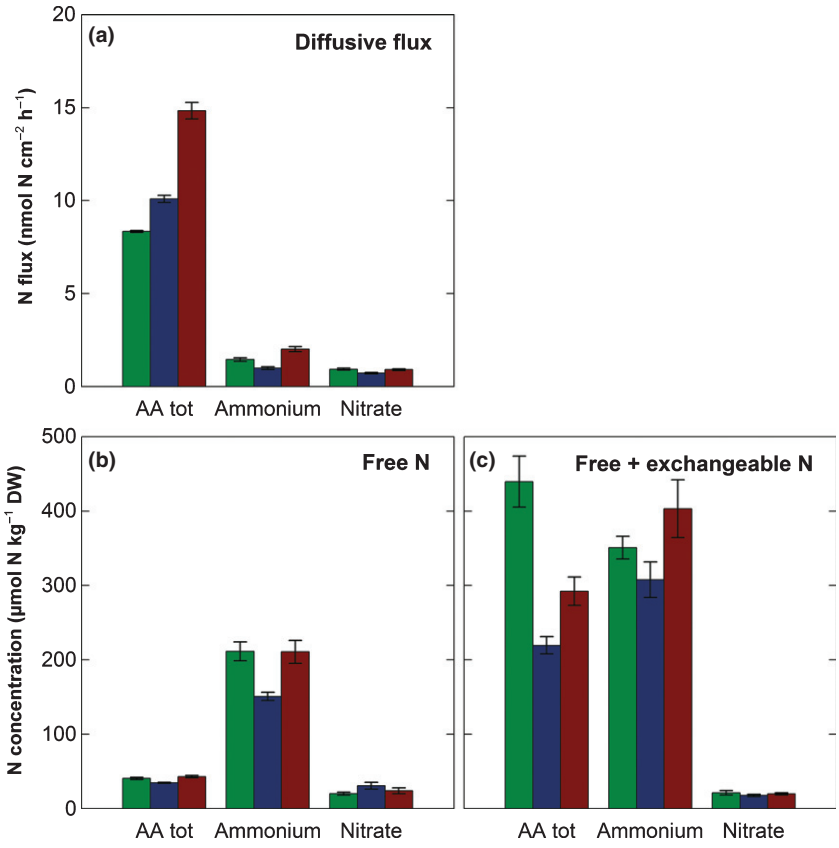

Fig. 2 Nitrogen (N) diffusive fluxes estimated by microdialysis sampling (a), free soil N concentrations estimated by water extraction (b), and free and exchangeable soil N concentrations (c) in boreal forest sites receiving different amounts of N fertilizer (green bars, 0 kg N; blue bars, 6 kg N; red bars, 50 kg N). Bars represent means ± SE of three forest sites (*n* for dialysis = 60, *n* for extracts = 27). AA tot, total amino acids.

acids (cf. Näsholm *et al.*, 2009). Hence, the available information suggests that any amino acid arriving at the membrane surfaces of roots or mycorrhizal hyphae will be absorbed at rates comparable to that of ammonium and appreciably higher than that of nitrate. Further, our study indicates that in boreal forests, amino acids dominate soil N supply. This result is in line with studies of Arctic (Kielland, 1994; Kielland *et al.*, 2007) and boreal ecosystems (Nordin *et al.*, 2001) but contrasts with several previous studies suggesting that inorganic N, particularly in the form of ammonium, is the major source of N available to plants and mycorrhizal fungi (Likens *et al.*, 1969; Robertsson, 1982; Kronzucker *et al.*, 1997; Rothstein, 2009).

Eutrophication of terrestrial ecosystems, resulting from fertilizer additions or anthropogenic N deposition, occurs mainly through input of mineral N forms (Vitousek *et al.*, 1997; Galloway *et al.*, 2008). The forests in our study are located in an area that receives very low background deposition of N (2 kg N ha^{-1} yr^{-1}) and we expected that in forests exposed to higher rates of mineral N input, plant-available N would shift towards ammonium and nitrate and that organic N would constitute a smaller proportion of this pool. Diffusive fluxes of soil N in non-fertilized plots in natural, N-limited forests were 8.3, 1.5 and 0.9 nmol N cm^{-2} h^{-1} for amino acid N, ammonium and nitrate, respectively (Fig. 2, Table S2). Surprisingly, long-term fertilization with NH_4NO_3 resulted in higher flux rates of amino acids, increasing from 8.3 nmol N cm^{-2} h^{-1} in unfertilized plots to 10.1 nmol N cm^{-2} h^{-1} at an addition rate of 6 kg N ha^{-1} yr^{-1} and 14.8 nmol N cm^{-2} h^{-1} at 50 kg N ha^{-1} yr^{-1}, while no such trend was observed for the two inorganic N forms (Fig. 2, Table S2). The fractions of N diffusing across the dialysis membrane that were accounted for by amino acids were, for the three N concentrations, 77.3, 81.3 and 83.6%, illustrating the potential dominance of amino acids as N sources for plants and mycorrhizal fungi in low-, medium- and high-N environments. This unexpected effect of inorganic N fertilizer application on supply rates of soil amino acids, but not on inorganic N, suggests that added N had a major effect on the overall N cycling in the forest soil by increasing the activity of soil microbes and roots. The fact that inorganic N remained at similar concentrations in all N treatments suggests that the later steps in the N mineralization pathway, such as ammonification and nitrification, may not limit production of available N (Schimel & Bennett, 2004; Kielland *et al.*, 2007; Wanek *et al.*, 2010) and instead emphasizes the importance of depolymerization of proteins to peptides and free amino acids as a major bottleneck in forest N cycling. By contrast, the effects of N fertilization on amino acid fluxes revealed by our *in situ* dialysis measurements were not detected after soils were sieved, homogenized and extracted in the laboratory according to standard techniques for characterizing soil N concentrations (Fig. 2, Tables S3, S4).

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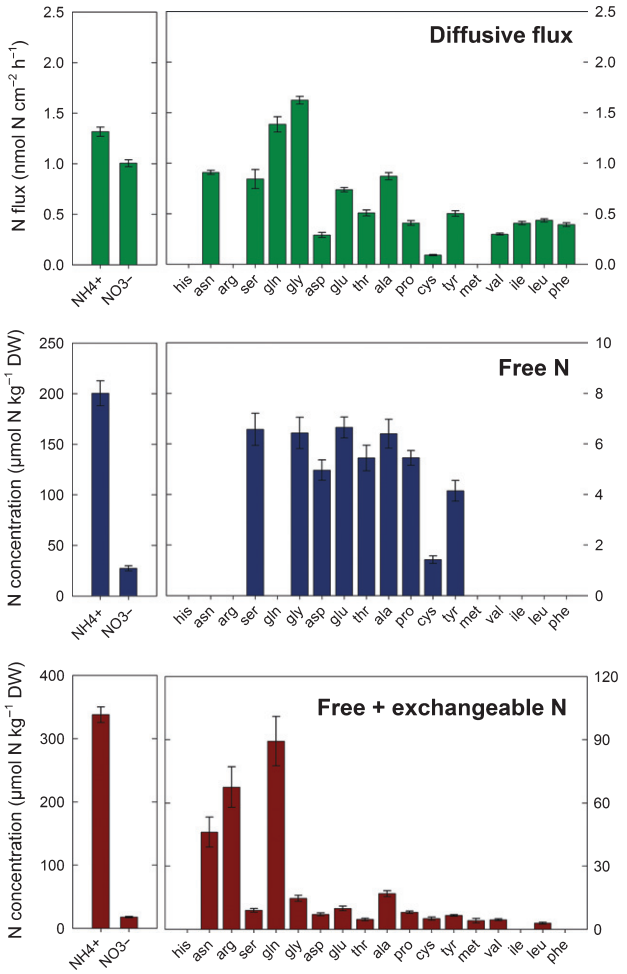

Fig. 3 Nitrogen (N) diffusion rates estimated by microdialysis sampling (top panel), free soil N concentrations estimated by water extraction (middle panel), and free and exchangeable soil N concentrations estimated by KCl (1 M) extraction in 10 boreal forest sites (bottom panel). Bars represent means ± SE (*n* for dialysis = 200, *n* for extracts = 90).

Conclusion

Plants may acquire N from a wide range of N compounds (Näsholm *et al.*, 2009), and, among the organic forms of N, amino acids are of special importance because of their relatively small size and rapid uptake rates. Here we show that induced diffusive flux of amino acids dominate over ammonium and nitrate in boreal forest soils, suggesting that such N forms play a critical role in the N nutrition of plants in this biome. Induced diffusive flux of amino acids was also responsive to long-term N fertilization, while no such response could be detected for either of the inorganic N sources, suggesting that amino acids may be primary N sources also in soils of higher fertility. Plant N acquisition, in particular in N-poor soils, depends on diffusion driven by the active uptake by plant roots and mycorrhizal hyphae. This fact, together with the apparent problem of using destructive techniques for studying compounds such as amino acids that exhibit high turnover rates in soils (Jones & Kielland, 2002), points to a need for new approaches in studies of plant–soil interactions, one of which is presented here.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Results from calibration of the individual microdialysis membranes.

Fig. S2 Relative contribution of individual N forms at each site.

Table S1 Detailed description of the 15 boreal forest sites

Table S2 Diffusive fluxes of individual N forms in N deposition plots

Table S3 Concentrations of individual N forms in soil water extracts

Table S4 Concentrations of individual N forms in soil KCl extracts

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